Manipulating cell wall biosynthesis in yeast and higher plants

by
Carl Ulrich Hörstmann

Thesis presented in partial fulfilment of the requirements for the degree Master of Science at the University of Stellenbosch

Supervisor: Prof Jens Kossmann
Faculty of Science
Department of Genetics

December 2010

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Declaration

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2010

Copyright © 2010 University of Stellenbosch

All rights reserved

Summary

Undeniably, changes in the environment and dwindling traditional energy resources have resulted in the search for viable, renewable energy alternatives such as biofuels. Cellulose is one of the most abundant polymers on earth and can be converted to simple sugars and fermented to ethanol biofuel fairly easily. Cellulose rich biomass that can serve to supply ethanol biofuel production can be sourced from unexploited agricultural waste. The main drawback to using vegetative tissue as opposed to harvested food stocks from crops results from the structural properties of plant cell walls. Although cellulose is abundant, the contaminating hemicellulose and lignin fibres within the cell wall matrix have a negative impact on the digestibility of the cellulose present. Thus, an important step in creating an effective biofuel production system from agricultural excess is developing crops with improved cell wall polymer characteristics that can be converted to ethanol more efficiently.

This project consisted of two parts. Firstly, the aim was to assess lignin production in transgenic sugarcane transformed with a construct aimed at down-regulating the 4-(hydroxyl) cinnamoyl CoA ligase (4CL) gene in the lignin biosynthesis pathway. The second part of the project revolved around discovering the mechanism of impared cell growth caused by expressing the gene encoding cellulose synthase from a marine invertebrate, *Ciona savignyi*, in the yeast *Saccharomyces cerevisiae*.

Several sugarcane lines that had been previously transformed with a hairpin RNAi construct aimed at down-regulating the 4CL gene in the monolignol biosynthesis pathway were subjected to analysis to determine if lignification had been reduced. Although the presence of the hairpin construct in the genomic DNA had been confirmed for all of the transgenic lines, there was no significant decrease in the lignin levels in any of the transgenic lines. PCR analysis of the mRNA and enzyme assays also confirmed that the 4CL gene was still being expressed. Ongoing work will determine the cause of the unsuccessful down-regulation.

Previously, it had been proven that the cellulose synthase gene from *C. savignyi* could be functionally expressed in *S. cerevisiae*. However, cellulose production resulted in extremely retarded growth of colonies and cultures, to the point of the apparent death of the cultures. The aim of this part of the project was to determine the mechanism (either

metabolic or physical) that causes this effect. To generate enough cell mass to perform metabolic analysis, several strategies to impede cellulose production in transgenic yeast were explored. Attempts to stop cellulose production and induce better growth by introducing Isoxaben (a traditional weed killer that targets cellulose synthases) into the growth medium used for the transgenic yeast proved unsuccessful. To control the expression of the transgene, it was attempted to clone the cellulose synthase gene into an expression system containing an inducible promoter. The cloning exercise proved extremely difficult and multiple attempts with several strategies proved unsuccessful. This process is still ongoing as the growth retarding process induced by cellulose production in yeast remains to be identified.

Opsomming

Dit is onontkenbaar dat veranderinge in die omgewing en minderwordende tradisionele energiebronne veroorsaak dat lewensvatbare en hernubare energiebronne soos biobrandstof gevind moet word. Sellulose is een van die mees volop polimere op aarde en kan redelik maklik omgeskakel word na eenvoudige suikers en gefermenteer word tot etanol-biobrandstof. Sellulose-ryk biomassa wat etanol-biobrandstof kan verskaf, kan herwin word van tot op hede ongebruikte landbou-afval. Die komplekse struktuur van plantselwande is die hoofstruikelblok in die omskakeling van vegetatiewe weefsel tot biobrandstof. Hoewel sellulose volop is, het die kontaminerende hemisellulose- en lignienvesels binne die selwand-matriks ’n negatiewe impak op die verteerbaarheid van die sellulose teenwoordig in die selwand. Daarom is ’n belangrike stap in die ontwikkeling van effektiewe biobrandstof-produksiesisteme vanaf landbou-afval om gewasse te ontwikkel met verbeterde selwandpolimeer-eienskappe wat etanol-produksie kan vergemakilik.

Hierdie projek het bestaan uit twee dele. Eerstens was die doel om vas te stel of die lignienproduksie geaffekteer is in transgeniese suikerriet getransformeer met ’n konstruk wat mik om die 4-(hidroksie)-cinnamoyl CoA ligase (4CL) geen te af-reguleer in die lignien-biosintese-padweg. Die tweede deel van die projek het daarop gefokus om die meganisme te ondek wat die belemmerde selgroei veroorsaak, as gevolg van die uitdrukking van die geen wat kodeer vir sellulose-sintase in ’n mariene ongewerwelde, *Ciona savignyi*, in *Saccharomyces cerevisiae*.

Verskeie suikerriet-lyne, wat voorheen getransformeer is met ’n haarnaald-RNAi-konstruk om die 4CL-geen te af-reguleer in die monolignol-biosintese-padweg, is onderwerp aan analise om vas te stel of lignifikasie verminder is. Hoewel die teenwoordigheid van die haarnaald-konstruk in die genomiese DNA bevestig is vir al die transgeniese lyne, was daar geen beduidende vermindering in die lignienvlakke in die transgeniese lyne nie. PKR-analise van die mRNA en ensiem-aktiwiteitstoetse het ook bevestig dat die 4CL-geen steeds uitgedruk word. Verdere ondersoek sal kan vasstel wat die oorsaak van die onsuksesvolle af-regulering is.

Voorheen is bewys dat die sellulose-sintase-geen van *C. savignyi* funksioneel uitgedruk kon word in *Saccharomyces cerevisiae*. Egter, selluloseproduksie het die gevolg gehad

dat groei in die transgeniese kolonies en kulture erg gestrem is, tot die punt dat die kulture dood voorgekom het. Die doel van hierdie deel van die projek was om vas te stel wat die meganisme (òf metabolies òf fisies) is wat hierdie verskynsel veroorsaak het. Om genoeg selmassa te genereer om metaboliese analise uit te voer, is verskeie strategieë om selluloseproduksie in transgeniese gis te verhinder, ondersoek. Pogings om selluloseproduksie te stop en om groei te verbeter deur Isoxaben by te voeg in die groeimedium gebruik vir transgeniese gis, was onsuksesvol. Isoxaben is ’n tradisionele onkruiddoder wat sellulose-sintases teiken en inhibeer. Om die uitdrukking van die transgeen te beheer, is ’n poging aangewend om dié sellulose-sintase-geen in ’n uitdrukking-sisteem te kloon met ’n induseerbare promotor. Die kloneringsoefening was uiters moeilik en veelvoudige pogings met verskeie strategieë was onsuksesvol. Hierdie proses moet verder gevoer word aangesien die groeistremmingsmeganisme veroorsaak deur selluloseproduksie in gis nog geïdentifiseer moet word.

Acknowledgements

I would like to thank Prof. Jens Kossmann and Dr Paul Hills for their supervision and guidance during this project.

I would like to thank the Institute for Plantbiotechnology, the National Research Fund and the South African Sugarcane Research Institute for the financial support that afforded me the opportunity to complete this study.

Thanks to Professor William Smith (Neuroscience Research Institute and Department of Molecular, Cellular and Developmental Biology, University of California) for the kind donation of the *Ciona savignyi* cellulose synthase gene.

Thanks also to Dr Michael Bester from the Institute for Wine Biotechnolgy at the University of Stellenbosch for supplying the pYES2 vector.

Thanks to the staff and fellow students of the IPB for their help and support were it was received.

An especial word of thanks to Dr Jan Bekker whom always had time for some excellent advise and due criticism were needed, Stanton Hector for advise and help in experimental ideas and execution and Kyle Willard for organizing chemicals, enzymes and other lab stocks on short notice and at obscure times.

The work presented in this document would never have possible with the constant support, help and motivation from my family. My mother, father and two brothers are forever my inspiration, I love you all.

My biggest word of thanks goes out to Liske, for literally being there every step of the way and making this an awesome experience. Thank you for putting up with all of my nonsense, keeping my head straight through the worst of it, and celebrating with me during the best of it. I love you and cherished every minute.

Preface

This thesis is presented as a compilation of four chapters. Chapter 1 serves as a general introduction to the study and establishes the aims and motivation, as well as reviewing literature pertinent to the study. The individual aims and outcomes of the study is described in Chapters 2 and 3 which presented in the format of a research article according to the Guidelines for authors of Plant Physiology. The outcomes of the study are discussed in Chapter 4 which serves as a general conclusion.

Chapter 1 General introduction and literature review

Chapter 2 Research Chapter: Evaluating lignin production in transgenic sugarcane modified to down-regulate 4-(hydroxyl)cinnamoyl CoA ligase

Chapter 3 Research Chapter: Expressing *Ciona savignyi* cellulose synthase in yeast

Chapter 4 General discussion and conclusion

Table of Contents

List of Abbreviations

4CL	4-(hydroxyl) cinnamoyl CoA ligase
ATP	Adenosine 5’-triphosphate
bp	base pairs
BSA	bovine serum albumin
C3H	p-coumarate 3-hydrolase
C4H	cinnamate 4-hydoxylase
CAD	cinnamyl alcohol dehydrogenase
CBM	carbohydrate binding modules
CCoAOMT	caffeoyl CoA *O*-methyltransferase
CCR	cinnamoyl CoA reductase
cDNA	complementary DNA
CesA	cellulose synthase A
CiCesA	*Ciona intestinales* cellulose synthase
CoA	coenzyme-A
COMT	caffeic acid/5-hydroxyferulic acid *O*-methyltransferase
CsCeS	*Ciona savignyi* cellulose synthase
CTAB	Cetryltrimethyl-amonium bromide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EST	epressed sequence tags
F5H	ferulate 5-hydroxylase
gDNA	genomic DNA
HCT	hydroxycinnamoyl CoA:quinate shikimate hydroxycinnamoyl-transferase
kb	kilobase pairs
KOR1	KORRIGAN1
LB	Luria-Bertani medium
PAL	phenylalanine ammonia-lyase
pCCoA3H	p-coumaryl CoA 3-hydroxylase
PCR	polymerase chain reaction

PGK	phosphoglycerate kinase
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
SAD	sinapyl alcohol dehydrogenase
SASRI	South African Sugarcane Research Institute
SEM	scanning electron microscope
SBM	structural biomass
TEM	transmission electron microscopre
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
UGT	uridine diphosphate-glucosyl transferase
WT	wild-type
YNB	yeast nitrogen base

1. General Introduction

1.1 Introduction

In the last couple of decades the changes in the environment have become un-ignorable, and the question of whether human industry is the cause or not, is moot. That modern life has a profound effect on the ecosystems of our planet is, however, not in question. The industries that flung human civilization into the new millennium can be felt in the pollutants that taint the rivers and seas of the world, and poisons floating in the air crucial for life. Following the global economic crisis of the 1970s, the demand for fossil fuels has more than tripled (Ragauskas et al., 2006) and projected energy needs are set to grow by 50% by the year 2025. Most of the current energy needs are met by burning non-renewable fossil fuels, forcing millions of tons of carbon dioxide into the environment, disrupting the planetary homeostasis. The explosion of a deep sea oil drilling project in April 2010 in the Gulf of Mexico and the massive and disastrous oil leak caused by this only re-enforced the need for research into viable alternatives to humankind's dependency on limited geological reserves as energy sources.

Depleting traditional energy sources, public pressure towards natural rather than synthetic products, and stricter regulations on greenhouse gas emissions and industrial waste have forced industry and science to investigate innovative and sustainable ways of capturing and distributing energy and producing renewable products (Reddy and Yang 2005).

Photosynthesis is the biggest and most effective "industry" on earth. Every year the earth receives nearly 4000 times the human energy usage predicted for 2050 in the form of solar energy (Somerville 2006a). Solar energy together with water and carbon dioxide are converted by photosynthesis into sugars and biopolymers, building the massive biomass that is plants. Although humans have been using plant biomass for a long time (e.g., wood burning for energy, raw as a building material, pulped to make paper, cotton in textiles and many other uses) it has only recently been investigated as a viable renewable resource. Currently many strategies are being investigated to exploit this seemingly vast resource. The biggest focus, however, is on biofuel development. In many countries over the world, biofuel in some form are already in everyday use. Around a quarter of the fuel used for ground based transportation in Brazil is derived from biomass. In countries such as

Australia, Austria, Canada, France and New Zealand the use of E10 (10% Ethanol/Petrol fuel blend) is optional. In Colombia, Costa Rica, India, Jamaica, Paraguay, Sweden and Thailand the use of low ethanol fuel blends are mandatory. The positive aspects of using and producing biofuel and other biomaterials do not just lie in controlling greenhouse gasses, but also lead to many economic and strategic advantages (Somerville 2006a).

Currently, the biggest source of biofuel is starch and sugarcane sucrose (Somerville 2007). However, concern has been raised whether basic human food stocks should be used for biofuel production. The rationale behind this is if the same product could be used for either fuel or food, the need for fuel could drive the cost of such stocks beyond the means of the poorest people who rely on it for food to survive. Thus pressure has been applied to find alternative sources for fuel production.

The solution may lie in lignocellulosic biomass from cell walls, which make up the bulk of terrestrial plant biomass and an as yet largely untapped carbon reservoir with plants producing nearly 180 billion tons of cellulose annually (Festucci-Buselli et al., 2007). Cellulose is the biggest component of plant cell walls together with hemicellulose and lignin, and is expected to be the largest source of renewable biofuel for the future (Somerville 2007; Mutwill et al., 2008; Demura and Ye 2010). Cellulosic biomass can be obtained from, what has been traditionally viewed as, agricultural waste in the form of stoves from cereal crops such as maize, rice, sorghum and sugarcane, pineapple and banana leaves; wood pulp from forestry; and other plants such as switch grass. Fast growing trees such as willow and poplar have also been suggested as possible sources of biomass for biofuel production. Industrial wastes and residues such as those from paper manufacturing can also be used, and together with the listed sources, are ideal, inexpensive and abundant sources of carbon for conversion to biofuel.

Extensive research on optimizing ethanol production of biofuel from cellulosic fibers has been done in recent times. Previously, cellulosic fibers have had to be pretreated enzymatically or chemically, reducing it to simple sugars to feed fermentation processes for ethanol production. In recent times several approaches to simplify this process have been researched and have led to the development of several transgenic organisms. During the late 1990s, transgenic yeast strains were developed with the ability to assimilate soluble cello-oligosaccharides comprising 2- to 6 glucose units (Cho et al., 1998; Van

Rensburg et al., 1998). Later research by Fujita et al. (2002) led to the development of a cellulose degrading yeast co-expressing two enzymes, a cellulytic enzyme and a glucosidase from *Trichodema reesii* and *Aspergillus aculeatus* respectively. These enzymes had previously been shown to work synergistically to degrade cellulose (Woodward, 1991) and their co-expression led to the first direct fermentation from barley β-glucans to ethanol without any pretreatment.

