SRA Final Report

Investigation of lignin biosynthesis in sugarcane for improved lignocellulosic ethanol production: final report 2010/068

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PART A

Section 1: Executive Summary

Second generation bioethanol is a renewable energy resource produced from lignocellulosic biomass with the potential to reduce reliance on oil-based energy. Sugarcane bagasse is an abundant source of lignocellulosic material available for bioethanol production. The utilisation of bagasse for biofuel production would be environmentally and economically beneficial, however, the lignin polymers restrict polysaccharide degradation by hydrolytic enzymes. Pretreatment is currently required to overcome the recalcitrant nature of lignin polymers, the cost of which prevents the cost-competitive production of bioethanol from lignocellulosic biomass. There is a strong consensus in published literature that reducing lignin content can increase glucose liberation during enzymatic hydrolysis from both wild type and genetically modified plants, including genetically modified sugarcane. Whilst lignin biosynthesis has received increasing research attention in some plant species, lignin biosynthesis and its manipulation in sugarcane has been explored only in recent publications and remains far from being fully understood. This thesis focuses on contributing to the limited knowledge available concerning lignin biosynthesis and secondary cell wall deposition in wild type sugarcane and the saccharification potential of genetically modified, lignin-reduced sugarcane bagasse through the completion of three specific aims.

The first aim was to develop a lignin biosynthesis profile in wild type sugarcane by correlating gene expression data with cell wall compositional data in sugarcane internodes. The expression levels of nine genes within the lignin biosynthesis pathway were quantified in five sugarcane stem sections of increasing maturity. Analysis found two distinct expression patterns in maturing stem tissue. The expression pattern of the nine genes did not appear linked with their position within the lignin biosynthesis pathway, suggesting the individual role of each gene may influence its expression pattern. Cell wall compositional analysis of the five stem sections found total lignin content significantly increased between the youngest stem tissue and more mature stem, whereas the structural carbohydrate content did not differ between young and mature stem sections.

The second aim of this thesis was to increase saccharification of sugarcane bagasse by reducing lignin content through the transgenic expression of two maize R2R3 MYB transcription factors. There were a number of plants produced that had reduced lignin. These plants underwent enzymatic hydrolysis and a number were found to have improved enzymatic hydrolysis, with significantly increased rates of glucose release after pre-treatment. The plants appeared phenotypically normal and did not have a reduction in juice sucrose production. Improved bagasse digestibility without negatively impacting juice sucrose levels increases the economic value of these plants to the sugarcane industry.

The third aim of this research was to generate lignin altered transgenic sugarcane with improved saccharification by specific RNAi targeting of three lignin biosynthetic pathway genes; CCoAOMT, F5H and COMT. As well as potentially reducing lignin content, targeting these genes may also affect the H:G:S lignin monomer ratios, which has been linked with changes in biomass digestibility. Initial analysis of sugarcane harbouring one RNAi cassette suggested down-regulation of the target genes was occurring and analysis of glasshouse grown plants found this trend continued during plant development. Gene down-regulation was greater in more mature tissue than young tissue and stronger in the CCoAOMT and F5H lines than in the COMT line. Across the three transgenic lines only one COMT-RNAi plant showed a reduction to lignin content. After 72 hours of enzymatic hydrolysis, the lignin reduced COMT-RNAi plant released significantly higher levels of glucose. Additionally, one plant from each RNAi line with no differences to lignin content also released significantly more glucose. No phenotypic differences were detected and all plants had juice sucrose levels equivalent to controls with two plants having significantly higher levels of sucrose.

