

**Isolation and characterisation of genes involved  
in carbon and chlorophyll metabolism in  
*Saccharum* species hybrids**

by

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## **Preface**

### **Abstract**

Sugarcane is a tropical perennial grass species belonging to the Poaceae (true grasses) family. Mature sugarcane is comprised mostly of sugarcane stalks, which accumulate high amounts of sucrose, a fact that has led to its wide cultivation of sugarcane for sucrose production. Sugar yields from sugarcane have been improved in the past by either creating transgenic sugarcane or through using traditional breeding methods. Increasing sugar yields in sugarcane is still of interest and new cisgenic strategies are being considered to alleviate consumer concerns over transgenic plants.

This thesis consists of two parts. The first was aimed at understanding the relation between trehalose-6-phosphate (T6P) synthesis and sucrose accumulation in sugarcane. In this study the *E. coli* genes involved in trehalose synthesis, *otsA* and *otsB*, were overexpressed in sugarcane in order to observe their effects on soluble sugar accumulation. Nine *otsA* and two *otsB* overexpressing lines were created, confirmed by gDNA insertion PCRs, sq-RT-PCR and immuno detection of encoded enzymes. Preliminary measurements of soluble sugars showed that four out of the nine *otsA* lines had significantly decreased and one line significantly increased sucrose concentrations. Correlating sq-RT-PCR results with soluble sugar measurements suggest that trehalose-6-phosphate synthase (TPS) expression affects sucrose levels in sugarcane, but further research of TPS activity is required before a conclusion can be reached. Further analysis of mature cane material in regard to relevant enzyme levels, carbohydrate levels and gene expression should contribute to more conclusive results.

Three novel sugarcane *TPS* encoding sequences were isolated and proven to be functional through complementation of the growth defect in *tps1Δ* yeast grown on glucose as a carbon source. Sugarcane *TPS* isoforms named *SoTPSa*, *SoTPSb* and *SoTPSc*, were isolated by successful application of 5' RACE alongside standard PCR using primers based on other monocotyledonous *TPS* sequences. The encoded *SoTPSa* contains a 25 amino acid insertion within the partial TPP domain. The encoded *SoTPSc* contains a 126 amino acid long N terminal truncation, which removes one of the thirteen amino acids found within the active site of the TPS domain. Future characterization of the encoded enzymes will determine the effects of these modifications on TPS activity.

The second part of this thesis describes initial efforts made in attempting to develop a cisgenic *in vitro* selectable marker system for sugarcane, *S. officinarum* callus, which uses a diphenylether type (DPE) herbicide as a selection agent and a sugarcane protoporphyrinogen oxidase (PPO) gene as a selection marker. Firstly the plastid targeted *PPO* from tobacco (*NtPPO-1*) was isolated and mutagenized, to mimic the double mutated *Arabidopsis* PPO, used by Li *et al.*, (2003) in maize. However, sugarcane calli transformed with the double mutated *NtPPO-1* and grown on media containing fomesafen herbicide, were incapable of regenerating. Future efforts will utilize a N-terminal sequence that is targeted to the plastid organelle, so as to ensure translocation of the enzyme to that subcellular location. Also, random mutations were induced in the *NtPPO-1* gene to screen for mutations that confer DPE herbicide resistance, however this work is currently on hold until a heme deficient *E. coli* can be obtained. Secondly, attempts were made to isolate a putative sugarcane plastid targeted *PPO* gene, so as to eventually use this in developing a cisgenic strategy. 5' RACE was successful in revealing additional nucleotide sequence adding 1006 bp to the already known partial sugarcane *PPO* sequence. However the fragment isolated was still a partial sequence.

## Samevatting

Suikerriet is 'n tropiese meerjarige gras spesie wat deel is van die Poaceae (ware grasse) familie. Volwasse suikerriet bestaan hoofsaaklik uit suikerrietstamme, wat hoë hoeveelhede sukrose akkumuleer, 'n feit wat gelei het tot die wye verbouing van suikerriet vir sukrose produksie. In die verlede is suikeropbrengste vanuit suikerriet verbeter deur die skep van transgeniese suikerriet óf die gebruik van tradisionele teelmetodes. Toenemende suiker opbrengste in suikerriet is steeds van belang en nuwe cisgeniese strategieë word oorweeg om verbruikerskommer oor transgeniese plante te akkommodeer.

Hierdie tesis bestaan uit twee dele. Die eerste deel is daarop gemik om die begrip van die verhouding tussen trehalose-6-fosfaat (T6P) sintese en sukrose ophoping in suikerriet te verstaan. In hierdie studie is die *E. coli* gene wat betrokke is in trehalose sintese, *otsA* en *otsB*, ooruitgedruk in suikerriet ten einde die uitwerking daarvan in die opgaar van oplosbare suiker te bestudeer. Nege *otsA* en twee *otsB* verhoogte uitdrukings lyne is geskep, bevestig deur gDNA bygevoegde PKR, sq-RT-PKR en immuno opsporing van geïnkripteerde ensieme. Voorlopige metings van oplosbare suikers toon dat vier van die nege *otsA* lyne 'n beduidende afname in sukrose vlakke en een lyn 'n beduidende toename in sukrose vlakke getoon het.