Cellulose conversion into ethanol biofuel has been extensively researched, but one of the biggest problems lies in exploiting the available plant biomass effectively. In higher plants, the blend of cellulose, hemi cellulose and lignin polysaccharides gives cell walls their strong and yet flexible properties. Although cellulose comprises the biggest part of plant derived biomass the complex structure of the plant cell wall makes the efficient extraction and use of the available cellulose difficult. To remedy this, many different research paths are being investigated, mainly: (1) to simplify the cell wall structure in such a manner that makes cellulose more available for degradation to simple sugars and (2) to produce cellulose with properties that is easily digestible with cellulase enzymes. The efficient conversion of lignocellulosic biomass to fermentable sugars, and subsequently to ethanol biofuel depends on optimizing genetic, molecular and macroscopic features of suitable sources.

Cellulose and hemicellulose can be fairly easily degraded enzymatically through a process called saccharification. However, the complex structures that form due to the presence of lignin in the cell wall matrix cause a problem. The manner in which lignin forms in the structure of the cell wall, renders both cellulose and hemicellulose inaccessible to hydrolytic enzymes, which compromises the efficiency of the hydrolytic enzymes to be used for biofuel production. Lignin's properties exaggerate the situation further. Through evolution plant cell walls have adapted to resist breakdown from pathogens and damage from mechanical forces (McCann and Carpita 2008) and lignin plays a key role in protecting cells in this regard: Lignin is highly resistant to degradation and strongly inhibits saccharification (Weng et al., 2008; Boerjan et al., 2003). In the past, fermentable sugars could only be released from lignocellulosic biomass by subjecting raw material to extreme mechanical, heat and chemical treatments (Hamelinck et al., 2005; McCann and Carpita, 2008, Weng et al., 2008). This is not an ideal strategy, not only because of the dangerous

and usually expensive methods, but because the processes either add additional compounds or alter the products, compromising the efficiency of downstream uses (Hamelinck et al., 2005). Thus the properties of biomass, especially with regard to its lignin content, will be one of the key points in determining its suitability as a source for biofuel production. As the levels of lignin present in biomass directly impact on the cost of pretreatment, its biosynthetic pathway has been extensively targeted for genetic modification through down-regulation (Simmons et al., 2010).

Lignin is the most abundant aromatic polymer in nature. It is a macromolecule of phenolic character, the dehydration product of three monomeric alcohols or lignols, namely: trans-p-coumaryl alcohol, trans-p-coniferyl alcohol and trans-p-sinapyl alcohol derived from p-cinnamic acid (Boerjan et al., 2003; Ralph et al., 2004). Lignin is an important part of biochemical evolution, carbon partitioning, cell differentiation and functional adaptation to water conduction, and the plant's ability to deal with biotic and abiotic stress (Soltani et al., 2006; Vanholme et al., 2008). Numerous genes are involved in biosynthesis, assembly and deposition of a modified cell wall template and of the lignin polymer itself (Boerjan et al., 2003), which give lignified cell walls their distinct structure and chemical properties. Lignin is one of the products of the phenylpropanoid biosynthetic pathway that also includes flavonoids and hydoxycinnamic acid conjugates. To date, the monolignol biosynthetic pathway is well described and some of the important enzymes include: 4-(hydroxy)cinnamoyl CoA ligase (4CL); p-coumarate 3-hydroxylase (C3H); cinnamate 4-hydroxylase (C4H); cinnamyl alcohol dehydrogenase (CAD); caffeoyl CoA O-methyltransferase (CCoAOMT); cinnamoyl CoA reductase (CCR); caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT); hydroxycinnamoyl CoA:quinate shikimate hydroxycinnamoyltransferase (HCT); ferulate 5-hydroxylase (F5H); phenylalanine ammonia-lyase (PAL); p-coumaryl CoA 3-hydroxylase (pCCoA3H); and sinapyl alcohol dehydrogenase (SAD) (Figure 1) (Humphreys and Chapple 2002; Boerjan et al., 2003; Vanholme et al., 2008).

Various genes in this pathway have been targeted for modified expression in several plant species such as tobacco, poplar, and alfalfa (Blaschke et al., 2003; Blee et al., 2003; Chabannes et al., 2001; Hu et al., 1999; Kajita et al., 1997; Pilate et al., 2002; Reddy et al., 2005). Down-regulation of several of these genes has had successful results in decreasing

the lignin content, or altering the digestibility of the transgenic biomass. The investigation of Hu et al. (1999) showed that the transgenic poplar trees had substantial decreases in their lignin levels and, surprisingly, significant increases in cellulose content. The successes in decreasing lignin levels in plants by down-regulating the 4CL gene in the lignin biosynthetic pathway made it a promising candidate target to decrease the lignin content of sugarcane. The goal of this project was to evaluate putatively transgenic sugarcane line containing a hairpin construct that was supposed to down-regulate the 4CL gene. Each putative transgenic sugarcane line was tested to determine the effect of the transgenic construct on its lignin level and its 4CL activity.

Another important branch of research regarding modifying plant cell walls for better use as a renewable resource for biofuel production lies in improving the digestibility of cellulose (Abramson et al., 2010). Cellulose micro fibrils consist of linear chains of (1→4)-linked β-D-glucose molecules. These micro fibrils are of indeterminate length, and vary highly in order and width, depending on the number of parallel chains (Taiz and Zeiger 2002).

In plants, cellulose exists in two forms or allomorphs that differ in the manner in which the parallel glucan chains are stacked. These cellulose allomorphs are known as Iα and Iβ. Cellulose is synthesized by six cellulose synthase subunits. The subunits are grouped together in the plasmid membrane and known as particle rosettes or terminal complexes. Cellulose synthases in higher plants are encoded by the Cellulose synthase A (CesA) gene family (Arioli et al., 1998; Taiz and Zeiger 2002) and have been thoroughly characterized in higher plants together with several other putative secondary wall components (Demura and Ye 2010; Somerville 2006b). It has been proposed that the amorphous regions of cellulose are more susceptible to cellulase enzymes (Abramson et al., 2010). Should this be true, it stands to reason that if plants could be made to produce less crystalline cellulose it could increase the efficacy with which cellulose is digested and thus positively impact on ethanol production.

Studies have shown that expressing microbial carbohydrate binding modules (CBM) in tobacco, poplar, Arabidopsis and rice reduces the crystallinity of cellulose produced in these plants (Abramson et al., 2010). Cellulose crystallinity in Arabidopsis has also been reduced by over-expressing a putative poplar ortholog of the Arabidopsis *KORRIGAN1* (*KOR1*) gene (Takahashi et al., 2009). Determining how altered crystallinity affects

cellulose degradation will greatly impact on research into streamlining biofuel production from cellulosic biomass. There are several non-traditional sources of cellulose that can be investigated for cellulose of novel nature.

Cellulose production is a trait primarily associated with higher plants and algae; however, there are several other organisms that produce cellulose. Fibers from these sources may have novel properties that can be exploited not only for biofuel production but also bio-polymer production. Most of the current knowledge on cellulose biosynthesis was determined from work on bacterial cellulose. Several bacteria species are able to produce cellulose; among these are *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) and *Agrobacterium tumefaciens*. Extensive research into cellulose production by *G. xylinus* and *A. tumefaciens* has led to the elucidation of cellulose biosynthesis and the understanding of the genetic factors involved (Matthysse et al., 1981; Matthysse et al., 1995; Swissa et al., 1980; Wong et al., 1990).

Research into exploiting alternative cellulose sources has yielded some interesting results. The pure and extremely fine cellulose fibers produced by *G. xylinus* have been used for the production of high-strength fiber composites (Nakagaito et al., 2005).

Another novel cellulose producing source is, unlikely enough, from a marine animal. Urochordates are a group of marine invertebrates that have been shown to be the only animals able to produce cellulose (Hirose et al., 1999; Kimura et al., 2001; Rånby 1952). Urochordates include three classes, namely, Appendicularia, which incorporate cellulose into its 'house', and Thaliacea and Ascidiacea (the latter two incorporate cellulose into their tunics). Nakashima et al. (2004) investigated the origin of cellulose production in *Ciona intestinalis,* an ascidian. It was found that *C. intestinalis* had a homolog of the cellulose synthase gene, and, further, that the *C. intestinales* cellulose synthase (Ci-CesA) gene is a fusion of both a cellulose synthase domain and a cellulase domain. Neither of these domains have any animal homolog. Research has shown strong similarities between the ascidian cellulose synthase gene, and that of bacterial origin. This might suggest that a bacterial cellulose synthase gene may have been laterally transferred to an extinct urochordate ancestor. *C. intestinales* was also shown not to contain the other constituent genes of bacterial operons required for cellulose production in bacteria (Nakashima et al., 2004). Ascidian cellulose synthesis also exhibit unique features alluding to some novel

processes in its mechanism of synthesis. Importantly it has been shown that the cellulose produced by ascidians are almost entirely of the Iβ allomorph produced by linearly arranged terminal complexes (Kimura and Itoh 1996) as opposed to bacterial cellulose comprising mostly the Iα allomorph.

In a previous study, it was shown that the cellulose synthase from the ascidian *Ciona savignyi* could be functionally expressed in *Saccharomyces cerevisiae* (Hörstmann and Kossmann 2007). During this study it was observed that the transgenic yeast grew extraordinarily slow. This led to the question of whether the production of cellulose by the transgenic yeast has led to some form of metabolic or physical restriction. The aim of this project was to evaluate the transgenic yeast together with other transgenic lines to determine the cause of the growth restriction, and to analyse the cellulose produced to establish if it has novel properties.

In summary, improving the quality of agricultural 'waste' biomass for use as a renewable stock for biofuel production is of great importance. This study (Chapters 2 and 3) represents some of the strategies being investigated and possible remedies. The focus is shifted to investigating a potential system of reducing the lignin content of sugarcane, and investigating the potential of expressing non native cellulose in novel expression systems. By investigating the potential of different technologies, the results may lie the foundation for the possible creation of an effective, economically viable and renewable agricultural source for biofuel production, with minimal impact on food stock production or available agricultural resources.

1.2 Project Aims and Outcomes

Chapter 2: Evaluating lignin production in transgenic sugarcane modified to down-regulate the 4CL enzyme.

Aim: To determine the effect of expressing a hairpin RNA sequence designed to silence the native *4CL* gene, on lignin production in transgenic sugarcane.

Outcomes: Sugarcane lines containing the hairpin construct, aimed to silence 4CL were analyzed, and had no effect on 4CL activity or lignin levels in the transgenic sugarcane lines.

Chapter 3: Investigating the effects of expressing *Ciona savignyi* cellulose synthase in yeast.

Aims: To determining the cause of the retarded growth patterns observed in transgenic *S. cerevisiae*, which expresses the cellulose synthase gene from *C. savignyi*, by metabolic analysis, and to evaluate the cellulose produced for novel properties.

Outcomes: Transgenic yeast lines ubiquitously expressing the *C. savignyi* cellulose synthase gene had such retarded growth that analysis proved impossible. Attempts to chemically inhibit cellulose synthase proved unsuccessful and difficulties in implementing an inducible expression system resulted in the unsuccessful characterization and metabolic analysis of cellulose synthase expressing yeast.

2. Evaluating Lignin Production in Transgenic Sugarcane Modified to Down-Regulate the 4CL Enzyme

2.1 Introduction

Lignin represents a definitive obstruction in the processing of plant material in human industry. In the last two decades extensive research into the biosynthesis of lignin has been done and a variety of transgenic plants modified in several steps of the phenylpropanoid and monolignol biosynthetic pathways have led to a better understanding of how these monomers are produced in plants (Figure 1) (Humphreys and Chapple 2002; Vanholme et al., 2008). As a result of this, several strategies have been developed to alter lignin production in plants to better suit human needs in future industry (Anterola and Lewis 2002; Boudet et al., 2003).

Biomass' properties regarding lignin content and digestibility have been of special concern in two industries, namely in the production of animal feeds, and in wood for manufacturing paper. As such, a great deal of research has gone into improving the lignin qualities of plant species used for these industries.

Research done by Reddy and Yang, (2005) to elicit changes in the lignin content of alfalfa (*Medicago sativa* L.), for use as animal feed, analysed several genes central to lignin production. Three enzymes involved in the synthesis of monolignols were targeted, namely C4H, C3H and F5H, and where the enzymes were down-regulated successfully ,plants had significantly reduced lignin levels.

Hybrid poplars which expressed antisense CAD transgenes improved the pulping characteristics and required less pre-treatment before pulping without having negative effects on the growth of the trees or insect interactions (Pilate et al., 2002). Lee et al. (1997) showed that the repression of 4CL activity by expressing the antisense gene is possible. Transgenic Arabidopsis lines showed as little as 8% 4CL activity and had correlating decreases in thioglycolic acid extractable lignin levels.

In 1999, Hu et al. produced transgenic aspen trees (*Populus tremuloides* Michx.) in which 4CL was successfully down-regulated by antisense expression of the *Pt4CL1* gene. The transgenic trees showed a reduction of up to 45% in lignin and a surprising 15% increase

in cellulose content. Structural integrity was preserved, and leaf, root and stem growth were enhanced. The combined lignin and cellulose mass was maintained between transgenic and wild type lines, thus indicating that the regulating factors compensate the lignin deficiency with cellulose alluding to high metabolic plasticity to sustain plant structure.

Further research on *P. tremuloides* has led to the production of transgenic trees expressing both antisense constructs of both the *4CL* and the *F5H* genes (Li et al., 2002). These trees showed as much as 52% reduction in lignin levels and a 30% increase in cellulose levels. Trees that had the *4CL* gene silenced showed slightly higher reduction in lignin levels and similar cellulose levels to those reported by Hu et al. (1999). Transgenic trees with only the F5H gene silenced, did not show a drop in the lignin levels, but exhibited a change in the ratio of the monolignols, syringyl and guaiacyl present. This suggests that although these genes had independent effects, they are contributed to overall lignin production.

Previous work resulted in the production of putative transgenic sugarcane lines. Annotated sequences from *Oryza sativa* and *Zea mays* was compared to sugarcane ESTs and homologous regions were used to design primers to amplify a putative sugarcane *4CL* gene (Table 2.1). From the work done, a DNA fragment that showed high homology to the *Z. mays 4CL* gene (Figure 2.1 and Figure 2.2) was used to construct a hairpin loop that contained a 613 bp fragment in both sense and antisense orientation with an 86 bp intron region dividing the two regions. The hairpin was cloned into the pU3Z sugarcane expression vector. The pU3Z vector allows for ubiquitous expression of the hairpin, courtesy of the maize ubiquitin (UBI) promoter and allows for ampicillin resistance in bacteria. The hairpin construct and a sugarcane selection plasmid were co-transformed into sugarcane using biolistics. From the transformed callus 13 putative lines were isolated and 11 lines were able to be hardened off to full plants growing under greenhouse conditions.

The objective of this study was to access lignin production in these 11 transgenic sugarcane lines expressing a hairpin construct targeted to silence the *4CL* gene. These plants were subjected to a range of tests to determine the effect of the presence of the hairpin construct targeting the 4CL gene on 4CL activity and on lignin levels.

2.2 Materials and Methods

2.2.1 Assessing Transgenic Lines

The 11 putative transgenic lines were evaluated for the presence of the hairpin construct. The youngest protruding leaves were harvested and milled in liquid nitrogen. The non-transformed NCo310 served as control. The processed tissue samples were stored at -80℃ and used for further analysis.