Current knowledge of lignin biosynthesis in monocots is limited, even more so in sugarcane. Increasing interest in the production of second generation bioethanol from lignocellulosic biomass, such as sugarcane bagasse, has led to an increase in lignin-focused research as lignin polymers are a major hurdle to the production of cost-competitive biofuel. The knowledge and findings of this thesis into the biosynthesis and deposition of lignin in sugarcane will be beneficial to the production or modification of cultivars with improved bioethanol production qualities. Additionally, there are no current reports of transcription factors being utilised to reduce the lignin content in transgenic sugarcane and the positive results in saccharification after MYB42 expression is of research interest. Furthermore, CCoAOMT and F5H have not previously been targeted by RNAi in sugarcane, and the combined results with COMT down-regulation suggest there may also be potential in further exploration of this avenue of research. The production of second generation bioethanol from sugarcane bagasse will have environmental benefits as they will reduce reliance on oil-based energy as well as economic benefits to the Australian sugarcane industry though product diversification.

Section 2: Background

Bioethanol is an energy source produced from the fermentation of plant-derived sugars. The utilisation of bioethanol as a sustainable transport fuel will have many environmental and social benefits that traditional fossil fuels cannot offer such as lower carbon emissions, improved economic stability and increased energy security. However, there are a number of challenges that must be overcome, including sourcing lignocellulosic biomass, optimising different approaches and conditions for pretreatment, hydrolysis and fermentation stages, before cellulosic ethanol will become economically competitive with fossil fuels.

Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin, and provides a large, untapped resource of carbohydrates for the production of bioethanol. Although lignin is necessary for plant development, water transport and defense, those same properties make it a physical and chemical barrier preventing access of hydrolytic enzymes to structural carbohydrates. Therefore pretreatment of lignocellulosic material to remove lignin is currently a crucial yet costly step in the enzymatic conversion of polysaccharides to simple sugars, which presents a major challenge to the production of cost-competitive, commercial scale second generation bioethanol. Using biotechnology to reduce the recalcitrance by decreasing the lignin content or altering its composition has shown success in some crop species, including sugarcane.

Numerous studies have shown it possible to reduce lignin content in plants through the down-regulation of genes in the lignin biosynthetic pathway. In general, there is a positive correlation between reduction of lignin biosynthetic gene transcription levels and an overall reduction of lignin content. It is hypothesised that the down-regulation of key genes controlling lignin biosynthesis in sugarcane will reduce the rate of lignin deposition, resulting in lignin-reduced sugarcane bagasse. In turn, this is expected to reduce the degree of pretreatment required, thus reducing the overall bioethanol production costs. It is further hypothesised that the down-regulation of specific genes within the lignin biosynthesis pathway could alter the lignin polymer composition by changing the H:G:S monomer ratio. Such an outcome may also improve pretreatment efficiency and therefore warrants further exploration.

Sugarcane is an excellent candidate for biofuels for a number of reasons. First, the bagasse is currently burnt inefficiently and could provide a large amount of carbohydrates for biofuels production. Also, the transport of sugarcane bagasse to a central location for the production of sugar would mean no additional transport costs for the biomass being used for biofuels production. The production of biofuels from bagasse would be a value added product to an existing industry. However, due to sugarcane's large and complex polyploid genome, combined with the long duration of traditional breeding, sugarcane is an ideal candidate for biotechnological improvement for the production of lignocellulosic biofuels.

Section 3: Outputs and Achievement of Project Objectives

Project objectives, methodology, results and discussion

The overall objective of this project was to modify lignin deposition in sugarcane to minimise the pretreatment requirements of second generation bioethanol production from bagasse. This project has added to the currently limited knowledge of lignin formation and modification in sugarcane through the three following aims:

1. *Transcriptional profile of lignin biosynthetic genes over the life cycle of the sugarcane plant.*

The aim of this research was to fill this knowledge gap for sugarcane, an important crop to the Australian economy and a potentially important crop for second generation bioethanol production. Sugarcane plants with lower lignin contents (wild type and genetically modified) have been found to release increased levels of glucose compared with sugarcane plants with higher lignin contents. Improved understanding of lignin biosynthesis and deposition in sugarcane will be of great value when deciding the most appropriate approaches to facilitate the development of commercial lines with increased saccharification potential.

Five mature wild-type sugarcane were harvested and tissue sampled from specific locations along the stem and root to create a developmental profile of sugarcane lignin biosynthesis. qPCR, acid hydrolysis and HPLC analysis were used to establish stem profiles of lignin gene expression and cell wall composition.