Korrelerende sq-RT-PKR resultate met oplosbare suikermetings dui daarop dat trehalose-6-fosfaat sintese (TPS) geenuitdrukking sukrose vlakke sal affekteer, maar verdere navorsing

van TPS aktiwiteit is nodig voordat 'n gevolgtrekking gemaak kan word. Verdere ontleding van volwasse riet materiaal met betrekking tot relevante ensiem vlakke, koolhidrate vlakke en geenuitdrukking, behoort by te dra tot meer volledige resultate.

In hierdie studie is drie nuwe suikerriet TPS gene geïsoleer en dit is bewys as funksioneel deur die komplimentering van die groeidefek van *tps1Δ* gis, gegroei op glukose as 'n koolstof bron. Suikerriet TPS isoforme, genoem SoTPSa, SoTPSb en SoTPSc, is geïsoleer deur die suksesvolle toepassing van 5 'RACE, in kombinasie met standaard PKR, deur van spesiaal ontwerpte primers, gebaseer op ander eensaadlobbige TPS gene, gebruik te maak. Die gekodeerde SoTPSa bevat 'n 25 aminosuur invoeging binne-in die gedeeltelike TPP domein. Die gekodeerde SoTPSc bevat 'n 126 aminosuur lange N terminaal afkapping, wat een van die dertien aminosure binne die aktiewe terrein van die TPS domein verwyder. Toekomstige karakterisering van hierdie geïnkripteerde ensiemes sal die effek van hierdie veranderinge op TPS aktiwiteit bepaal.

Die tweede deel van hierdie tesis beskryf die aanvanklike probeerslae wat gemaak is in 'n poging om 'n cisgeniese *in vitro* selekteerbare merker vir suikerriet, *S. officinarum* kallus te ontwikkel. Hierin word gebruik gemaak van 'n difenylether tipe (DPE) onkruidodder as 'n seleksie agent, en 'n suikerriet protoporphyrinogen oksidase (PPO) geen as 'n seleksie merker. In 'n poging om dit te bewerkstellig is daar eerstens plastied geteikende PPO van tabak (NtPPO-1) geïsoleer en geteikende mutagenese suksesvol daarop uitgevoer. Mutasies wat geïnduseer is, is gegrond op die dubbele gemuteerde *Arabidopsis* PPO, wat gebruik was in mielies deur Li *et al.*, (2003). Alhoewel die suikerriet kallus getransformeer is met die dubbele gemuteerde NtPPO-1 konstruksie en geselekteer is op media wat fomesafene onkruidodder bevat, was die kallus nie in staat om te regenerer nie. In toekomstige pogings sal probeer word om 'n N-terminale volgorde, geteiken op 'n plastied organel, te benut sodat translokasie van die ensiem aan die plastied organel verseker kan word. So ook is toevallige mutasies veroorsaak in die *NtPPO-1* gene om te soek vir nuwe mutasies wat DPE onkruidodderweerstand verleen, maar hierdie werk is tans gestop totdat 'n heem gebrekkige *E. coli* mutant verkry kan word. Tweedens, is pogings aangewend om 'n vermeende suikerriet plastied geteikende PPO gene te isoleer, om uiteindelik te gebruik in die ontwikkeling van 'n cisgeniese strategie in suikerriet. 5 'RACE was suksesvol in die onthulling van bykomende nukleotiede volgorde deur 1006 bp by te voeg by die reeds bekende gedeeltelike suikerriet PPO fragment. Nie teenstaande is die fragment wat nuut geïsoleer is, steeds slegs 'n gedeeltelike volgorde volgens vergelykings met ander bekende plant PPO gene.

## References

Li, X. Volrath, S.L. Nicholl, D.B.G. Chilcot, C.E. Johnson, M.A. Ward, E.R. Law, M.D. (2003). Development of protoporphyrinogen oxidase as an efficient selection marker for *Agrobacterium tumefaciens*-mediated transformation of maize. *Plant Physiology*, 133:736-747.

## Aim

The first aim of this study is to take initial steps in understanding the link between trehalose-6-phosphate and sucrose accumulation in sugarcane, so as to potentially utilize this in increasing sucrose accumulation in sugarcane. This is to be achieved by isolating and characterizing a functional sugarcane TPS gene. *E. coli* TPS and TPP genes are also to be overexpressed in sugarcane, to observe the affects thereof on sucrose accumulation in sugarcane.

The second aim is to develop a cisgenic selectable marker system for sugarcane callus, using a plant PPO gene and a DPE herbicide as a selectable marker. As a proof of concept, the double mutated tobacco PPO-1 gene is to be overexpressed in sugarcane callus and tested if these can be selected on media containing fomesafen herbicide. A sugarcane PPO-1 gene is also to be isolated and characterised, to develop this as a cisgenic selectable marker.

## Layout of thesis

Each chapter has an introduction, materials and methods, results and discussion section. This thesis is divided into the following chapters:

**Chapter 1** Is a survey of literature focussing on sugarcane and the two enzyme encoding genes studied in this thesis. The first part of the chapter focuses on sugarcane as an economically important crop. The second part focuses on literature concerning protoporphyrinogen oxidase. The third part focuses on literature concerning trehalose-6-phosphate and the enzyme responsible for its synthesis.