DNA was extracted with a method modified from McGarvey and Kaper (1991). Briefly, to 400 µl extraction buffer [50 mM Tris–HCl (pH8); 0.7 M NaCl; 10 mM EDTA (pH 8); 1.0 % w/v CTAB; 0.1 % v/v β–Mercaptoethanol], 10 to 20 mg of frozen plant material was added, vortexed for 10 seconds and incubated at 60℃. After 1 hour 400 µl of 24:1, Chloroform: Isoamyl-Alcohol (v/v) was added, again vortexed for 10 seconds and centrifuged at 4℃ for 5 minutes at 13 000 rpm in a Hermle Z233 MK-2 desktop centrifuge. The top aqueous layer was then removed to a new tube, 1 volume ice cold isopropanol added and incubated for 30 minutes at -20℃. After incubation the samples were centrifuged at 13 000 rpm for 15 minutes at 4℃ and the supernatant was aspirated off. The DNA pellet was then washed in 70% ethanol twice and the pellet dried and resuspended in ddH_2O.

The prepared DNA samples for the 11 putative transgenic lines and the control line was then subjected to PCR analysis with a single primer designed to amplify across the intron of the hairpin construct (Tables 2.1 and 2.2). The PCR products of the 11 lines and the NCo310 control were separated with electrophoresis on a 0.8% agarose gel to confirm the presence of the hairpin construct in the genome of the putative transgenic lines.

2.2.2 Assessing Lignin Content in Transgenic Sugarcane Lines

For assessing the lignin content of the transgenic and control sugarcane lines, frozen and processed leaf tissue samples were used form greenhouse grown transgenic and non-transgenic lines, and from samples taken from the seventh and thirteenth internodes of 5 transgenic lines and a control NCo310 line grown in a field trial environment at the South African Sugarcane Research Institute (SASRI) terrace in KwaZulu Natal, South Africa. The internode samples were harvested from the field trial plants, frozen and milled, and stored

at -80℃ for further use. Lignin was isolated and determined according to methods modified from Brinkmann et al. (2002).

The structural biomass (SBM) was isolated from all samples by suspending 0.5 g to 1.0 g of the frozen and milled plant material in 20 ml Washing Buffer [100 mM K_2HPO_4/KH_2PO_4 (pH 7.8); 2% (v/v) Triton X-100] and agitated for 30 minutes at room temperature. The samples were then centrifuged for 20 minutes at 5 500 x g and the washing step repeated. The samples were then washed four times in 20 ml 96 % ethanol while incubating in an 80℃ water bath for 30 minutes. After final incubation the tubes were again centrifuged at 5 500 x g for 20 minutes and ethanol removed. The pellets were dried for 12 hours in an 80℃ oven and stored in airtight containers at room temperature.

Lignin levels were determined in triplicate for each line. The dried SBM (5 mg) was weighed into microfuge tubes, 1.5 ml 2 M HCl and 0.3 ml concentrated Thioglycolic acid (Sigma-Aldrich) were added, and were incubated for 4 hours at 95℃. After incubation, the samples were rapidly cooled on ice and centrifuged in a desktop centrifuge for 10 minutes at 13 000 rpm. The supernatant was carefully aspirated and samples were washed three times with dH_2O. The pellets were incubated at room temperature for 18 hours in 1 ml 0.5 M NaOH with shaking. After incubation, the samples were centrifuged for 10 minutes at 13 000 rpm and the supernatant removed to a new tube. The pellet was then resuspended in 0.5 ml 0.5 M NaOH, thoroughly vortexed, and centrifuged for 5 minutes at 13 000 rpm. The supernatant was removed and combined with the previous supernatant. Concentrated HCl (0.3 ml) was added and the samples were then incubated at 4℃. After a 4 hour incubation the samples were centrifuged at 13 000 rpm for 10 minutes and the supernatant discarded. The resulting pellets were resuspended in 1 ml 0.5M NaOH and lignin levels determined by spectrophotometry using a Powerwave X Microplate Spectrophotometer with KC4 Kineticalc version 2.7. Lignin levels were determined at 280 nm. A standard curve was prepared using lignin (alkali) from Sigma-Aldrich to determine the lignin levels in the extracts of the transgenic and wild type sugarcane.

2.2.3 Assessing 4CL Activity in Transgenic Sugarcane Lines

To determine 4CL activity in transgenic and control 4CL plants, frozen leaf tissue samples were used. A crude protein extraction was performed, modified from Tsai et al. (1998) and

Knobloch and Hahlbrock (1977). In 1.2 ml extraction buffer (200 mM Tris-HCl pH 7.5; 2.5 % w/v PVPP), 200 – 300 mg of milled plant material was added, vortexed and centrifuged for 20 minutes at 13 000 rpm at 4℃ in a desktop centrifuge. The supernatant was removed to a new tube and sterile glycerol added to a final concentration of 30% and frozen at -80℃ for further use. The protein concentration was determined spectrophotometrically using the Bio-Rad Protein Assay dye. The protein concentration was calculated using a standard curve prepared with Bovine Serum Albumin (BSA).

Enzyme activity was determined spectrophotometrically (Knobloch and Hahlbrock 1977). The reaction mixtures contained 0.5 mM 4-hydroxycinnamic acid (Merck), 0.3 mM Coenzyme A (Sigma), 5 mM ATP and 5 mM $MgCl_2$ in 400 mM Tris-HCl (pH 7.8). Each reaction contained 6 µg of the crude protein extract and was measured at 310 nm over 30 minutes at 1 minute intervals to measure the decrease in 4-Hydroxycinnamic acid (p-coumaric acid) levels over time.

2.2.4 PCR Analysis of 4CL Transcription Levels in Transgenic Sugarcane

cDNA was generated from RNA extracted using standard lab protocols from frozen leaf material. RNA was extracted from tissue samples by adding 200 mg frozen and milled leaf tissue to 1.2 ml Extraction buffer [2 % (w/v) CTAB; 2 % (w/v) Polyvinylpyrrolidone (PVP); 100 mM Tris-HCl (pH 7.5); 25 mM EDTA; 2 M NaCl; 0.5 g/l Spermidine, and 3% (v/v) β-Mercaptoethanol] pre-warmed to 65℃ and vortexed immediately. The extraction mixture was incubated at 65℃ for 15 minutes and centrifuged at 13 000 rpm for 10 minutes at 4℃ in a desktop centrifuge. The supernatant was removed to a new tube, 1 volume 24:1 Chloroform: Isoamyl alcohol added and vortexed for 30 seconds. The samples were then centrifuged at 13 000 rpm for 15 minutes at 4℃ and the upper aqueous layer removed to a new tube and the Chloroform: Isoamyl alcohol extraction repeated and the upper layer again removed to a new tube. Lithium Chloride (8 M) was added to a final concentration of 2 M and samples incubated at 4℃ overnight. After the overnight incubation, the samples were centrifuged at 13 000 rpm for 60 minutes at 4°C and supernatant discarded. The pellet was washed in 70 % ethanol, dried and resuspended in dH_2O and kept at -80℃. RNA concentrations and quality was determined by nanodrop and gel electrophoresis respectively.

cDNA was generated from total RNA extraction using the RevertAid™ First Strand cDNA Synthesis Kit from Fermentas according to manufacturer's guidelines and used for PCR amplification. Primers were designed to amplify a 192 bp fragment of the gene targeted for silencing (Tables 2.1 and 2.2). If silenced, the gene should not amplify from the transgenic sugarcane lines but should amplify from the wild type NCo310 control line.

2.3 Results

2.3.1 Assessing Transgenic Sugarcane Lines

gDNA was isolated successfully from all 11 transgenic lines and from the NCo310 control line. Results from the PCR amplification across the intron are shown in the gel electrophoresis image in Figure 2.3. Amplification in all eleven putative transgenic lines and not in the wild type NCo310 line confirms the successful transformation of the sugarcane lines.

2.3.2 Assessing Lignin Content in Transgenic Sugarcane Lines

The SBM was isolated from the leaves of all 11 transgenic sugarcane lines and of the NCo310 control line grown under greenhouse conditions. SBM was also extracted from internode 7 and 13 of field grown sugarcane lines 1, 7, 10, 11 and 12 and a control NCo310 line. Thioglycolic lignin extractions were prepared with each milliliter of extract containing the lignin from 5 mg of processed tissue. The standard curve used to calculate the lignin levels from the absorption values of the lignin extractions is shown in Figure 2.4. The levels of lignin present in the extracts made from the leaves of greenhouse grown transgenic plants as well as wild type sugarcane are given in Table 2.3 and graphically represented in Figure 2.5. A two tailed t-test showed that there was no significant difference in the lignin levels between the transgenic sugarcane lines and the wild type NCo310 line. The lignin levels in the seventh and thirteenth internodes of field grown transgenic lines 1, 7, 10, 11 and 12, as well as wild type NCo310 control lines are represented in Figure 2.6 and the values shown in Table 2.4. Only transgenic sugarcane lines 10 and 12 showed lignin levels that are significantly lower than those of the wild type NCo310 line (p value lower than 0.05) in the seventh internode. Interestingly, the transgenic lines 1, 7, 10, 11 and 12 showed a significant increase in lignin levels over the

wild type NCo310 sugarcane (p values lower than 0.05) in extracts made from the thirteenth internode.

2.3.3 Assessing 4CL Activity in Transgenic Lines

The BSA standard curve (Figure 2.7) was used in order to calculate the protein concentrations of the crude extracts in the 11 transgenic sugarcane lines and the control NCo310 line and is shown in Table 2.5. The standard curve to determine p-coumaric acid concentration at 310 nm to assess 4CL enzyme activity is shown in Figure 2.8. The change in OD_{310} over 30 minutes was used to calculate the rate of p-coumaric acid conversion. The 4CL activity for each transgenic line as well as the control NCo310 line is shown in Figure 2.9 and the values given in Table 2.6. Results show that the enzyme activity for all of the transgenic lines hover within 2 percent of the activity of the wild type NCo310 line.

2.3.4 PCR Analysis of 4CL Transcription Levels in Transgenic Sugarcane

RNA samples were extracted successfully from the young leaf samples of each of the eleven transgenic lines as well as the control NCo310 line (Figure 2.10) and used to generate cDNA. PCR amplification revealed that the target fragment was expressing in all of the transgenic lines as well as wild type NCo310 (Figure 2.11), confirming that the targeted 4CL gene was still expressing in all of the transgenic lines.

2.4 Discussion

Extensive analysis have been done to determine if transgenic sugarcane containing a hairpin construct targeted to silence the 4CL gene in the monolignol biosynthetic pathway has had any effect on lignin levels. Although it was proven that the hairpin construct was successfully integrated into the DNA of the transgenic sugarcane by PCR verification, initial experimentation on lignin levels in the leaves of transgenic sugarcane showed that there was no significant decrease in lignin levels. Further experimentation on tissue samples derived from stalks from field grown transgenic lines confirmed that there was no decrease in lignin. In fact, the lignin levels seemed to have increased in the thirteenth internode of four of the tested transgenic sugarcane lines. An enzyme assay performed on the youngest leaves of the transgenic sugarcane revealed that 4CL activity in the

transgenic sugarcane lines was comparable to that of wild type sugarcane. PCR analysis of RNA extracted from the same tissues confirmed that the targeted gene encoding 4CL was still expressing in these tissues.

Although targeted silencing of the 4CL gene has led to the sometimes dramatic decrease in the lignin levels of other plant species (Hu et al., 1999) it has not been successful in this study. It has been proven that this hairpin construct targeted at 4CL has not led to the down-regulation of the 4CL enzyme and consequently not reduced the total lignin levels present in the transgenic sugarcane. In a study conducted by Baucher et al. (1999) the CAD gene in alfalfa was targeted in an attempt to improve the digestibility of alfalfa. The transgenic alfalfa did not show a change in lignin levels but did have an altered monolignol ratio. As the aim of this study was to decrease overall lignin content, the monolignol ratios were not investigated and no comment can be made as to the possibility of changes in the monolignol composition of the transgenic sugarcane. It does seem, however, that the target enzyme was unaffected, and any subsequent change in lignin quality or composition is unlikely. The increase in stalk lignin of some of the transgenic lines is also an interesting phenomenon and the possible mechanisms behind this may be a topic for investigating in a further study.

Future work on decreasing lignin levels in sugarcane may include targeting the 4CL gene for silencing with a different fragment or complete antisense expression of the gene. Complete antisense suppression of the 4CL gene has proven extremely successful in other silencing studies (Hu et al., 1999; Li et al., 2002). Several other genes in the lignin biosynthetic pathway have been successful in down-regulating lignin production. Silencing peroxidases specific to the lignin biosynthetic pathway, involved in the final steps of lignin synthesis, have proven successful in decreasing lignification of the plant cell wall. In a study by Blee et al. (2003) a peroxidase isoenzyme was down-regulated by antisense inhibition, the resulting transgenic tobacco plants yielded lignin reductions of 40 to 50% below that of wild type plants without displaying any phenotypic difference. Peroxidases are but one of many enzymes in the lignin biosynthetic pathway that have been successfully down-regulated. Any of these genes may be targeted for, either singularly or in combination with 4CL in an attempt to decrease the lignin content of sugarcane to improve cellulose digestibility for the purposes of ethanol biofuel production.

3. Expressing *Ciona savignyi* Cellulose Synthase in Yeast

3.1 Introduction

Ciona savignyi belongs to the ascidian class of the urochordate subphylum and produces cellulose in its epidermis to form the tough outer coat or tunic. Like another member of the ascidian class, *Ciona intestinales*' cellulose producing ability has also been researched (Matthysse et al., 2004). A functional gene was found and the further research by Matthysse et al. (2005) showed that the cellulose synthase gene from *C. savignyi* could be functionally expressed in *Agrobacterium tumefaciens.* In *celA* and *chvB* cellulose deficient *A. tumefaciens* mutants, root colonization and biofilm production were negatively impacted. The over-expression of the ascidian cellulose synthase gene in the mutants restored cellulose production in *celA* and *chvB* mutants and could partially restore biofilm production in the latter. This proves that transgenic expression of this gene is possible.

The source of this cellulose synthse is unique therefore, it is possible that the cellulose produced might have novel traits that could be of use for biofuel or biopolymer production. In a previous study the introduction and expression of the *C. savignyi* cellulose synthase (CsCeS) gene (GenBank accession no. AY504665) into *Saccharomyces cerevisiae* was investigated. The present study was undertaken to determine if this cellulose synthase gene could be functionally expressed in a novel eukaryotic system. A *S. cerevisiae* laboratory strain was transformed successfully with a constitutive expression vector containing the CsCeS gene, pYCeS2007 (Figure 3.1). It was shown that the CsCeS gene could be functionally expressed in yeast by immunoblot testing against cellulose (Figure 3.2), but that cellulose production had dramatic side effects. The transgenic yeast exhibited extremely retarded growth patterns with very slow growth on plates and little or no growth beyond the first striping on new medium. Two possible theories for this phenomenon were proposed, namely that either cellulose production imposes metabolic stress on normal cell functions, or that extracellular cellulose somehow physically restricts the cell growth and division.

Thus, the objectives of this study were: to further investigate the effects of the expression of *C. savignyi* cellulose synthase in transgenic yeast lines; to examine enzyme activity, cellulose production and transgenic cellulose characteristics; and, finally, to investigate the effect of the gene expression had on cellular growth and development to determine the mechanism of the metabolic control. To this end both ubiquitous and inducible promoters together with cellulose synthase inhibiting substances (Isoxaben) were employed to control cellulose production.