The trends in the stem expression data (Figure 1) dichotomise the lignin biosynthesis genes: expression decreases with tissue age (PAL, CCR, 4CL, COMT and CAD) or expression remains constant during maturation (C3H, F5H, C4H and CCoAOMT). The position of the genes within the lignin biosynthesis pathway and their expression pattern do not appear linked suggesting the function of the gene may influence its expression more than its location within the biosynthetic pathway.

The composition of the cell wall changes as a plant matures as a result of secondary cell wall deposition. After cell elongation has ceased, the secondary cell wall is formed which involves the initial deposition of cellulose and hemicellulose, followed by the lignification of this polysaccharide matrix. Within sugarcane, rapid elongation of young internode cells precedes cell wall thickening, including lignification, which is indicative of internode maturation. No significant differences were seen in carbohydrates throughout the stalk, but there was significant higher lignin content between the youngest section of the stem and the older stem, which may reflect the order of the formation of the secondary cell wall. As lignin is incorporated after polysaccharide deposition, the secondary cell walls in the young section may have been under construction when harvested, compared to mature cell walls in older sections. Polysaccharide components had been fully deposited but lignin deposition was ongoing.

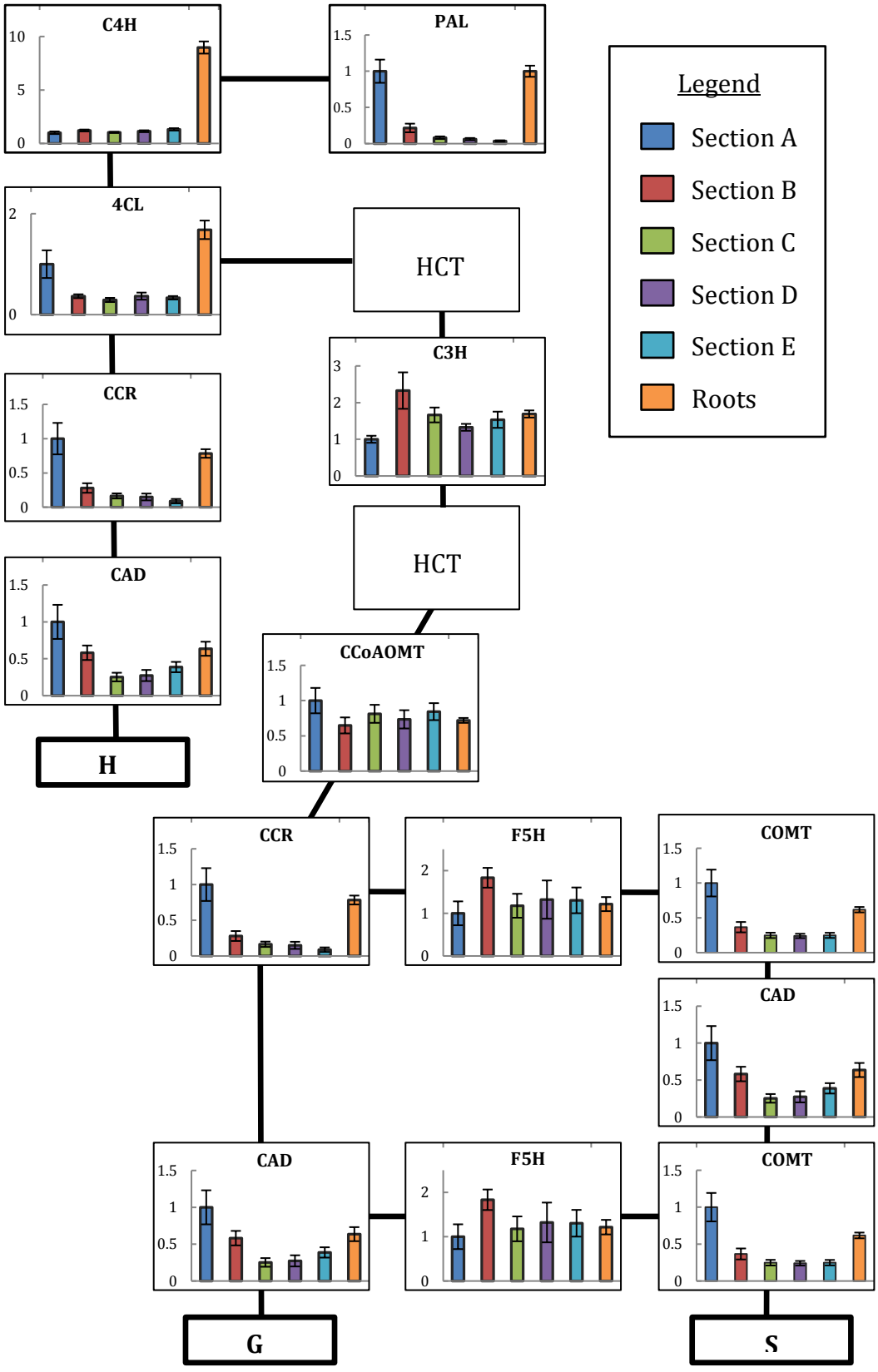


FIGURE 1: qPCR expression levels of lignin biosynthesis genes in stem sections A – E (youngest to most mature) and root tissue as seen in Figures 4.1 and 4.2 laid out as lignin biosynthesis pathway based on Hisano *et al.* (2009). Expression levels are normalised to section A for each gene.

2. *Analysis of transgenic sugarcane plants expressing maize MYB transcription factors 31 and 42, two genes known to exert down-regulatory control over lignin biosynthesis.*

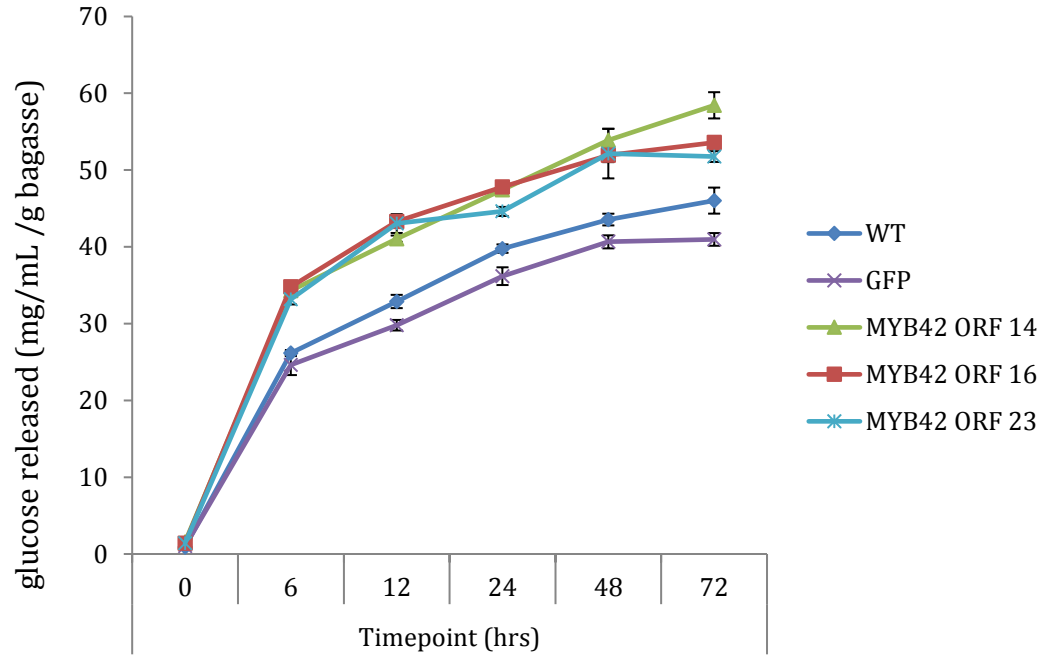
The expression of *Zm*MYB31 and *Zm*MYB42 in other species has resulted in improved saccharification. If expression in sugarcane were to emulate these previous and improve saccharification, this would benefit the production of second generation bioethanol from, and increase the monetary value of sugarcane bagasse. The purpose of the aim is to assess whether the overexpression of two MYB transcription factors can impact lignin gene expression, thereby decreasing lignin content and increasing the availability and ease of access to structural carbohydrates without having a negative effect on soluble sucrose production. The assessment of this possibility required the production and analysis of transgenic plants. Plants were assessed for transgene transcript level (qPCR), cell wall composition (acid hydrolysis), saccharification (enzymatic hydrolysis) and juice sucrose levels (HPIC) to confirm no deleterious effects on sugar production.