**Chapter 2** describes work done in understanding the link between trehalose-6-phosphate and sucrose accumulation in sugarcane. The first part describes work done in creating *otsA* and *otsB* overexpressing sugarcane lines and the initial measurements of soluble

sugars performed on these lines. The second part describes efforts made to isolate a putative sugarcane gene, resulting in the isolation of three sugarcane TPS genes, where these were shown to be functional in yeast complementation assays.

**Chapter 3** describes work done in developing a selectable marker system for sugarcane callus. The first part describes efforts to isolate a putative sugarcane PPO-1 gene. The second part describes the creation of a double mutagenized tobacco PPO-1 construct and testing whether this could be used to select for transformed sugarcane callus, selected on media containing fomesafen. Also described is the effort made in trying to identify other tobacco PPO mutations that confer herbicide resistance.

**Chapter 4** presents general conclusions of the results obtained in chapters 2 and 3 and future prospects which may result in conclusive findings for this study

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## **List of Abbreviations**

### **Chemicals and Molecules**

2,4-D	2,4-dichlorophenoxyacetic acid
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CaCl <sub>2</sub>	Calcium chloride
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
dNTP	Deoxyribonucleotide
DPE	Diphenylether type herbicides
EDTA	Ethylenediamine tetra acetic acid
G6P	Glucose-6-phosphate
GC-MS	Gas chromatography mass spectrometry
gDNA	Genomic DNA
HK	Hexokinase
Inv	Invertase

KOH	Potassium hydroxide
LB	Luria Bertani medium
LBA	Luria Bertani medium with agar
LiAc	Lithium acetate
LiCl	Lithium chloride
MgCl <sub>2</sub>	Magnesium chloride
MS	Murashige and Skoog medium
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PCIA	Phenol:chloroform:isoamyl-alcohol
Protogen	Protoporphyrinogen IX
Proto	Protoporphyrin IX
PGI	Phosphoglucoisomerase
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
S23142	<i>N</i> -(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide
SDS	Sodium dodecyl sulphate
Tris-HCL	Tris-hydroxymethyl aminomethane
T-DNA	Transfer DNA

Temed	Tetramethylethylenediamine
T6P	Trehalose-6-phosphate
TBE	Tris-Borate-EDTA buffer
UDPG	Uridine diphospho-glucose
YP	Yeast peptone medium
YPA	Yeast peptone medium with agar

### Genes and Enzymes

<i>adg1-1</i>	ADP glucose pyrophosphorylase small subunit 1
AGPase	ADP-glucose pyrophosphorylase
<i>als</i>	Acetolactate synthase
<i>ApL3</i>	<i>Arabidopsis</i> AGPase encoding gene
<i>AtTPS1</i>	<i>Arabidopsis thaliana</i> trehalose-6-phosphate synthase 1
<i>AtTPS6</i>	<i>Arabidopsis thaliana</i> trehalose-6-phosphate synthase 6
<i>bar</i>	Phosphinotricin acetyltransferase
bZIP11	Basic motif leucine region zipper motif transcription factor 11
<i>CIF1</i>	Encodes small subunit of TPS/TPP complex
DNase I	Deoxyribonuclease I
<i>ELIP</i>	Early light induced protein
FastAp	Fast alkaline phosphatase
<i>FDPI</i>	Mutation causes yeast to not grow on glucose
G6PDH	Glucose-6-phosphate dehydrogenase
<i>hemH</i>	Earlier name of prokaryotic protoporphyrinogen oxidase gene

<i>hemG</i>	Prokaryotic protoporphyrinogen oxidase gene
<i>hemY</i>	Earlier name of prokaryotic protoporphyrinogen oxidase gene
<i>hpt</i>	Hygromycin phosphotransferase
<i>HSP70</i>	Heat shock protein 70
<i>HXK1</i>	Hexokinase I
<i>HXK2</i>	Hexokinase II
<i>nptII</i>	Neomycin phosphotransferase
<i>NtPPO-1</i>	<i>Nicotiana tabacum</i> protoporphyrinogen oxidase I
<i>NtPPO1-DM</i>	Double mutated <i>NtPPO-1</i> containing S316L and Y437M mutations
<i>otsA</i>	Trehalose-6-phosphate synthase from <i>E. coli</i>
<i>otsB</i>	Trehalose-6-phosphate phosphatase from <i>E. coli</i>
<i>OsTPS1</i>	<i>Oryza sativa</i> trehalose-6-phosphate synthase 1
<i>PMSF</i>	phenylmethylsulfonyl fluoride
<i>PNK</i>	T4 polynucleotide kinase
<i>PPO-1</i>	Protoporphyrinogen oxidase I
<i>PPO-2</i>	Protoporphyrinogen oxidase II
<i>PMI</i>	Phosphomannose isomerase
<i>SbTPS</i>	<i>Sorghum bicolor</i> trehalose-6-phosphate synthase
<i>SbPPO-1</i>	Protoporphyrinogen oxidase I
<i>SlTPS1</i>	<i>Selaginella lepidophylla</i> trehalose-6-phosphate synthase 1
<i>SnRK1</i>	Sucrose non-fermenting 1 related protein kinase
<i>SNF-1</i>	Sucrose non-fermenting kinase 1
<i>SoTPS1</i>	Sugarcane partial trehalose-6-phosphate synthase