3.2 Materials and Methods

3.2.1 Wild Type and Transgenic Yeast Strains used for Experimentation

Three *S. cerevisiae* yeast laboratory strains were used for transformation experiments namely: BY4247 (selectable for L-histidine, L-leucine, L-lysine and uracil), YHUM and ∑1278 (both selectable for L-histidine, L-leucine, L-tryptophan and uracil).

During previous work the pYCeS2007 expression construct was created, which allows for the constitutive expression of the *C. savignyi* cellulose synthase gene courtesy of *PGK* promoter and allows for selection by complementing uracil deficiency in yeast and ampicillin selection in bacteria. For initial experimentation, transgenic YHUM *S. cerevisiae* lines were created. Transgenic lines were selectable on minimal media consisting of Yeast Nitrogen Base (YNB; 6.7 g/l) L-histidine, L-leucine and L-tryptophan at the described concentrations (Lundblad and Struhl 2003) and glucose (20 g/l).

For further experimentation, the BY4247 and ∑1278 *S. cerevisiae* yeast stains were transformed with the pYCeS2007 construct using a lithium acetate based chemical transformation method (Lundblad and Struhl 2003) and selected on minimal media containing YNB, glucose, L-histidine, L-leucine and L-lysine as described above (BY4247 strain).For the ∑1278 strain, L-lysine was replaced with L-tryptophan. Control plates contained YNB, glucose, and the four required amino acids, and uracil at the prescribed concentration (Lundblad and Struhl 2003).

3.2.2 Growth Curves

Growth curves were determined by inoculating 5 ml YPD medium (10 g/l yeast extract; 20 g/l bacterial peptone; 20 g/l glucose) with a single colony and allowed to grow overnight at

28℃. After the 5 ml culture reached an OD_{600} of 0.5 it was added to 200 ml YPD in an Erlenmeyer flask and grown at 28℃ with shaking. Two hours after inoculation, a 500 ul sample was removed every 60 min and measured spectrophotometrically until the readings became stationary.

3.2.3 Electron Microscopy Analysis of Transgenic Yeast

For electron microscope analysis, a three day old single transgenic ∑1278 colony was removed from a selective media plate and suspended in 5 ml liquid YPD medium and incubated with shaking at room temperature overnight. For a control sample a similar sized three day old wild type ∑1278 colony was removed from a control plate. The samples were sent to the Electron Microscope Unit at the University of Cape Town for scanning electron microscope (SEM) and transmission electron microscope (TEM) analysis.

3.2.4 Inhibition of Cellulose Synthase with Isoxaben

For this portion of the study transgenic and control yeast lines were grown on YPD media containing Isoxaben. A 10 mM Isoxaben stock solution was prepared by dissolving Isoxaben (Sigma-Aldrich) in water containing Tween 20. The stock solution was diluted in YPD media to a final concentration of 20 µM, 10 µM and 2 µM respectively. Wild type ∑1278 *S. cerevisiae* and transgenic ∑1278 *S. cerevisiae* containing the pYCeS2007 expression vector were grown on plates at 28℃ for three days and growth monitored.

3.2.5 Constructing an Inducible Vector for Expressing CsCeS in Yeast

A yeast specific inducible expression system, the pYES2 expression vector from Invitrogen was chosen for this part of the study. The pYES2 expression system allows for inducible expression of the inserted gene by including galactose in the media as the carbon source. This expression is controlled by the *GAL1* promoter. The plasmid allows for ampicillin resistance in bacteria and uracil deficiency selection in yeast by way of the *URA3* gene.

Two strategies were used to clone the *C. savignyi* cellulose synthase gene into the pYES2 expression system.

First, the *C. savignyi* cellulose synthase gene was restricted from the source plasmid with *Eco* RI (Fermentas) and separated by gel electrophoresis on a 0.8% agarose gel. The

separated DNA fragment was excised and purified from the gel using the Fermentas GeneJET™ Gel Extraction Kit. The pYES2 vector was prepared by opening the multiple cloning site with *Eco* RI (Fermentas), separating on an agarose gel and also purified with the Fermentas GeneJET™ Gel Extraction Kit. The purified vector was thereafter treated with Shrimp Alkaline Phosphatase (SAP; Promega). The cellulose synthase gene DNA fragment was then ligated into the opened and dephosphorylated pYES2 vector using T4 DNA Ligase (Fermentas).

Secondly, the target gene was amplified with PCR from the source plasmid with *Pfu* polymerase (Fermentas) and KAPA HiFi polymerase (KAPA Biosystems) using primers flanking the start codon and the end terminus and introducing *Eco* RI and *Not* I restriction sites to the start and end respectively (Table 3.1). The amplified PCR product was cloned into pJET using the Fermentas CloneJET™ PCR Cloning Kit and transformed into *E. coli* using a heat shock transformation protocol and was plated out on LB agarose plates containing 50 µg/ml Ampicillin and allowed to grow overnight at 37℃. Several colonies were selected and overnight cultures prepared in 5 ml LB medium containing 50 µg/ml Ampicillin. Plasmid extractions were prepared using the Fermentas GeneJET™ Plasmid Miniprep Kit. The plasmid preparations were subjected to restriction digest (*Eco* RI and *Not* I; Fermentas) and evaluated by electrophoresis on a 0.8% agarose gel. Lines were identified that contained the target gene and the plasmid digests were restricted, and the gene separated and isolated from an agarose gel. The isolated, restricted gene was then ligated into pYES2 opened with *Eco* RI and *Not* I as described above.

The pYES2 – CsCeS ligation mixtures were used to transform DH5α *E. coli* cells. Two types of transformation protocols, heat shock and electroporation, were followed. For electroporation, the Bio-Rad Gene Pulser Xcell™ Electroporation System was used and electrocompotent DH5α was prepared according to the manufacturer's recommendation, frozen and stored at -80℃. Electroporation transfo rmation mixtures were prepared with 0.5 µl ligation mixtures and 20 µl electrocompotent cells and electroporated in 0.2 mm Bio-Rad Electroporation cuvettes. Transformation mixture was plated out on LB agarose plates containing 50 µg/ml Ampicillin and grown overnight at 37℃.

Putative transformed colonies were selected and characterized by two methods: colony PCR targeting the gene of interest and restriction digests of plasmid preparations. For colony PCR characterizations two sets primers were designed: one set that would target an internal fragment (499 bp) of the *C. savignyi* cellulose synthase gene and the other to determine orientation (Table 3.1 and 3.2). Of the latter primer pair the forward primer anneals to the T7 site in the promoter region of the plasmid and the reverse primer anneals 300 base pairs (bp) down-stream of the start codon and amplifies a 380 bp fragment if the gene is in the correct orientation.

Restriction digests were performed on plasmid preparations from 5 ml overnight cultures. To determine the inclusion and orientation of the gene two digests were performed. First, a *Bam* HI (Fermentas) was performed to cut the plasmid once, and evaluated by electrophoresis on a 0.8% agarose gel to determine overall plasmid size. The lines that showed the correct plasmid size (about 10.5 kb) could then subjected to a *Bgl* I (Fermentas) digest to confirm orientation in the plasmid. Plasmids that contain the CsCeS gene in the sense orientation should yield two fragments of 7.7 kb and 2.6 kb. If the CsCeS gene was present in the antisense orientation the *Bgl* I digest would yield fragments of 5.4 kb and 4.9 kb.

3.3 Results

3.3.1 Wild Type and Transgenic Yeast Strains used for Experimentation

All three yeast strains were transformed successfully with the pYCeS2007 expression vector and propagated on suitable media. The transgenic ∑1278 yeast line proved marginally easier to propagate on solid media and was subsequently used for electron microscopy analysis and testing for cellulose synthase inhibition by Isoxaben treatment.

3.3.2 Growth Curves

The yeast cultures were monitored for a 24 hour period after inoculation to measure growth. The wild type yeast strains entered log stage growth within 7.5 to 8 hours of inoculation and reached the stationary phase within 14 hours of inoculation. The transgenic yeast lines showed no real growth beyond the initial inoculation levels over the

entire period, even when additional glucose was added to the cultures. All measured growth curves are displayed in Figure 3.3.

3.3.3 Electron Microscopic Analysis of Transgenic Yeast

The results of the electron microscope analysis are shown in Figures 3.4 and 3.5. SEM analysis revealed no clear difference in size, form or outer morphology of the transgenic and wild type ∑1278 *S. cerevisiae* cultures (Figures 3.4A and 3.4B). Further analysis of yeast cross sections on the TEM revealed no obvious differences in the cell wall morphology between the transgenic and wild type yeast lines (Figures 3.5A and 3.5B).

3.3.4 Inhibition of Cellulose Synthase with Isoxaben

Wild type and transgenic ∑1278 *S. cerevisiae* were allowed to grow on YPD plates with between 2 and 20 µM Isoxaben concentrations. The presence of Isoxaben in the media appeared to have no effect on the growth pattern of the transgenic yeast but neither did it have a negative effect on the wild type yeast growth.

3.3.5 Constructing an Inducible Vector for Expressing CsCeS in Yeast

The *C. savignyi* gene was successfully isolated from the donor plasmid through digestion with *Eco* RI and the pYES2 vector was digested and dephosphorylated (Figure 3.6). Ligation of the restricted cellulose synthase gene into the *Eco* RI site of the pYES2 vector proved difficult, however, with several ligation reaction ratios and reaction sizes being tested. Successful ligation of the CsCeS gene into pYES2 has not yet been achieved. The second strategy for cloning the CsCeS gene into pYES2 relied on introducing an *Eco* RI restriction site and a *Not* I restriction site at the 5' and 3' ends of the gene respectively by PCR amplification with appropriate primers. Attempts to amplify the gene from the plasmid has also proved difficult, several annealing temperatures and salt concentration combinations were tested with both polymerases. The results of the amplification attempts range from no amplification to nonspecific amplification. Amplification at 68.3℃ with KAPA HiFi polymerase resulted in a DNA fragment of the correct size (Figure 3.7) and was used for cloning into the CloneJET cloning system and transformed. Several colonies were tested and the positive colonies were selected for plasmid preparation. The preparations were used for restriction digests and several attempts at directionally cloning the CsCeS

gene into the pYES2 vector using the *Eco* RI and *Not* I restriction sites was unsuccessful so far.

3.4 Discussion

Although the CsCeS gene has previously been functionally expressed in yeast, it remains difficult to study. Both of the additional *S. cerevisiae* yeast strains that were used for transformation with the pYCeS2007 expression vector exhibited the same extremely retarded growth patterns that were observed during a previous study (Hörstmann and Kossmann 2007). In all three strains, the confirmed transgenic lines showed close to no growth in liquid cultures when compared to their wild type counterparts. The slow growth was so severe that no experimentation could be performed to determine the effect that cellulose production had on the yeast strains' metabolism. Analysis by both scanning electron microscopy and transmission electron microscopy failed to reveal any obvious differences in the internal or exterior structures between the wild type and transgenic yeast lines. Thus no explanation for the slow growth patterns could be gained from the electron microscope results. Subsequently the aim was to control or inhibit cellulose synthesis and the expression of the cellulose synthase to allow the yeast to grow enough to allow for proper metabolic testing.

Attempts to control cellulose production in the transgenic yeast lines by introducing Isoxaben into the medium have however also proven unsuccessful. Isoxaben is used in weed killers and is believed to interfere with cellulose synthesis by inhibiting cellulose synthase in higher plants (Scheible et al., 2001). The presence of the isoxaben in the medium did not visibly improve the growth of the transgenic yeast lines and it can thus be concluded that there is no disruption of the growth limiting effects perceived in the cellulose producing transgenic lines. The final experiment that aimed to counter the negative effects of cellulose production in the yeast by inducing cellulose synthase expression only after significant growth of the culture was achieved, could not be concluded. Cloning the *C. savignyi* cellulose synthase gene into the pYES2 vector proved extremely difficult and numerous attempts with several strategies had proven unsuccessful at the conclusion of this study. Efforts to clone the gene into the pYES2 expression system are currently ongoing. Future work, once the successful cloning has been achieved, will

include the metabolic assessment of the cellulose producing yeast. The transgenic yeast can then be subjected to protein and metabolic analysis and the changes determined and investigated. Determining why the expression of this gene affects the growth of the yeast in such a profound way may shed a little light on some of the regulatory mechanisms present in yeast metabolism. This knowledge in turn can be applied to a variety of systems for industrial and scientific use.

Future work will also include expressing the CsCeS gene in higher plants and examining the effect this has on plant growth, biomass production and biomass properties with regard to digestibility. Investigating the effects of expressing a novel cellulose synthase gene in plants could prove important for the development of biofuel production systems. Expressing the gene could have a number of effects on biomass production. It could either lead to an overall increase in cellulosic biomass, an improvement in the digestibility of the biomass, a combination of the latter two, or a completely novel effect.

4. General Discussion and Conclusion

4.1 Discussion and Conclusion

Biofuels are essential to developing a society independent of fossil fuels and will enable industry to be driven in a 'carbon neutral' fashion. To make this vision of the future a reality, systems need to be developed for the efficient production, processing and utilization of available, renewable resources. The focus of this study was to research cell wall manipulation in sugarcane and yeast for the purposes of contributing knowledge to the search for efficient biofuel production systems. The study consisted of two parts, firstly, accessing transgenic sugarcane for lowered lignin production, and secondly, evaluating cellulose production in transgenic yeast.

The evaluated transgenic sugarcane was modified to express a hairpin RNA loop, targeted to silence the *4CL* gene in an effort to lower the lignin content present in the cell walls. The plants were shown to contain the hairpin construct in their gDNA, but evaluation of plant tissues showed that it had no real effect on lowering the lignin levels, RNA transcription levels or the enzyme activities of the transgenic lines when compared to the wild type control sugarcane lines. The reasons for this lack of suppression are still being investigated and these results will determine the course of future work. The hairpin construct will be sequenced to determine if either a sequence error or an incorrect DNA fragment might have been cloned into the construct. Should this prove to be the case, it would explain the lack of silencing even in the presence of the transgenic hairpin construct.

Transgenic *Saccharomyces cerevisiae* yeast strains expressing the *Ciona savignyi* cellulose synthase gene exhibited altered growth patterns. The aim of this part of the study was to assess the mechanisms that resulted in this phenotype. Transgenic yeast lines ubiquitously expressing the CsCeS gene could not grow sufficiently to allow for metabolic analysis. To counteract the negative effect on growth resulting from cellulose production several strategies to inhibit cellulose production were tested. An attempt to inhibit cellulose production by including Isoxaben into the media was unsuccessful, and no improvement in the growth of transgenic yeast was perceived. The second strategy chosen to control cellulose production involved transforming the selected yeast strains with an inducible expression system. The expression of the CsCeS gene would then be induced after

sufficient growth of the transgenic culture had been achieved and would allow for the production of sufficient cell biomass to perform metabolic analysis. This could not be achieved due to the inability to incorporate the CsCeS gene into the inducible expression system. This process is continuing in an effort to find more results. Due to time constraints, and because it is a next step in this experimentation, the final results of this process cannot be reported in this manuscript.

4.2 Future Work

At the core of biofuel production is the conversion of cellulose derived from biomass to sugars that can be fermented to ethanol for fuel. Much more research can be done on modifying and increasing cellulose mass plant and commercial crops.