Of the lines analyzed (selected based on qPCR results), only one MYB 31 line had significantly decreased lignin levels. However six MYB42 lines had decreased lignin content. For all of the MYB42 lines this translated into higher glucose yields from enzymatic hydrolysis after the samples were pretreated (Figure 2). None of the lines with improved saccharification results showed reduced soluble sucrose levels suggesting that the cell wall properties of sugarcane can be significantly improved for biofuels production without negatively impacting the primary production of sugar.

This research highlights MYB42 as a transcription factor of interest for improving the production of second generation bioethanol from sugarcane bagasse, and directly shows the gains that can be made using biotechnology.

3. *Down-regulation of CCoAOMT, F5H and COMT lignin biosynthetic genes in transgenic sugarcane via RNA interference.*

The goal of this aim was to target lignin content or polymer composition by altering the expression of genes involved in controlling the production of each of the three individual lignin monomers. By modifying lignin polymer composition without reducing lignin content, it was hypothesised that the lines would have improved saccharification without a negative effect on plant phenotype. The purpose of the aim is to assess whether using RNAi can impact expression of specific lignin genes (CCoAOMT, F5H and COMT*)*, thereby reducing or altering lignin composition and increasing the availability and ease of access to structural carbohydrates without having a negative effect on juice sucrose production. The assessment of this possibility required the production and analysis of transgenic plants. Plants were assessed for transgene transcript level (qPCR), cell wall composition (acid hydrolysis), saccharification (enzymatic hydrolysis) and juice sucrose levels (HPIC) to confirm no deleterious effects on sugar production.