<i>SoTPSa</i>	Sugaracne trehalose-6-phosphate synthase isoform a
<i>SoTPSb</i>	Sugaracne trehalose-6-phosphate synthase isoform b
<i>SoTPSc</i>	Sugaracne trehalose-6-phosphate synthase isoform c
<i>RAB16C</i>	Dehydrin RAB16C Recombinant Protein
<i>rbcS</i>	Ribulose-1,5-bisphosphate carboxylase small subunit gene promoter
RNase	Ribonuclease
TPS	Trehalose-6-phosphate synthase
TPP	Trehalose-6-phosphate phosphatase
<i>tps1Δ</i>	W303-1A <i>tps1Δ::TRP1</i> yeast
<i>tps2Δ</i>	W303-1A <i>tps2Δ::LEU2</i> yeast
<i>tps1Δ + tps2Δ</i>	W303-1A MATa <i>tps1Δ::TRP1 tps2Δ::LEU2</i> yeast
TPSP	Bi-functional fusions of TPS and TPP
<i>treC</i>	<i>E. coli</i> Trehalose-6-phosphate hydrolase
<i>treF</i>	<i>E. coli</i> Trehalase
<i>UBI</i>	Maize ubiquitin promoter
<i>WS118</i>	<i>WS118</i> promoter region in <i>Oryza sativa</i>
<i>ZmTPS1</i>	<i>Zea mays</i> trehalose-6-phosphate synthase 1

## Terms

A220V	Alanine to valine substitution at amino acid position 220
bp	Base pairs
BLAST	Basic local alignment search tool
blastn	BLAST search a nucleotide database with nucleotide query



blastx	BLAST search a protein database with translated nucleotide query
CAF	Central Analytic Facility, Stellenbosch University
CDD	Conserved domain database
G221S	Glycine to serine substitution at amino acid position 221
GMO	Genetically modified organism
ISAAA	International service for the acquisition of agri-biotech applications
kDA	Kilo daltons
NCBI	National Centre for Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
S316L	Serine to leucine substitution at amino acid position 316
S305L	Serine to leucine substitution at amino acid position 305
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sq-RT-PCR	Semi quantitative real time PCR
U	Unit
UV	Ultraviolet
(v/v)	volume per volume
(w/v)	weight per volume
WT	Wild type
Y426C	Tyrosine to cysteine substitution at amino acid position 426
Y426M	Tyrosine to methionine substitution at amino acid position 426
Y437M	Tyrosine to methionine substitution at amino acid position 437

# Chapter 1

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## Literature Review

## 1.1 Modification of agricultural traits in sugarcane

Sugarcane is largely cultivated for sugar production and it is estimated that roughly 75% of sugar produced worldwide is from sugarcane agriculture, with the remaining 25% being harvested from sugar beet. Breeding methods have been successful in the past in creating sugarcane cultivars with increased sucrose yields and other traits beneficial for sugarcane cultivation. However, recent progress in creating further improvement in sugarcane cultivars has been slow and this has been attributed to a lack in genetic diversity (Dal-Bianco *et al.*, 2012; Zhou *et al.*, 2013).

Alternatively, the creation of genetically modified sugarcane has been used with the same goal of improving agricultural traits in sugarcane. Techniques for genetically modifying sugarcane were developed in the early 1990's and have been refined since then (Franks and Birch, 1991; Bower and Birch, 1992; Van der Vyver *et al.*, 2013). These techniques include protocols for *in vitro* regeneration, transforming gene constructs into sugarcane and the selection of transformed plants. Potential genes for modification of sugarcane have been identified, influencing agricultural traits such as insect resistance, herbicide resistance, drought tolerance and increased sugar accumulation (Wu and Birch, 2007; Groenewald and Botha, 2008; Arruda, 2012). Additionally, herbicide resistance genes have been developed for use in *in vitro* selecting of putative transformed sugarcane clones (Negrotto *et al.*, 2000; Jain *et al.*, 2007; Van der Vyver *et al.*, 2013). Some problems have been encountered with genetic modification strategies, such as somaclonal variation and difficulties in backcrossing transgenic lines with parental cultivars (Dal-Bianco *et al.*, 2012)

Another strategy that is receiving more attention recently is the use of cisgenics, rather than transgenics to further enhance agricultural traits in crop plants. Transgenic crop plants are genetically modified plants that contain genes from different species. One example of a transgenic strategy is the targeted expression of a bacterial sucrose isomerase to the vacuoles of sugarcane, which was done by Wu and Birch, (2007). Commercialization of such transgenic plants is difficult due to regulations and public concerns about expressing genes from different species in crop plants. Cisgenic plants differ from this as they are altered using genes from their own genome and not from different species (Schubert and Williams, 2006; Jacobsen and Schouten, 2009). An example of this cisgenic sugarcane created by Vickers *et al.*, (2005), where overexpression of the sugarcane polyphenol oxidase gene in sugarcane lead to enhanced browning of sugar. Another successful use of cisgenics, was the silencing of fructose-6-phosphate 1-phosphotransferase (PFP) activity in sugarcane by Groenewald and

Botha, (2008) by constitutively expressing a sugarcane PFP gene, resulting in increased sucrose accumulation.