Also important, however, is to modify crops for increased biomass production and yield and to engineer cell wall structures so as to overcome the natural resistance to degradation. The complex cell wall structure resulting from entwined and cross linked lignin and hemicellulose fibers need to be modified in such a manner as to enable the efficient liberation of sugars from cellulose without affecting the structural integrity of the plants.

In a review by Demura and Ye (2010) the possibilities for increasing biomass production in plants by either manipulating and regulating developmental genes, or genes involved in hormone production, were discussed. An increase in overall plant biomass production should directly correlate to an increase in biomass available for fuel production. Recent studies into the signaling pathways that regulate plant development have yielded positive results. Research by Salehi et al. (2005) found that over-expression of the *FLOWERING LOCUS C* gene from Arabidopsis in tobacco, flowering could be delayed. The prolonged vegetative state of the transgenic tobacco plants resulted in both an increase in leaf size and an increased biomass production.

In Chapter 2 several enzymes that have been targeted for down-regulation in the lignin biosynthetic pathway to increase cell wall degradability are discussed. There are a number of other strategies that can be followed in an effort to improve the digestibility of the cell wall. Another group of plant cell wall polymers that have been targeted for down-regulation to improve degradation for increased biofuel production are xylans.

Xylans, like lignins, are polymers that form a substantial part of the secondary cell walls as glucuronoxylans in woody plants and arabinoxylans in grasses (Taiz and Zeiger 2002, York and O'Neill 2008). Although it is known that xylans affect the digestibility of cellulose from cell walls, much of the biosynthetic pathway remains to be fully elucidated. Recent progress in determining how xylans are produced has made it possible to investigate the possibilities of reducing xylan levels to improve digestibility of cell wall biomass (York and O'Neill 2008).

A recent study by Lee et al. (2009) targeted a glycosyl transferase (PoGT47C) essential for glucuronoxylan production in poplars for down-regulation. The successful down-regulation of this enzyme resulted in substantial increases in the digestibility by cellulase in some of the transgenic tree lines. Digestibility is thus aided substantially by the lowered production of xylan and the subjective effect of lowered cross linking in the cell wall structures. This increased digestibility, like that of plants with lowered lignin levels, translates directly to increased sugar liberation and ethanol production potential.

An additional strategy for improving cell wall degradability targets the lignin cross linkages for degradation. In the cell walls of grasses ferulic acids are esterified to arabinoxylans and oxidatively coupled to lignin to form ferulate-polysaccharide-lignin complexes (Hatfield et al., 1999). These complexes form an extensive cross-linked network in the cell wall that inhibits cell wall degradation (Hatfield et al., 1999). Recently, a study investigated the effects of expressing a ferulic acid esterase from the fungus *Aspergillus niger* in *Festuca arundinacea*, a forage grass (Buanafina et al., 2008). The transgenic enzyme was targeted to the vacuole and released upon cell death. The results of this study showed an increase in the digestibility and a reduction of esterified phenolics present in the cell wall. The reported study was aimed at improving the forage quality of the grass, but the same principles apply for the production of biofuel and the technology can be applied to cereals for this purpose.

Finalizing the work on cellulose producing yeast should also yield interesting results. If the mechanisms that affected the transgenic yeasts' growth in such a substantial manner could be determined they might be of modified for use by science and industry. Also, if the mechanism could be counteracted or the inducible system allows for the sufficient

production of cellulose, the novel cellulose may be analysed to determine potential uses for biofuel and the possible expression in other biosystems such as crops.

4.3 Final Word

Previously, it has been stated that the sun produces more than enough energy to drive human activity (Somerville 2006a). Harnessing that energy should now be the main concern. Plants are far more efficient at capturing and storing solar energy than any artificial system of human design could be, and requires very little input to do this. Of the vast tonnage of agricultural biomass generated for food production very little eventually ends up in the mouths of people and animals. Converting the balance of that biomass to biofuels and biomaterials will enable us to realize a future industry that functions wholly within a closed carbon cycle and contribute substantially to the longevity of humankind's existence on this planet.

Producing bioproducts such as biofuels and biomaterials from agricultural biomass without impacting on food production will require innovative solutions. The land acreage available for agriculture in most first world countries is decreasing at an alarming rate, thus the question of allocating agricultural surface to both food production *and* fuel production separately is not an option. While third world countries (especially in Africa) have a lot to offer in this regard, they have their own hurdles. Apart from the political hurdles such as unstable governments, there is a substantial lack of infrastructure to support commercial agriculture in an environment that is harsh and generally hostile to commercial crops. The potential of Africa to become the world food source also needs to be balanced with the preservation of wilderness and wild live, as Africa largely remains one of the few havens were wildlife can roam freely, live and breed (mostly) unrestricted by human activity.

Thus the optimal use of the limited available resources will rely on designer crops tailored for optimal crop yield, disease resistance, stress tolerance and fuel production potential with minimal input. For most scientists this is a necessary and tangible future. Perhaps the greatest obstacle in achieving this is the refusal of the general consuming public to accept genetically modified (read improved) crops. The refusal to accept GM crops can also be interpreted as a refusal to accept the need for them when one considers the greater scope of the situation concerning society, agriculture and the environment. The need to develop

crops that can grow more efficiently and that produce biomass that can be used more efficiently should also be kept in mind by scientists. Wyman (2007) highlighted the subject of focusing the limited funds and resources at the disposal of researchers on primarily developing biofuel and bioproduction systems in a manner that results in viable, valuable, efficient and commercially applicable technologies. This will be achieved mainly by developing technologies for altering the way in which plant cell walls are synthesized to ensure the optimum efficiency of carbon liberation for conversion to ethanol biofuel.

References

Abramson M, Shoseyov O, Shani Z (2010) Plant cell wall reconstruction towards improved lignocellulosic production and processability. Plant Science 178: 61 -72

Anterola AM, Lewis NG (2002) Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry 6: 221 – 294

Arioli T, Peng L, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Höfte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Williamson RE (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science. 279:717-720

Baucher M, Bernard-Vailhé MA, Chabbert B, Besle J-M, Opsomer C, Van Montagu M, Botterman J (1999) Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the effect on lignin composiyion and digestibility. Plant Molecular Biology 39: 437 - 447

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annual Revew of Plant Biology 54: 519 - 546

Boudet AM, Kajita S, Grima-Pettenati J, Goffner D (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends in Plant Science 8: 576 – 581

Blaschke L, Legrand M, Mai C, Polle A (2003) Ligninfication and structural biomass production in tobacco with suppressed caffeic/5-hydroxy ferulic acid-*O*-methyl transferase activity under ambient and elevated CO_2 concentrations. Physiologia Plantarum 121: 75 - 83

Blee KA, Chai JW, O'Connell AP, Schuch W, Lewis NG, Bolwell GP (2003) A lignin-specific peroxidase in tobacco whose antisense suppression leads to vascular tissue modification. Phytochemistry 64: 163 – 176

Brinkmann K, Blaschke L, Polle A (2002) Comparison of different methods for lignin determination as a basis for calibration of near-infrared reflective spectroscopy and implications of lignoproteins. Journal of Chemical Ecology 28: 2483 – 2501

Buanafina MM de O, Langdon T, Hauck B, Dalton S, Morris P (2008) Expression of fungal ferulic acid esterase increases cell wall digestability of tall fescue (*Festuca arundinacea*). Plant Biotechnology Journal 6: 264 – 280

Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM (2001) Strong decrease in liginin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. The Plant Journal 28: 257 – 270

Cho KM, Yoo YJ, Kang HS (1998) δ-Integration of endo/exo-glucanase and β-glucosidase genes into the yeast chromosomes for direct conversion of cellulose to ethanol. Enzyme and Microbial Technology 25: 23 – 30

Demura T, Ye Z-H (2010) Regulation of plant biomass production. Current Opinion in Plant Biology 13: 299 - 304

Festucci-Buselli RA, Otoni WC, Joshi CP (2007) Structure, organization, and functions of cellulose synthase complexes in higher plants. Brazilian Journal of Plant Physiology 19: 1 - 13

Fujita Y, Takahashi S, Ueda M, Tanaka A, Okada H, Morikawa Y, Kawaguchi T, Arai M, Fukuda H, Kondo A (2002) Direct and efficient production of ethanol from cellulosic material with yeast strain displaying cellulolytic enzymes. Applied and Environmental Microbiology 68: 5136 – 5141

Hamelinck CN, van Hooijdonk G, Faaij APC (2005) Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy 28: 384 – 410

Hatfield RD, Ralph J, Grabber JH (1999) Cell wall cross-linking by ferulates and diferulates in grasses. Journal of the Science of Food and Agriculture 79: 403 – 407

Hirose E, Kimura S, Itoh T, Nishikawa J (1999) Tunic of pyrosomas, doliolids and salps (Thaliacea, Urochordata): Morphology and cellulosic components. The Biological Bulletin 196: 113 – 120

Hörstmann CU, Kossmann JM (2007) Investigating the potential of producing novel biopolymers in different expression systems. BScHons Thesis. University of Stellenbosch, Stellenbosch

Hu W-J, Harding SA, Lung J, Popko Jl Ralph J Stokke DD, Tsai CJ, Chaing VL (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. Nature Biotechnology 17: 808 – 812

Humphreys JM, Chapple C (2002) Rewriting the lignin roadmap. Current Opinion in Plant Biology 5: 224 – 229

Kajita S, Hishiyama S, Tomimura Y, Katayama Y, Omori S (1997) Structural characterization of modified lignin in transgenic Tobacco Plants in which the activity of 4-courmarate:coenzyme A ligase is depressed. Plant Physiology 114: 871 – 879

Kimura S, Itoh T (1996) New cellulose synthesizing complexes (terminal complexes) involved in animal cellulose biosynthesis in the tunicate *Metandrocarpa uedai*. Protoplasma 194: 151 – 163

Kimura S, Ohshima C, Hirose E, Nishikawa J, Itoh T (2001) Cellulose in the house of the appendicularian *Oikopleura rufescens*. Protoplasma 216: 71 – 74

Knobloch K-H, Hahlbrock K (1977) 4-Coumarate:CoA ligase from cell suspension cultures of *Petroselinum hortense* Hoffm. Archives of Biochemistry and Biophysics 184: 237 – 248

Lee D, Meyer K, Chapple C, Douglas CJ (1997) Antisense suppression of 4-coumarate:Coenzyme A ligase activity in Arabidopsis leads to altered lignin subunit composition. The Plant Cell 9: 1985 – 1998

Lee C, Teng Q, Huang W, Zhong R, Ye Z-H (2009) Down-regulation of PoGT47C expression in poplar results in a reduced glucuronoxyan content and an increased wood digestibility by cellulase. Plant and Cell Physiology 50: 1075 – 1089

Li L, Zhou Y, Cheng X, Sun j, Marita JM, Ralph J, Chiang VL (2002) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. Proceedings of the National Academy of Sciences 100: 4939 – 4944

Lundblad V, Struhl K (2003) Unit 13: Yeast. *In* Current Protocols in Molecular Biology. John Wiley and Sons Inc. http://onlinelibrary.wiley.com.ez.sun.ac.za/doi/10.1002/0471142727.mb1300s92/full

Matthysse AG, Holmes KV, Gurlitz RHG (1981) Elaboration of cellulose fibril by *Agrobacterium tumefaciens* during attachment to carrot cells. Journal of Bacteriology 145: 583 – 595

Matthysse AG, Thomas DL, White AR (1995) Mechanism of cellulose synthesis in *Agrobacterium tumefaciens*. Journal of Bacteriology 177: 1076 – 1081

Matthysse AG, Deschet K, Williams M, Marry M, White AR, Smith WC (2004) A functional cellulose synthase from ascidian epidermis. Proceedings of the National Academy of Sciences 101: 986 - 991

Matthysse AG, Marry M, Krall L, Kaye M, Ramey BE, Fuqua C, White AR (2005) The effect of cellulose overproduction on binding and biofilm formation on roots by *Agrobacterium tumefaciens*. Molecular Plant - Microbe Interactions 18: 1002 – 1010

McCann MC, Carpita NC (2008) Designing the deconstruction of plant cell walls. Current Opinion in Plant Biology 11: 314 – 320

McGarvey P, Kaper JM (1991) A simple and rapid method for screening transgenic plants using PCR. Biotechniques 11: 425 – 432

Mutwil M, Debolt S, Persson S (2008) Cellulose synthesis: A complex complex. Current Opinion in Plant Biology 11: 252 – 257

Nakagaito AN, Iwamoto S, Yano H (2005) Bacterial cellulose: the ultimate nano-scalar cellulose morphology for the production of high strength composites. Applied Physics A 80: 93 – 97

Nakashima K, Yamada L, Satou Y, Azuma J-I, Satoh N (2004) The evolutionary origin of animal cellulose synthase. Development Genes and Evolution 214: 81 – 88

Pilate G, Guiney E, Holt K, Petit-Conil M, Lapierre C, Leplé J-C, Pollet B, Mila I, Webster EA, Marstorp HG, Hopkins DW, Jouanin L, Boerjan W, Schuch W, Cornu D, Halpin C (2002) Field and pulping performances of the transgenic trees with altered lignification. Nature Biotechnology 20: 607 – 612

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr., Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T (2006) The path forwards for biofuels and biomaterials. Science 311: 484 – 489

Ralph J, Lundquist K, Brunow G, Lu K, Kim H, Schatz PF, Marita JM, Hatfield RD, Ralph SA, Christensen JH, Boerjan W (2004) Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl propanoids. Phytochemistry Reviews 3: 29 – 60

Rånby BG (1952) Physico-chemical investigations on animal cellulose (Tunicin). Ark Kemi 4: 241 - 248

Reddy MSS, Chen F, Shadle G, Jackson L, Aljoe H, Dixon RA (2005) Targeted down-regulation for forage quality improvement in alfalfa (*Medicago sativa* L.). Proceedings of the National Academy of Sciences 102: 16573 – 16578

Reddy N, Yang Y (2005) Biofibers from agricultural byproducts for industrial applications. Trends in Biotechnology 23: 22 - 27

Salehi H, Ransom CB, Oraby HF, Seddighi Z, Sticklen MB (2005) Delay in flowering and increase in biomass of transgenic tobacco expressing the Arabidopsis floral repressor gene *FLOWERING LOCUS C*. Journal of Plant Physiology 162: 711 – 717

Scheible W-R, Eshed R, Richmond T, Delmer D, Somerville C (2001) Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis lxr1* mutants. Proceedings of the National Academy of Sciences 98: 10079 – 10084

Simmons BA, Loqué D, Ralph J (2010) Advances in modifying lignin for enhanced biofuel production. Current Opinion in Plant Biology 13: 313 – 320

Somerville C (2006a) The billion-ton biofuels vision. Science 312: 1277

Somerville C (2006b) Cellulose synthesis in higher plants. Annual Review of Cell and Developmental Biology 22: 53 – 78

Somerville C (2007) Biofuels. Current Biology 17: R115 – R119

Soltani BM, Ehlting J, Hamberger B, Douglas CJ (2006) Multiple *cis*-regulated elements regulate distinct and complex patterns of developmental and wound-induced expression of *Arabidopsis thaliana 4CL* gene family members. Planta 224: 1226 – 1238

Swissa M, Aloni Y, Weinhouse M, Benziman M (1980) Intermediary steps in *Acetobacter xylinum* cellulose synthesis: Studies with whole cells and cell-free preparations of the wild type and a celluloseless mutant. Journal of Bacteriology 143: 1142 – 1150

Taiz L, Zeiger E (2002) Plant Physiology, Ed 3rd, Sinauer Associates, Inc, Sunderland.