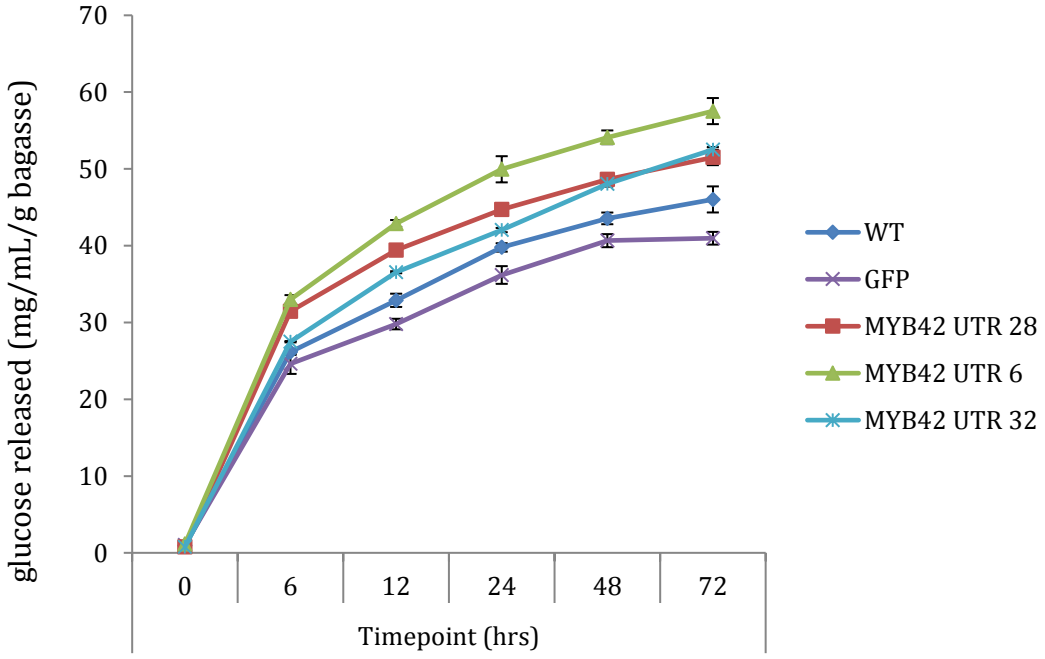


FIGURE 2: Total glucose concentration in enzymatic hydrolysis solution (mg/mL) per gram (g) of bagasse showing standard error of the mean measured at six time points over a period of 72 hours incubation for MYB42 ORF plants (A); and MYB42 UTR plants (B). WT n = 3; GFP n = 3, n = individually analysed plants. WT: wild type control group; GFP: transgenic control group.

Plants were regenerated harboring one of the three RNAi constructs, and lines were recovered that had decreased expression of each gene of interest. There was one COMT line with decreased lignin content, and one of each F5H and CCoAOMT that had increased lignin content. Few changes in structural carbohydrate content were detected, with the majority of modified plants being comparable to controls. The COMT-RNAi plant with significantly reduced lignin content released significantly more glucose than all other RNAi and controls plants (Figure 3). An additional three plants, one from each RNAi line, with no changes in lignin content also had significant improvements to glucose release after enzymatic hydrolysis when compared with transgenic controls (Figure 3). There was no deleterious effect on soluble sucrose. Further work is ongoing to characterise the lignin monomer composition of these lines to include in publications.

This research highlights COMT as a potential target for improving the production of second generation bioethanol from Australian sugarcane bagasse, and is supported by recent publications in which COMT was down regulated by RNAi in a North American cultivar. The results of this aim directly show the gains that can be made using biotechnology.

Conclusions

The findings presented in this thesis, through the completion of three specific aims, contribute to the current knowledge of lignin biosynthesis in sugarcane and provide proof of concept for the continued use of biotechnology in sugarcane towards the production of economically viable second generation bioethanol which will have global, as well as local, economic and environmental benefits.

Additionally, it was confirmed that biotechnology, in the form of MYB transcription factors and RNAi vectors, could be applied to an important Australian sugarcane cultivar to improve the digestion of bagasse, an important advancement towards decreasing the production costs of second generation bioethanol. In common, future research for the three research projects presented in this thesis includes analysis of field grown plants. This represents a large, but necessary step towards the realisation of GM sugarcane being grown for the dual purposes of sucrose and biofuel production. Assessing the reproducibility of the results presented in the thesis in field grown plants is a key step towards commercialisation.