Although regulations still present difficulties in developing and utilizing new genetically modified crop plants, some countries have approved the use of transgenic and cisgenic crops. Recently, the US approved the use of a cisgenic potato line called Innate, which was developed by Simplot Plant Sciences (New York Times, Pollack, 2014). This line expresses a gene from a wild potato relative, conferring traits such as reduced acrylamide content, reduced water usage and reduced bruising of potatoes during transport (Jones *et al.*, 2014; New York Times, Pollack, 2014). Also, Indonesia has scheduled the planting of a drought tolerant genetically modified sugarcane line for the year 2014 (Waltz, 2014). During field trials in drought conditions, a sugarcane line expressing the glycine betaine gene from *Rhizobium meliloti* showed increased sucrose accumulation in comparison with wild type sugarcane (Waltz, 2014)

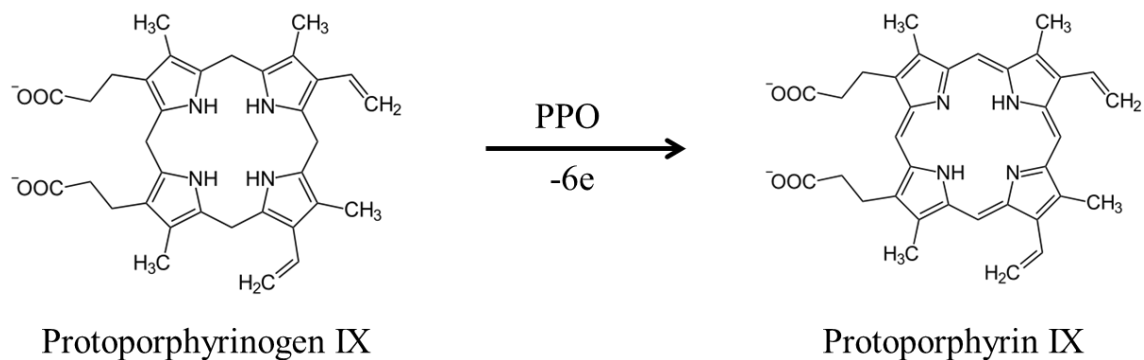
Future research would be focused on attaining endogenous genetic sequences from sugarcane and using these for further genetic modification of sugarcane. It is hoped that the creation of cisgenic sugarcane cultivars would ease regulation and aid in commercialization of these new cisgenic cultivars (Jacobsen and Schouten, 2009).

## 1.2 Protoporphyrinogen oxidase

The enzyme protoporphyrinogen oxidase (PPO) (EC 1.3.3.4; BRENDA: <http://www.brenda-enzymes.org/>) catalyses the oxidation and conversion of protoporphyrinogen IX (Protogen) to protoporphyrin IX (Proto) (Narita *et al.*, 1996). PPO enzymes can be found in both prokaryotic and eukaryotic organisms such as animals, bacteria, fungi and plants. Within all these organisms, protoporphyrin IX is the last common intermediate shared by both the heme and chlorophyll biosynthetic pathways (Nishimura *et al.*, 1995; Narita *et al.*, 1996; Li and Nicholl, 2005; Patzoldt *et al.*, 2006). PPO deficiency has been linked to variegate porphyria in humans and poor aerobic growth and light sensitivity in bacteria (Nishimura *et al.*, 1995; Narita *et al.*, 1996). Diphenylether type (DPE) herbicides are inhibitors of PPO activity in plants, causing cellular death, via light dependant mechanisms (Narita *et al.*, 1996; Li and Nicholl, 2005).

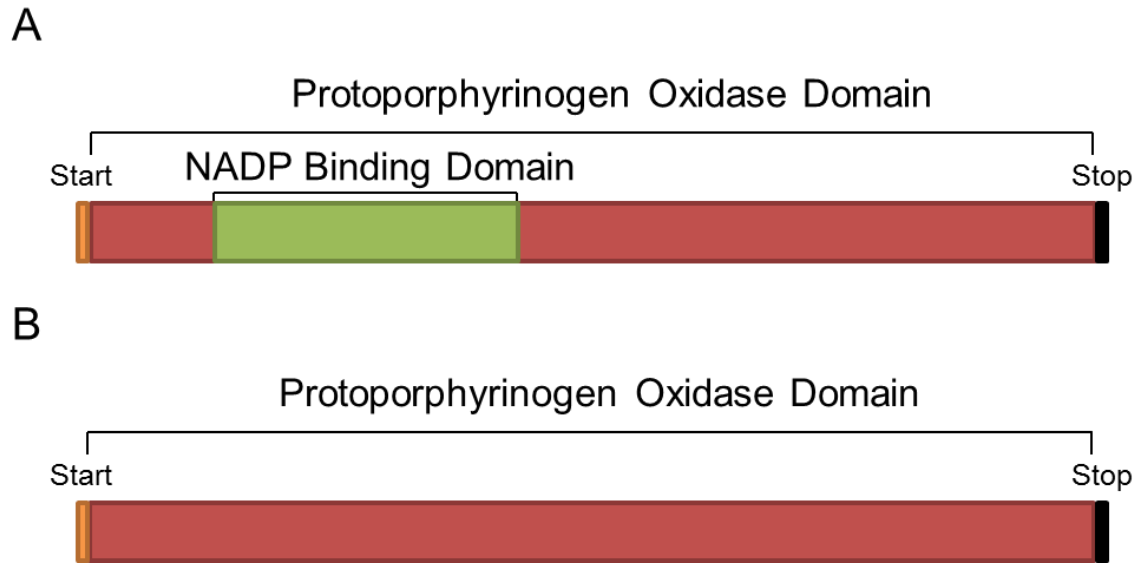
### 1.2.1 Conserved domain and enzyme structure