Takahashi J, Rudsander UJ, Hedenström M, Banasiak A, Harholt J Amelot N, Immerzeel P ,Ryden P, Endo S, Ibatullin FM, Brumer H, Del Campillo E, Master ER, Vibe Scheller H, Sundberg B, Teeri TT, Mellerowicz EJ (2009) *KORRIGAN1* and its Aspen homolog *PttCel9A1* decrease cellulose crystallinity in Arabidopsis stems. Plant and Cell Physiology 50: 1099 – 1115

Tsai C-J, Popko JL, Mielke MR, Hu W-J, Podila GK. Chiang VL (1998) Suppression of *O*-methyltransferase gene by homologous sense transgene in Quaking Aspen causes red-brown wood phenotypes. Plant Physiology 117: 101 – 112

Van Rensburg P, Van Zyl WH, Pretorius IS (1998) Engineering yeast for efficient cellulose degradation. Yeast 14: 67 – 76

Vanholme R, Morreel K,Ralph J, Boerjan W (2008) Lignin engineering. Current Opinion in Plant Biology 11: 278 – 285

Weng J-K, Li X, Bonawitz ND, Chappple C (2008) Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. Current Opinion in Biotechnology 19: 166 – 172

Wong HC, Fear AL, Calhoon RD, Eichinger GH, Mayer R, Amikam D, Benziman M, Gelfand DH, Meade JH, Emerick AW, Bruner R, Ben-Bassat A, Tal R (1990) Genetic organization of the cellulose synthase operon in Acetobacter xylinum. Proceedings of the National Academy of Sciences 87: 8130 – 8134

Woodward J (1991) Synergism in cellulase systems. Bioresource Technology 36: 67 – 75

Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. Trends in Biotechnology 25: 153 – 157

York WS, O'Neill MA (2008) Biochemical control of xylan biosynthesis – which end is up? Current Opinion in Plant Biology 11: 258 – 265

Figures

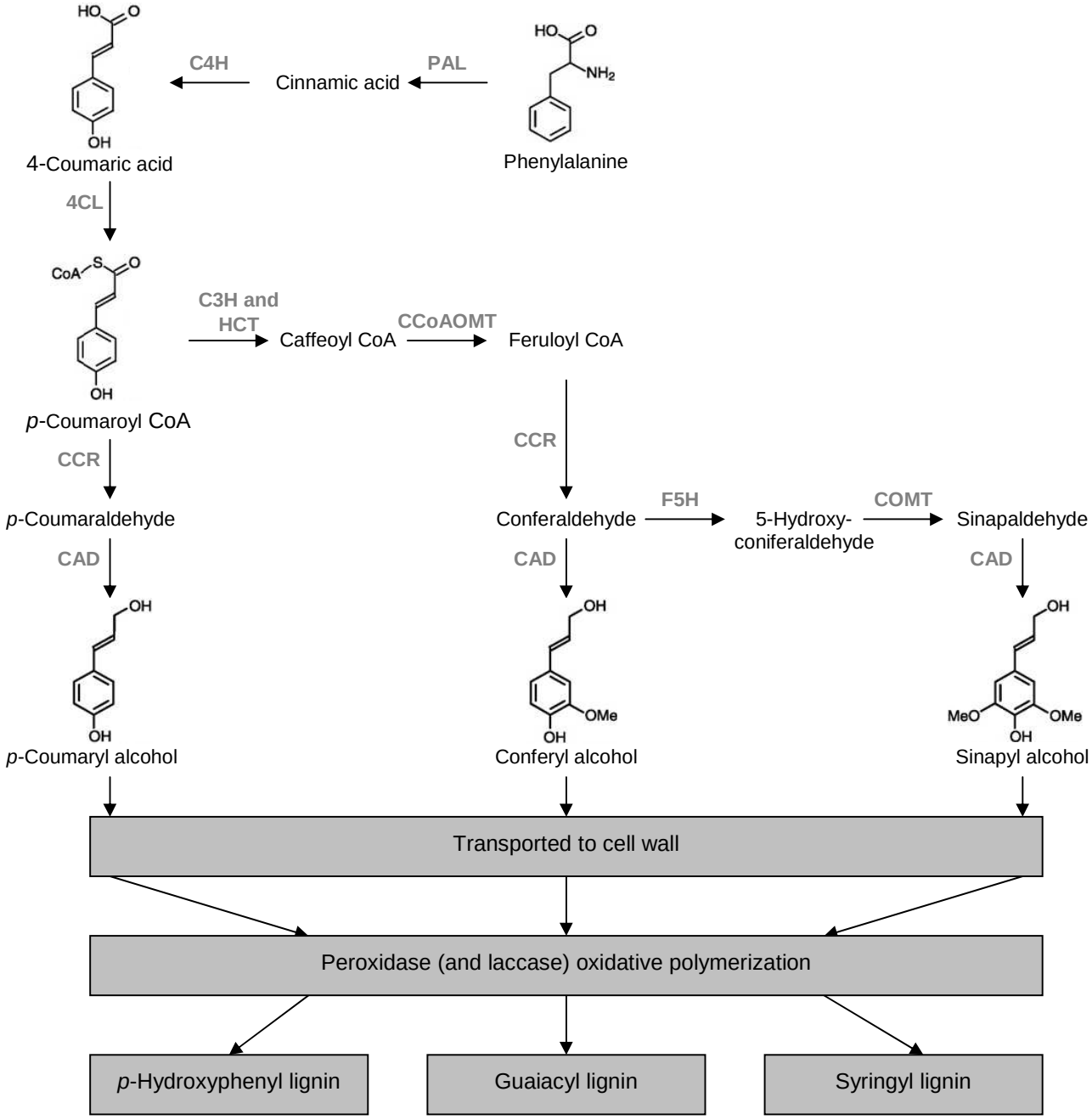

Figure 1 A simplified scheme of the most common lignin biosynthesis pathway in angiosperms. The implication of laccases in monolignol oxidation have yet to be elucidated (Boudet et al., 2003).

1 – 50	**GGCGAGAACCCGAACCTG**TACTTCAGCAAGGACGACGTGCTGCTGTGCCT
51 – 100	GCTGCCGCTGTTCCACATCTACTCGCTCAACTCGGTGCTGCTGGCGGGGC
101 – 150	TGCGCGCGGGCTCCACCATCGTGATCATGCGCAAGTTCGACCTGGGCGCG
151 – 200	CTGGTGGACCTGGTGCGCAAGCACGCCATCACCATCGCGCCCTTCGTGCC
201 – 250	GCCCATCGTGGTGGAGATCGCCAAGAGCCCCCGCGTGACCGCCGCCGACC
251 – 300	TCGCCTCCATCCGCATGGTCATGTCCGGCGCCGCGCCCATGGGCAAGGAG
301 – 350	CTCCAGGACRCCTTCATGACCAAGTCCCCAACGCCGTCCTCGGGCAGGGG
351 – 400	TACGGGATGACGGAGGCG**GGGCCC**GTGCTGGCGATGTGCCTGGCGTTCGC
401 – 450	CAAGGAGCCGTTCCAGGTCAAGTCCGGGTCGTGCGGCACCGTGGTGCGGA
451 – 500	ACGCGGAGCTGAAGATCGTCGACCCCGACACCGGCGCCGCCCTCGGCCGG
501 – 550	AACCAGCCCGGCGAGATCTGCATCCGCGGGGAGCAGATCATGAAAGGTTA
551 – 600	CCTGAACGACCCCGAGTCGACAAAGAACACCATCGACAAGGACGGCTGGC
601 – 650	TGCACACCGGCGACATCGGCTACGTCGACGACGACGACGAGATCTTCATC
651 – 700	GTCGACAGGCTCAAGGAGATCATCAAGTACAAGGGGTTCCAGGTGCCCCC
701 – 750	GGCGGAGCTCGAGGCGCTCCTCATCACGCACCCGGAGATCAAGGACGCCG
751 – 800	CCGTCGTGTCAATGAAGGATGATCTTGCTGGTGAAATCCCTGTCGCCTTC
801 – 850	ATCGTGCGGACCGAAGGCTCTGAAGTCACCGAGGATGAGATCAAGCAATT
851 – 900	TGTCGCCAAGGAGGTGGTTTTCTACAAGAAGGTACACAAGGTTTTCTTCA
901 – 950	CCGAATCCATCCCCAAGAACCCGTCCGGCAAGATCCT**AAGGAAGGACTTG**
951 - 957	**AGAGCCA**

Figure 2.1 Nucleotide sequences of the fragment amplified from sugarcane mRNA using 4CL2Fand 4CL1R primers.

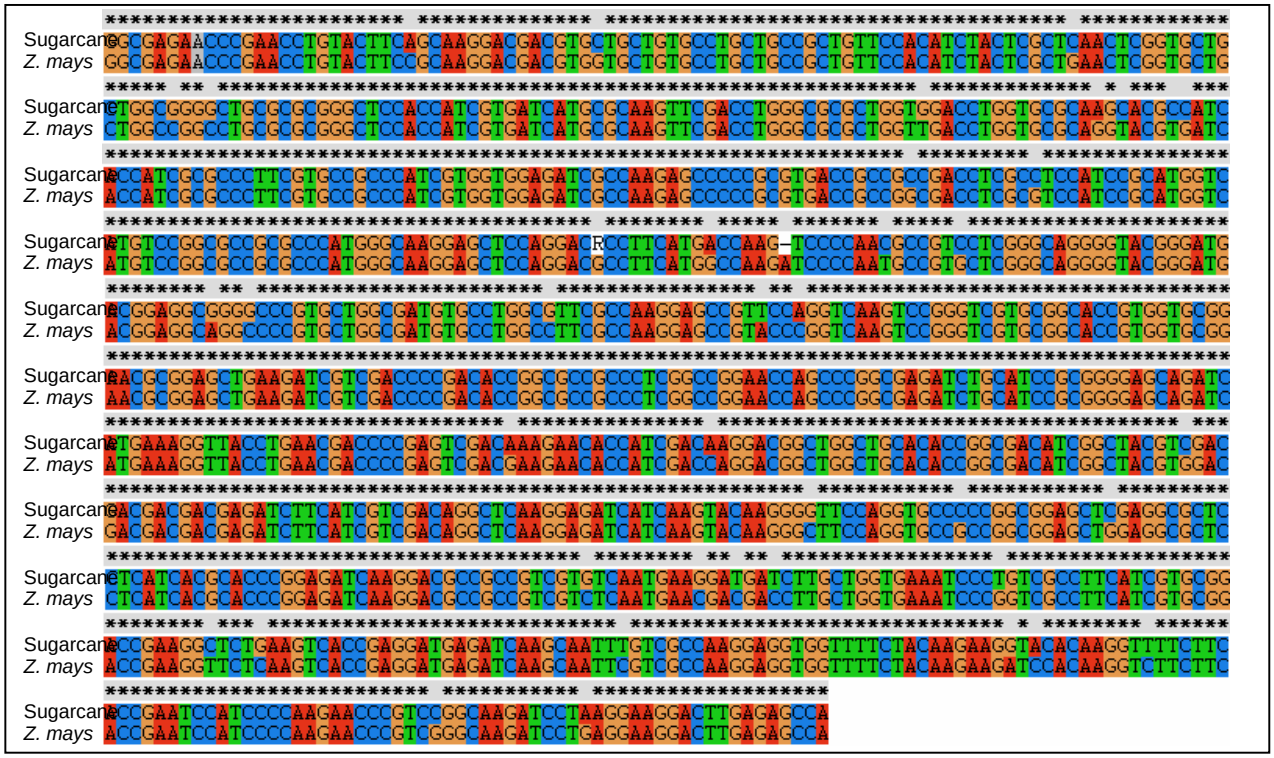

Figure 2.2 Comparison of putative 4CL fragment from sugarcane to that of Z. mays (GenBank accession no. AY566301). Complete amplified fragment (957 bp) aligned to Z. mays sequence shown from bp 688 to bp 1645.

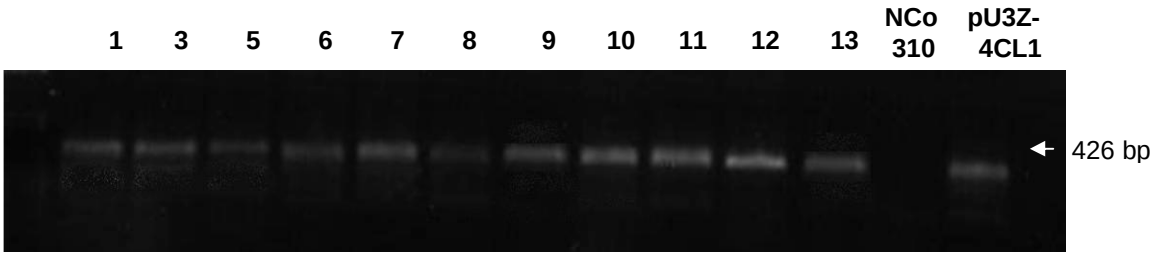

Figure 2.3 Confirmation of 4CL hairpin presence in sugarcane genome. PCR product, using the 4CL Int primer to amplify across the intron of the hairpin.

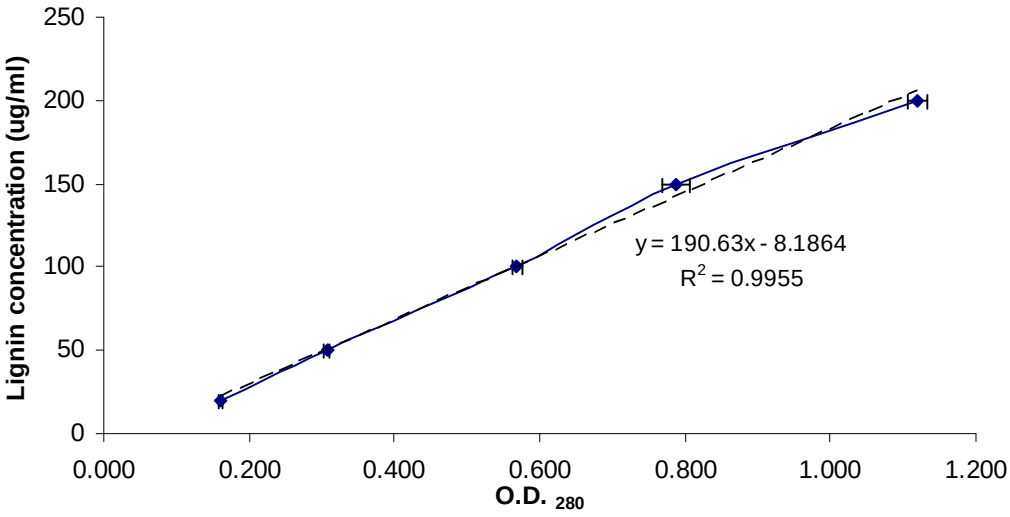

Figure 2.4 Lignin standard curve, determined at 280nm.