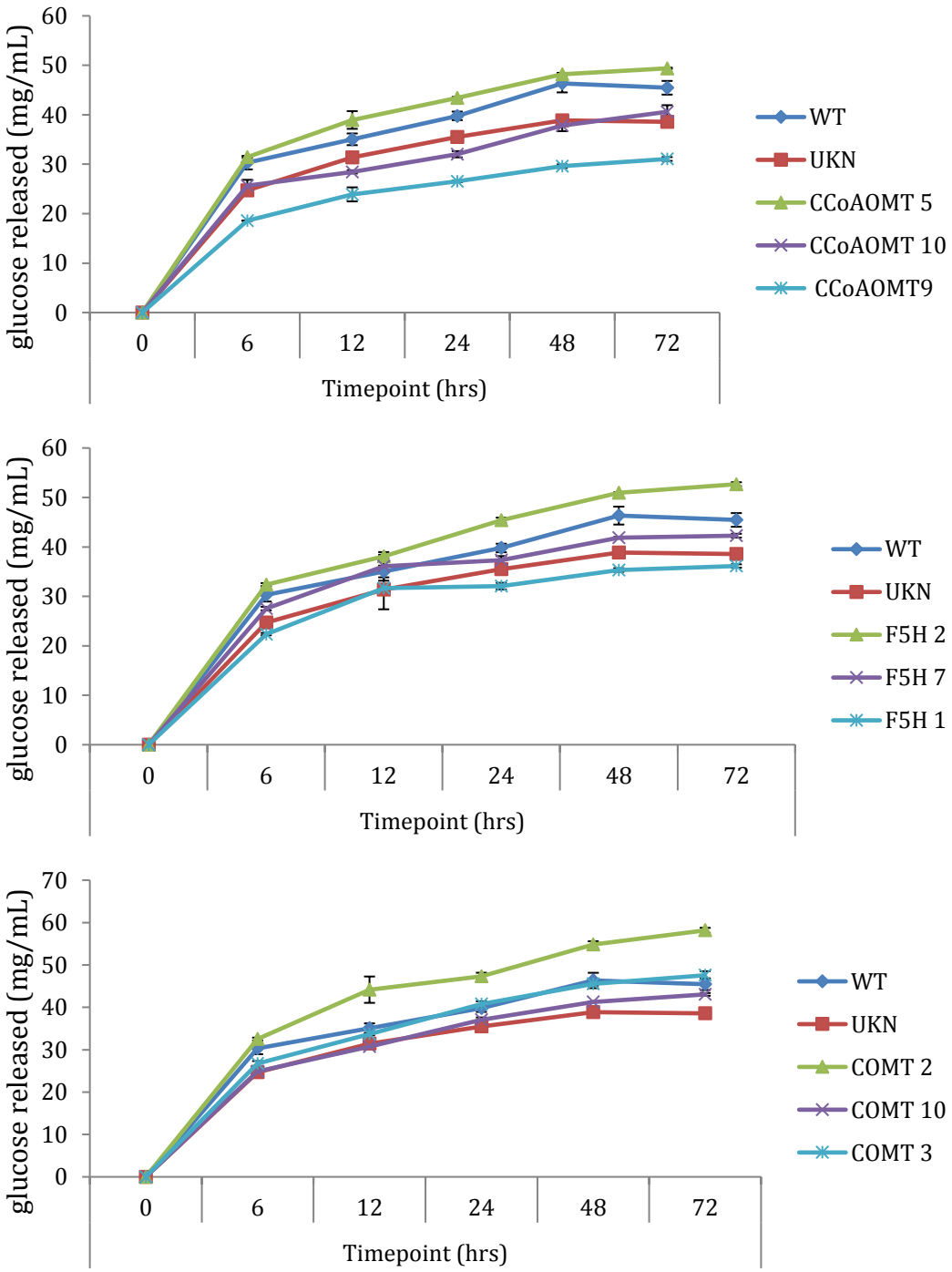


FIGURE 3: Total glucose concentration in enzymatic hydrolysis solution (showing standard error of the mean) measured at six time points over a period of 72 hours incubation based on weight of bagasse hydrolysed (mg/mL/g bagasse) for CCoAOMT RNAi (A); F5H RNAi (B); and COMT RNAi (C). WT n = 3; UKN n = 3, with n = individual plants analysed. WT: wild type control group; UKN: transgenic control group.

Section 4: Outputs and Outcomes

This project has resulted in one thesis and will be the subject of three scientific papers yet to be written.

Section 5: Intellectual Property (IP) and Confidentiality

N/A

Section 6: Industry Communication and Adoption of Outputs

N/A

Section 7: Environmental Impact

N/A

Section 8: Recommendations and Future Industry Needs

The results from the three aims of the project are currently being written up as three individual scientific papers. The work will be published in high impact scientific journals. Continued work on altering the cell wall of sugarcane is needed, as well as increased genomic resources to support this work.

Section 9: Publications

The student's thesis is attached to this report. Also on QUT ePrints database.

http://eprints.qut.edu.au/86088/