When the PPO enzyme is present it is responsible for catalysing the oxidation and removal of six electrons from the protogen ring structure, to form protoporphyrin IX. This oxidation allows further modification of the porphyrin ring structure contained within the protoporphyrin IX molecule (Figure 1.1) (Nishimura *et al.*, 1995; Narita *et al.*, 1996; Li and Nicholl, 2005; Patzoldt *et al.*, 2006).



**Figure 1.1:** Oxidation of protoporphyrinogen IX to porphyrinogen IX by protoporphyrinogen oxidase enzyme.

PPO enzymes possess a general architecture with variations in amino acid sequences between organisms. In all eukaryotic, and some prokaryotic organisms, PPO enzymes possess a NAD(P)-binding Rossmann-like domain situated near the N terminus of the PPO protein. PPO enzymes with an NAD(P)-binding Rossmann-like domain tend to use oxygen as the final electron acceptor, while prokaryotic PPO enzymes without this domain, use the cell's respiratory chain as a final electron acceptor (Figure 1.2) (Dailey *et al.*, 1994; Dailey and Dailey, 1996; Kuk *et al.*, 2005; Marchler-Bauer *et al.*, 2013).



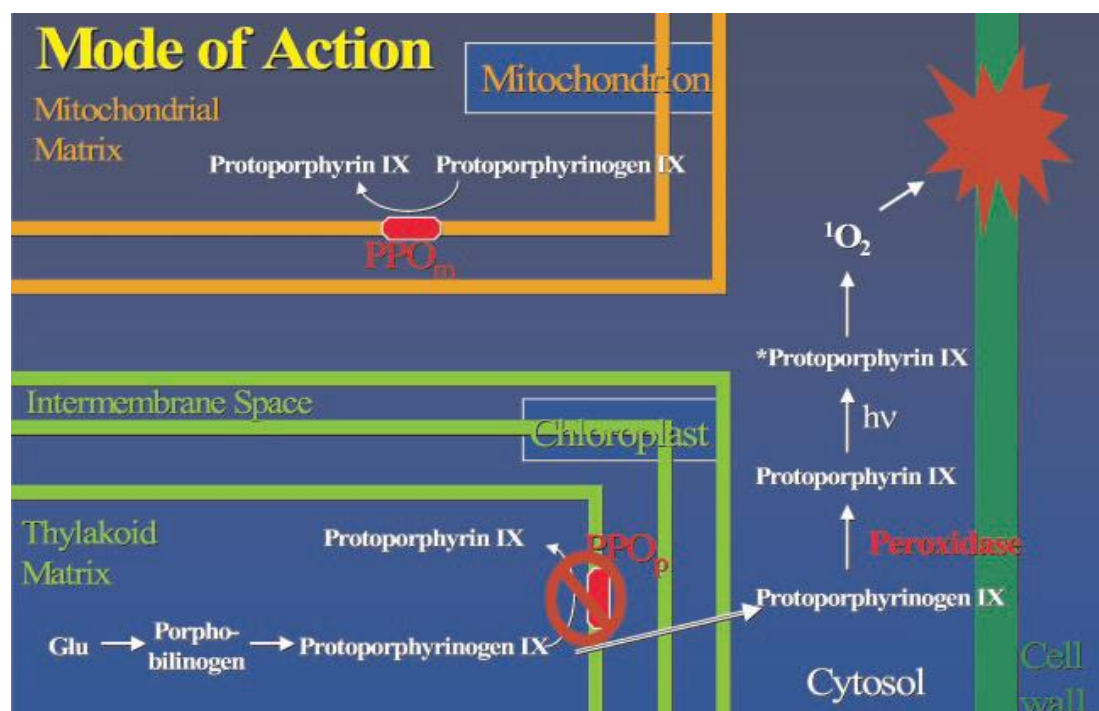
**Figure 1.2:** General conserved domain structure of PPO enzymes in eukaryotes and prokaryotes (**A**) General conserved domain structure of PPO enzymes that utilize oxygen as a final electron acceptor. This conserved domain structure is found in most eukaryotic PPO enzymes and certain bacteria PPO enzymes, such as *B. subtilis*. (**B**) General conserved domain structure of most prokaryotic PPO enzymes, which tend to use an electron transport chain as an electron acceptor.