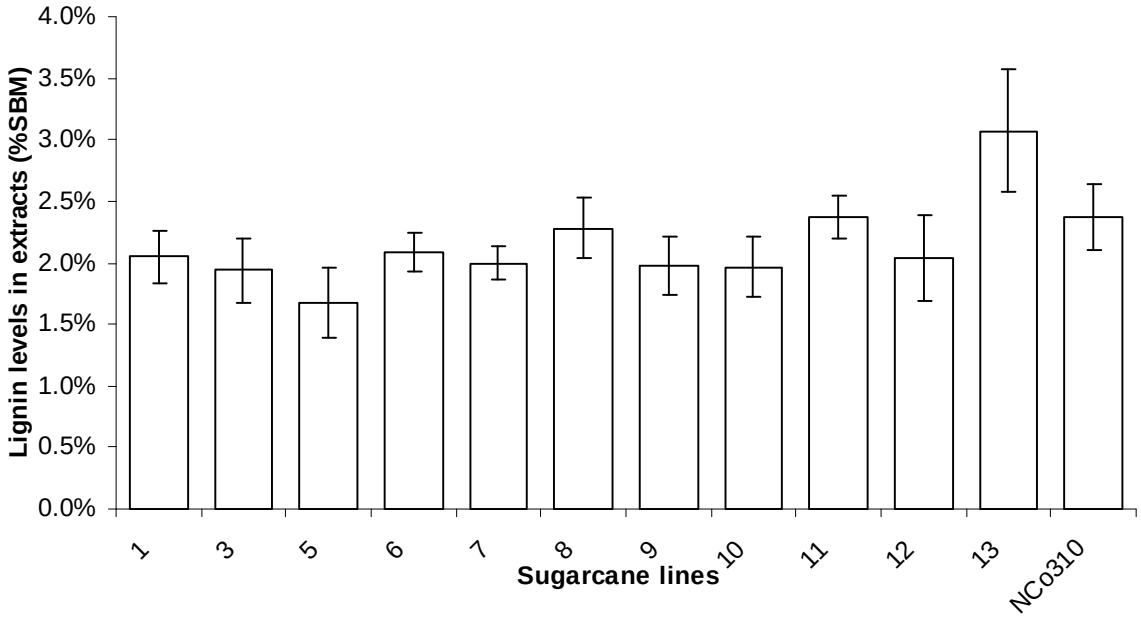

Figure 2.5 Lignin levels in the leaves of transgenic and WT (NCo310) sugarcane grown under glasshouse conditions.

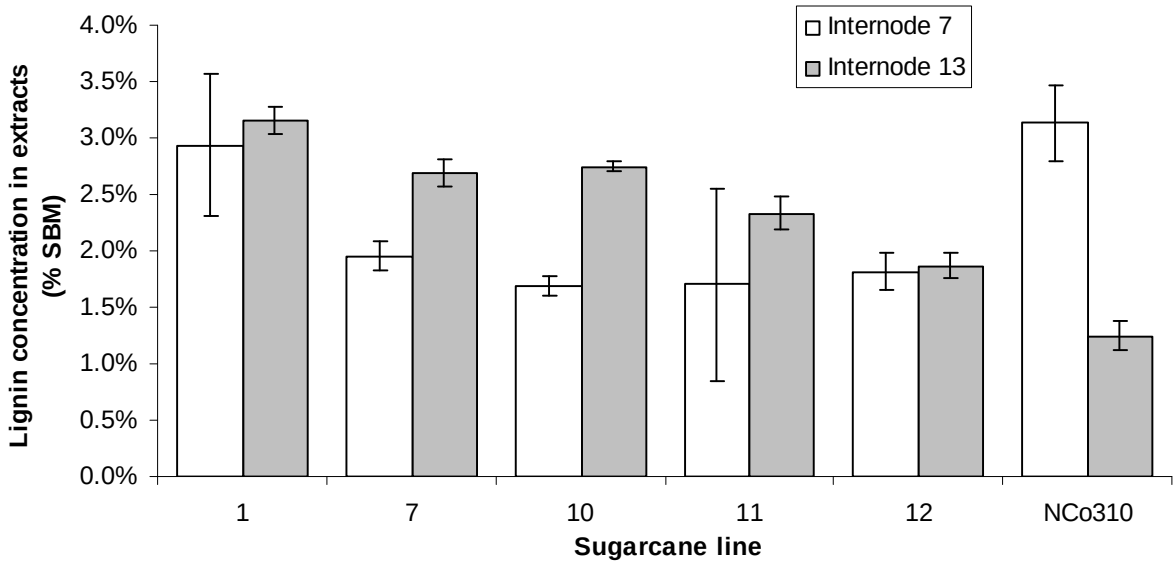

Figure 2.6 Lignin levels in the 7th and 13th internodes of transgenic and WT sugarcane grown under field trial conditions.

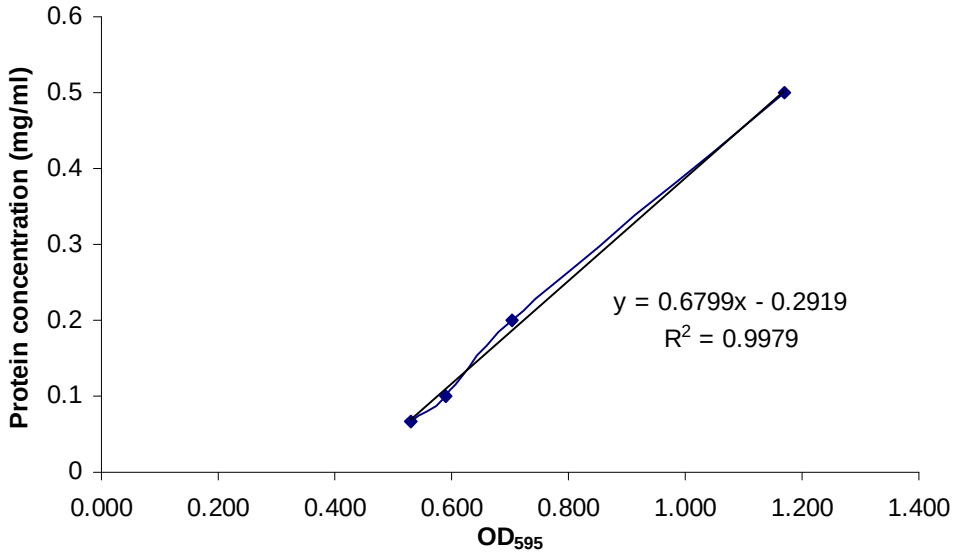

Figure 2.7 BSA standard curve.

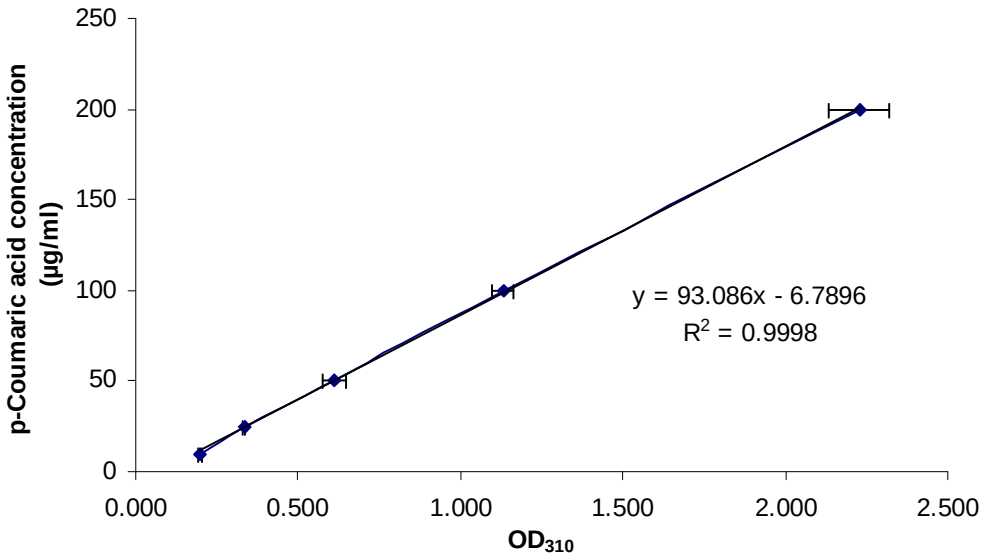

Figure 2.8 p-Coumaric acid standard curve.

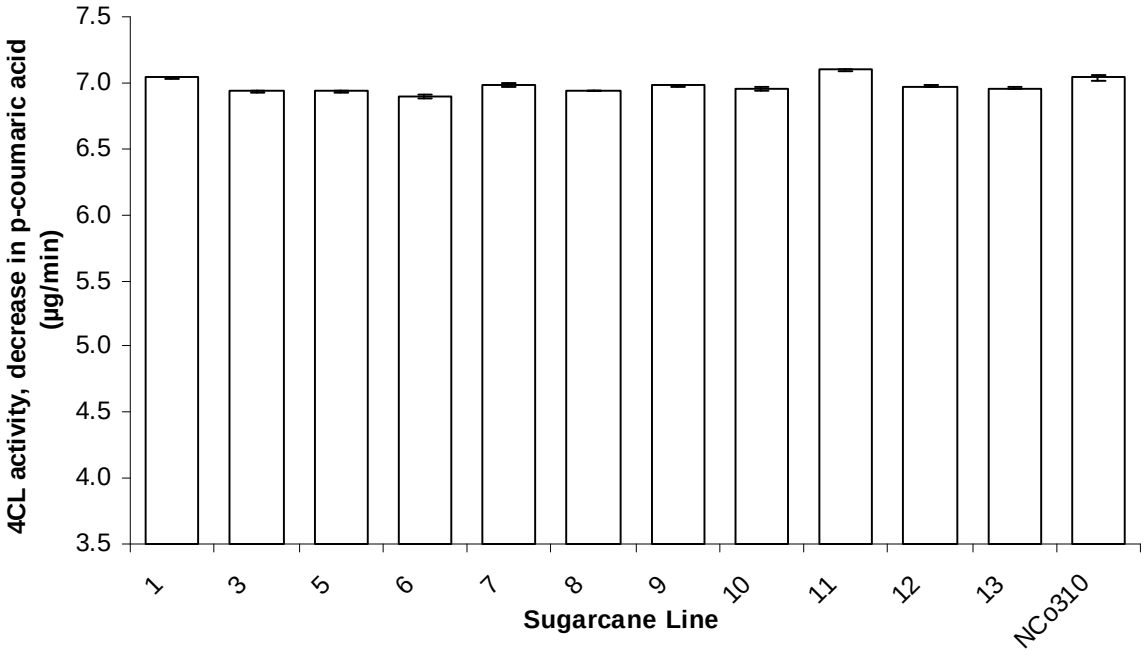

Figure 2.9 4CL activity measured in transgenic and WT sugarcane measured as a decrease in p-coumaric acid levels over time. Determined spectrophotometrically at 310 nm.

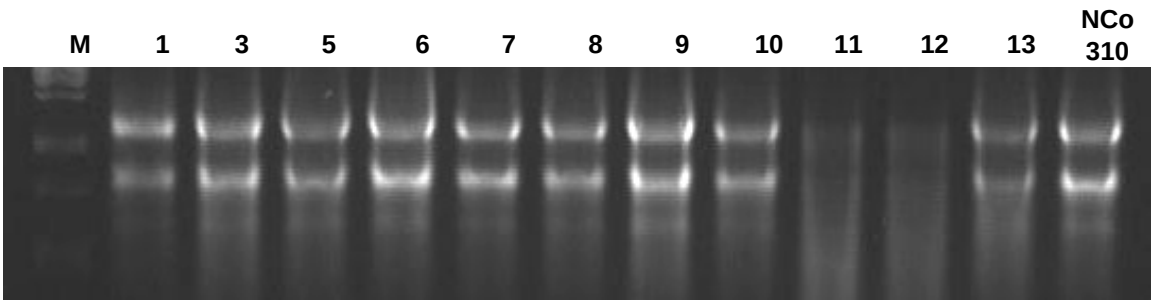

Figure 2.10 RNA extractions from the young leaves of transgenic and WT sugarcane from plants grown under glasshouse conditions. Fragments were separated on a 0.8 % agarose gel.

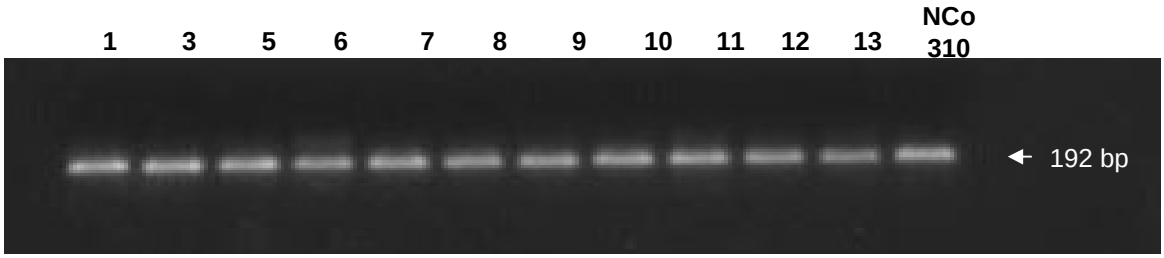

Figure 2.11 Confirmation of 4CL expression in transgenic and WT sugarcane. PCR product amplified with 4CL1F and 4CL1R primers from cDNA generated from RNA extracted from young leaf material. Fragments were separated on a 0.8 % agarose gel.

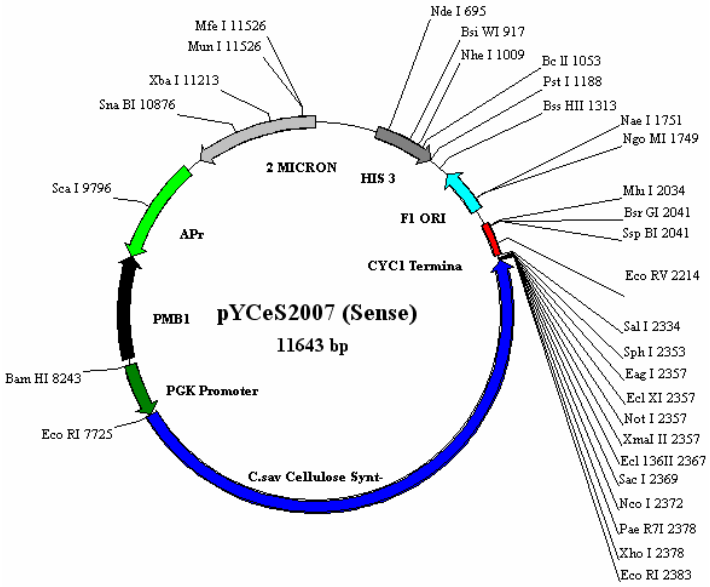

Figure 3.1 Diagram of the pYCeS2007 expression vector containing the *C. savignyi* cellulose synthase gene.

Figure 3.2 Cellulose targeted immunoblot results. Quarter 1 representing a WT YHUM *S. cerevisiae* sample tests negative for cellulose production and quarters 2 to 4 representing different transgenic lines which tested positive for cellulose production.