### 1.2.2 Protoporphyrinogen oxidase's role in heme and chlorophyll synthesis

Eukaryotic PPO enzymes are known to occur in both the mitochondrial and chloroplastic membranes (Jacobs and Jacobs, 1987; Camadro *et al.*, 1991). At that point, protoporphyrin IX is formed as a result of PPO activity and can be modified to form either a heme or chlorophyll structure (Nishimura *et al.*, 1995). Hemes usually contain an iron molecule situated in the centre of the porphyrin ring structure, while chlorophyll contains a magnesium ion instead. Hemes form prosthetic groups in cytochromes, which are required for the functioning of electron chain transports and aid in the indirect generation of ATP (Nishimura *et al.*, 1995). They also form parts of co-factors in catalases, cytochromes, haemoglobin, oxygenases and peroxidases (Chiu *et al.*, 1989; Nishimura *et al.*, 1995). Chlorophyll synthesis on the other hand is used for the absorption and utilization of radiant light energy, which is used to drive photosynthetic processes in algae, cyanobacteria and plants. The synthesis of protoporphyrin IX is, therefore, essential for the synthesis of chlorophyll as a light harvesting pigment and for the synthesis of heme, which serves as an essential cofactor in cytochromes and other enzymes (Nishimura *et al.*, 1995; Li and Nicholl, 2005; Patzoldt *et al.*, 2006).

### 1.2.3 Diphenylether type herbicides and inhibition of PPO activity

DPE class herbicides have been shown to have a strong inhibitory effect on PPO enzymes, binding to the catalytic site of the enzyme and blocking access to its substrate, protoporphyrinogen IX (Camadro *et al.*, 1991; Jacobs *et al.*, 1991). Prior to 1991, it was known that treating plants with DPE herbicides resulted in the accumulation of intracellular photo-reactive protoporphyrin IX, resulting in cellular death and damage via light dependant mechanisms (Camadro *et al.*, 1991; Jacobs *et al.*, 1991). Jacobs *et al.*, (1991) characterised DPE inhibition in plants and described a mode of action for DPE inhibition in plants (Figure 1.3). Briefly, when DPE herbicides inhibit chloroplastic PPO enzymes, this results in the accumulation of protoporphyrinogen IX, which leaks into the cytosol and is oxidised by DPE insensitive peroxidases to form protoporphyrin IX. Accumulated protoporphyrin IX in the cytosol reacts with light to form reactive oxygen species, leading to membrane damage and cell death (Li *et al.*, 2003).



**Figure 1.3:** DPE inhibition in plants. DPE inhibition as described by Jacobs *et al.*, (1991) and illustrated by Li *et al.*, (2003), indicating the synthesis of protoporphyrin IX in both the mitochondria and chloroplast by the PPO enzyme. When the PPO enzyme activity is blocked, protoporphyrinogen IX leaks into the cytosol where it is oxidised to form protoporphyrin IX, which reacts with reactive oxygen species leading to membrane damage and cellular death.

## 1.2.4 PPO isolation and characterization

### 1.2.4.1 PPO isolation and characterization in prokaryotes

Prokaryotic PPO genes have been isolated from *Bacillus subtilis*, *Desulfovibrio gigas*, *Escherichia coli* and *Myxococcus xanthus* as well as various other bacteria (Klemm and Barton, 1987; Hansson and Hedestedt, 1992; Sasarman *et al.*, 1993; Dailey *et al.*, 1994; Nishimura *et al.*, 1995; Dailey and Dailey, 1996). Unlike other prokaryotic PPO enzymes, the *B. subtilis* and *M. xanthus* PPO enzymes use oxygen as a final electron acceptor (Dailey *et al.*, 1994; Dailey and Dailey, 1996). Following the identification of the *E. coli* PPO gene came the isolation of PPO deficient mutants used in screening for eukaryotic and plant PPO genes (Nishimura *et al.*, 1995). Uniquely, the *B. subtilis* PPO was shown to be only weakly inhibited by acifluorfen, which is a DPE class herbicide that strongly inhibits both animal and plant PPO enzymes (Dailey *et al.*, 1994; Dailey and Dailey, 1996; Li and Nicholl, 2005; Kuk *et al.*, 2005). This unique ability of the *B. subtilis* PPO gene led to efforts to express this enzyme in plants, in order to engineer plants that are resistant to PPO inhibiting herbicides (Lee *et al.*, 2000).

### 1.2.4.2 PPO isolation and characterization in eukaryotes

Protoporphyrin oxidase activity has been characterised in mammals as early as 1976 (Poulson, 1976). The interest in isolating and characterising PPO genes in eukaryotes was mainly to understand associated diseases caused by deficient PPO activity, such as variegate porphyria (Nishimura *et al.*, 1995; Narita *et al.*, 1996). To date, PPO encoding genes have been isolated and characterised in animals, fungi, plants, humans and mice (Nishimura *et al.*, 1995; Camadro and Labbe, 1996; Dailey *et al.*, 2002).

### 1.2.4.3 PPO isolation and characterization in plants

An *A. thaliana* PPO cDNA sequence was first isolated by Narita *et al.*, (1996), using the *hemG* deficient mutant VSR-800 *E. coli* strain, to screen an *A. thaliana* cDNA library (Narita *et al.*, 1996). The isolated *A. thaliana* PPO cDNA (NCBI accession number: D83139.1; NCBI: <http://www.ncbi.nlm.nih.gov/>) was 1.7 kb and encoded a 537 amino acid protein with an additional putative leader peptide responsible for transport into the mitochondria (Narita *et al.*, 1996).