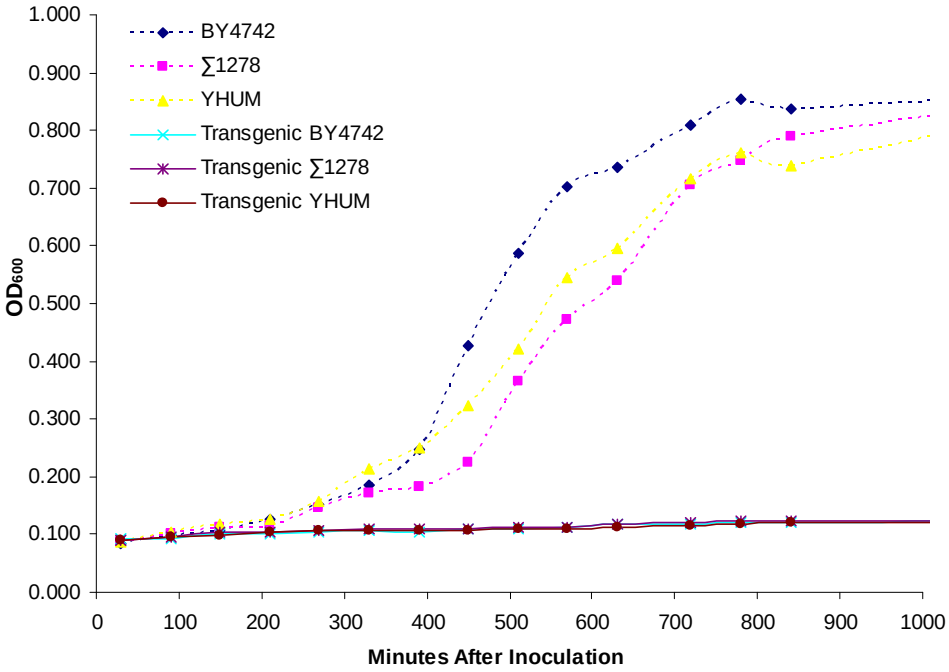

Figure 3.3 Growth curves of WT and transgenic *S. cerevisiae* strains.

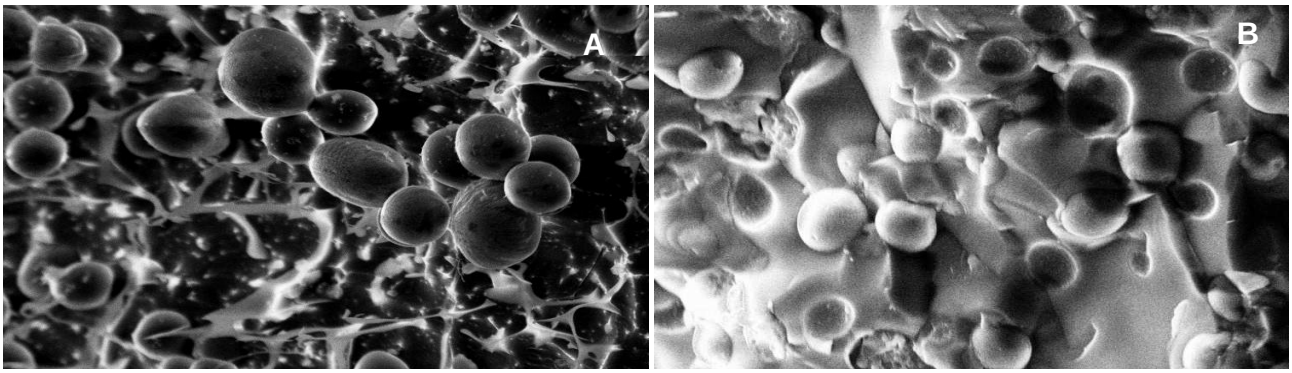

Figure 3.4 SEM images of WT (A) and transgenic (B) ∑1278 *S. cerevisiae* yeast cells.

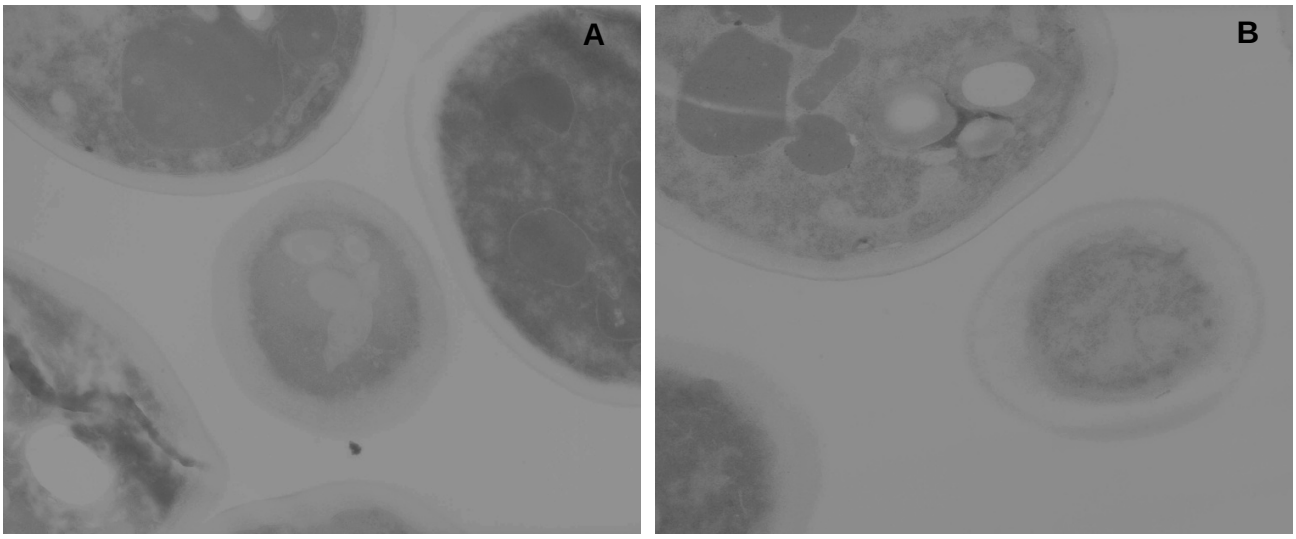

Figure 3.5 TEM images of WT (A) and transgenic (B) ∑1278 *S. cerevisiae* yeast cells.

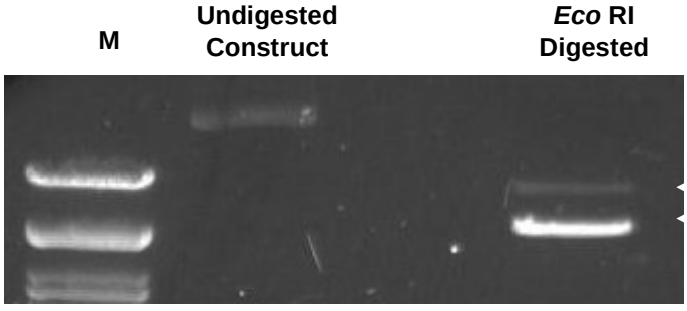

Figure 3.6 Undigested and *Eco* RI digested samples of the donor plasmid containing the CsCeS gene fragments seperated on a 0.8% agarose gel. The lower band of the digested sample correlates to the CsCeS gene.

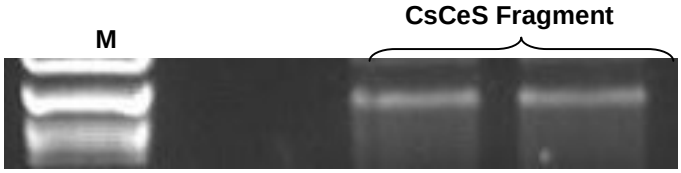

Figure 3.7 CsCeS DNA fragment obtained by PCR amplification from the donor plasmid to introduce a *Eco* RI restriction site and a *Not* I restriction site respectively to the 5' and 3' ends of the gene as shown on a 0.8% agarose gel.

Tables

Table 2.1 Primers used to amplify the putative 4CL gene fragment from sugarcane and binding sites relative to *Z. mays* gene.

Primer	Sequence	Binding site (bp; from - to)	Relative *Z. mays* binding site (bp; from - to)
4CL1F	AGG ATG ATC TTG CTG GTG AAA	766 - 786	1454 - 1474
4CL2F	GGC GAG AAC CCG AAC CTG	1 -18	688 - 705
4CL1R	TGG CTC TCA AGT CCT TCC TT	938 - 957	1626 - 1645
4CL Int	GCT CTG AAG TCA CCG AGG AT	817 -836	1505 - 1524

Table 2.2 PCR product sizes from primer combinations.

Primer pairs	Product size
4CL1F - 4CL1R	191 bp
4CL2F - 4CL1R	957 bp
4CL Int*	426 bp

* Amplification across the intron of the hairpin construct

Table 2.3 Lignin levels of in leaf tissue sample extracts of glasshouse grown transgenic sugarcane lines, and control (NCo310) plants.

Line		1	3	5	6	7	8	9	10	11	12	13	NCo 310
Lignin Levels (% SBM)	Rep 1	1.68%	1.44%	1.38%	1.76%	2.12%	1.81%	1.55%	1.51%	2.16%	1.84%	2.62%	1.84%
	Rep 2	2.41%	2.33%	2.24%	2.24%	2.15%	2.63%	2.36%	2.34%	2.71%	2.73%	4.08%	2.59%
	Rep 3	2.06%	2.05%	1.39%	2.26%	1.73%	2.41%	2.02%	2.04%	2.23%	1.56%	2.53%	2.69%
	Average	2.05%	1.94%	1.67%	2.09%	2.00%	2.28%	1.97%	1.97%	2.37%	2.04%	3.07%	2.37%
	StdErr	0.002	0.003	0.003	0.002	0.001	0.002	0.002	0.002	0.002	0.004	0.005	0.003
	p Value	0.18	0.06	0.15	0.11	0.41	0.47	0.10	0.08	0.99	0.50	0.28	

Table 2.4 Lignin levels in tissue samples from internode 7 and 13, harvested from transgenic sugarcane lines and the control (NCo310) stalks.

Line		1	7	10	11	12	NCo310
Internode 7	Lignin Concentraion (% SBM)	2.36%	1.91%	1.53%	2.45%	1.59%	2.46%
		2.28%	1.74%	1.82%	2.66%	1.72%	3.40%
		4.18%	2.21%	1.71%	0.00%	2.13%	3.53%
	Average Concen-tration (% SBM)	2.94%	1.96%	1.69%	1.70%	1.81%	3.13%
	Standard Error	0.006	0.001	0.001	0.009	0.002	0.003
	p Value	0.75	0.07	0.03	0.32	0.03	
Internode 13	Lignin Concentraion (µg/ml)	2.92%	2.49%	2.66%	2.19%	2.03%	1.11%
		3.20%	2.66%	2.82%	2.63%	1.92%	1.14%
		3.32%	2.91%	2.75%	2.17%	1.66%	1.49%
	Average Concen-tration (ug/ml)	3.15%	2.69%	2.75%	2.33%	1.87%	1.25%
	Standard Error	0.001	0.001	0.000	0.001	0.001	0.001
	p Value	0.00	0.00	0.01	0.04	0.11	

Table 2.5 Protein concentrations of crude protein extracts in young leaf tissue harvested from greenhouse grown transgenic sugarcane lines containing the 4CL hairpin construct.

Sugarcane Line	1	3	5	6	7	8	9	10	11	12	13	NCo 310[b]
Sample readings[a] (OD_{595})	1.224	1.011	0.893	0.982	1.052	1.221	1.055	0.968	0.910	0.928	0.826	0.859
	1.175	0.922	0.935	0.945	1.096	1.226	1.070	0.962	0.920	0.936	0.832	0.840
	1.185	1.014	0.999	1.023	1.071	1.235	1.106	0.992	0.904	0.935	0.826	0.884
Extract Protein Concentrations (mg/ml)	0.540	0.395	0.315	0.376	0.423	0.538	0.425	0.366	0.327	0.339	0.270	0.292
	0.507	0.335	0.344	0.351	0.453	0.542	0.436	0.362	0.334	0.344	0.274	0.279
	0.514	0.398	0.387	0.404	0.436	0.548	0.460	0.383	0.323	0.344	0.270	0.309
Average Concentration (mg/ml)	0.520	0.376	0.349	0.377	0.438	0.543	0.440	0.370	0.328	0.342	0.271	0.293
Standard Error	0.010	0.021	0.021	0.015	0.009	0.003	0.010	0.006	0.003	0.002	0.001	0.009

[a] The BSA standard curve was used to calculate the protein concentration
[b] NCo310 = wild type sugarcane

Table 2.6 4CL Activity in transgenic sugarcane lines and NCo310 control.

Sugarcane Line	1	3	5	6	7	8	9	10	11	12	13	NCo 310
Mean Change in [a] mOD310/min	-2.66	-1.53	-1.55	-1.33	-2.12	-1.61	-1.96	-1.63	-3.20	-2.08	-1.93	-2.17
	-2.81	-1.50	-1.50	-1.26	-1.81	-1.62	-2.07	-1.48	-3.23	-1.85	-1.65	-2.83
	-2.51	-1.58	-1.54	-0.76	-2.19	-1.70	-2.02	-2.03	-3.49	-1.95	-1.91	-2.94
Activity (µg/min)	-7.04	-6.93	-6.93	-6.91	-6.99	-6.94	-6.97	-6.94	-7.09	-6.98	-6.97	-6.99
	-7.05	-6.93	-6.93	-6.91	-6.96	-6.94	-6.98	-6.93	-7.09	-6.96	-6.94	-7.05
	-7.02	-6.94	-6.93	-6.86	-6.99	-6.95	-6.98	-6.98	-7.12	-6.97	-6.97	-7.06
Average Activity (µg/min)	7.038	6.934	6.933	6.894	6.981	6.943	6.978	6.950	7.099	6.973	6.961	7.037
Standard Error	0.008	0.002	0.001	0.017	0.011	0.003	0.003	0.015	0.009	0.006	0.008	0.022
p Value	0.97	0.04	0.05	0.06	0.17	0.04	0.10	0.06	0.07	0.15	0.11	
Reaction as Percentage of Wild Type	100%	99%	99%	98%	99%	99%	99%	99%	101%	99%	99%	100%

[a] p-Coumaric acid conversion measured at OD_{310} over a 30 min interval

Table 3.1 Primers used to amplify *C. savignyi* cellulose synthase from donor plasmid and to confirm insertion and orientation.

Primer	Sequence	Binding site* (bp; from - to)
CsCelSynth F EcoRI	CGG AAT RCG CAA TGA GCA GGG ATA	-3 - 16
CsCelSynth R NotI	TTG CGG CCG CGG ATT ATT GGG TTC	4520 - 4533
CsCeSyI F	AGT ATC GCT GCT TCG GAA AA	1459 - 1478
CsCeSyI R	CTA CCT CAA GCG GTT CCT TG	1932 - 1957
CsCeS Rev	ATT GAA ATT GTC CTT CTG TT	280 - 299
T7	GTA ATA CGA CTC ACT ATA GGG	474 - 494**

*Binding site relative to *C. savignyi* gene starting at ATG start codon.

** Binding site on pYES2 vector

Table 3.2 PCR product sizes from primer combinations for *C. savignyi* cellulose synthase.

Primer pairs	Product size
CsCelSynth F EcoRI - CsCelSynth R NotI	4554 bp
CsCeSyI F - CsCeSyI R	499 bp
T7 - CsCeS R	378 bp