Lermontova *et al.*, (1997) reported the isolation of two tobacco *PPO* cDNA's, using the *hemG* deficient VSR-751 *E. coli* strain, derived from the VSR-800 strain (Narita *et al.*, 1996). The first sequence (1644 bp; 59.13 kDa protein; NCBI accession number: Y13465) contained a 50 amino acid putative transit peptide that enabled transport to the chloroplast and was termed *PPO-1*. The second sequence, termed *PPO-2* (1515 bp; 55.4 kDa protein; NCBI accession number: Y13466) was found to be targeted to the mitochondrial membrane. Both proteins only share 27.2% identity in amino acid sequence when aligned with one another (Lermontova *et al.*, 1997). They also noted that the tobacco *PPO-1* and *PPO-2* shared 71.2% and 24.6% sequence identity with the *Arabidopsis PPO* sequence (Narita *et al.*, 1996; Lermontova *et al.*, 1997).

The full length plastid targeted *PPO-1* cDNA sequence for spinach (1689 bp; 59.9 kDa protein; NCBI accession number: AB029492) was isolated and characterised by Che *et al.*, (2000) using the *E. coli* BT3 strain ( $\Delta hemG::Kmr$ ). The translated spinach *PPO-1* was 78% similar to the *Arabidopsis PPO-1* and 71% similar to the tobacco *PPO-1* proteins and contained a 49 amino acid N terminus transit peptide (Che *et al.*, 2000).

The spinach *PPO-2* cDNA sequence (1593 bp; 58.4 kDa protein; NCBI accession number: AB046993.1) was isolated by Watanabe *et al.*, (2001) and was found to have two in-frame start codons that code for two proteins, where one protein is targeted to the chloroplast and the other is targeted to the mitochondria. The sequence displayed 70% identity to the tobacco *PPO-2* cDNA sequence and 28% identity to the spinach *PPO-1* cDNA and a unique 5' 26 amino acids sequence that aligned with the 33 amino acid N terminal sequence also found in maize *PPO-2* cDNA (Figure 1.4). Immunoblot analysis of spinach leaf extracts showed that two products of approximately 59 kDa and 55 kDa, were present in chloroplast and mitochondrial organelles, respectively (Watanabe *et al.*, 2001). This dual targeting peptide would later be shown to also be present in the sorghum hypothetical *PPO-2* as well as in waterhemp (Figure 1.5) (Patzoldt *et al.*, 2006; Rousonelos, 2010).

		N-terminal extension			
Spinach	1	MVILPVSQ	STNL-GLSLVSP----	TKNNPV--MGNVSE	RNOVNOPI-SAKRVAV 47
Maize	1	MLALTASASSASSHPYR	HASAHTRRPRLRAVLAMAG-SDDPRAA-PARS---	VAV	50
Arabidopsis	1	-----	-----MAS-GAVADHQIEAVSG	KRVAV	21
Tobacco	1	-----	-----MAP-SAGEDKH---	SSAKRVAV	18
Potato	1	-----	-----MAP-SAGEDKQNC	P---KRVAV	18
Soybean	1	-----	-----MAS-SATDDN-PRS-V-	KRVAV	18
Yeast	1	-----	-----MLL-PLTK---	LKP-RAK-VAV	16
Mouse	1	-----	-----MGR-T-	-----VIV	7
Human	1	-----	-----MGR-T-	-----VVV	7
				* . . . . .	
Spinach	48	VGAGVSGLA	AAYKPKSNGLVN--TLFEAD--SRAGGK	LKTIVVKD-GLIWDEGANT	97
Maize	51	VGAGVSGLA	AAYRLRQSGVNV--TVFEAA--DRAGGK	IRTNS-EGGFVWDEGANT	100
Arabidopsis	22	VGAGVSGLA	AAYKPKSRGLNV--TVFEAD--GRVGGK	LRSMQON-GLIWDEGANT	71
Tobacco	19	IGAGVSGLA	AAYKPKIHGLNV--TVFEAE--GKAGGK	LRSVSQD-GLIWDEGANT	68
Potato	19	IGAGVSGLA	AAYKPKIHGLNV--TVFEAEFTGRAGGK	LRSLSQD-GLIWDEGANT	70
Soybean	19	VGAGVSGLA	AAYKPKSHGLDV--TVFEAE--GRAGGK	LRSVSQD-GLIWDEGANT	68
Yeast	17	VGGVSGLC	FYFLSKLRPDVEITLFE	SQ--NRTGGWIYSCNTRDMSGNPIMLEK	69
Mouse	8	LGGGISGLA	ASYHLIRGSPPKVILVEGS--KRTGGW	IRSIRGSDGAIF-ELGPR	59
Human	8	LGGGISGLA	ASYHLRSPCPPKVVLE	SS--ERLGGWIRSVRGPNGAIF-ELGPR	59
				* . . . . .	

**Figure 1.4:** Alignment of the N terminal regions of PPO amino acid sequences from various eukaryotic species. Sequences are from spinach (NCBI accession number: BAB60710.1), Arabidopsis (NCBI accession number: BAA11820.1), tobacco (NCBI accession number: CAA73866.1), potato (NCBI accession number: CAA12401.1), soybean (NCBI accession number: BAA76348.1), yeast (NCBI accession number: NP\_010930.1), and humans (NCBI accession number: BAA07538.1) (Watanabe *et al.*, 2001).

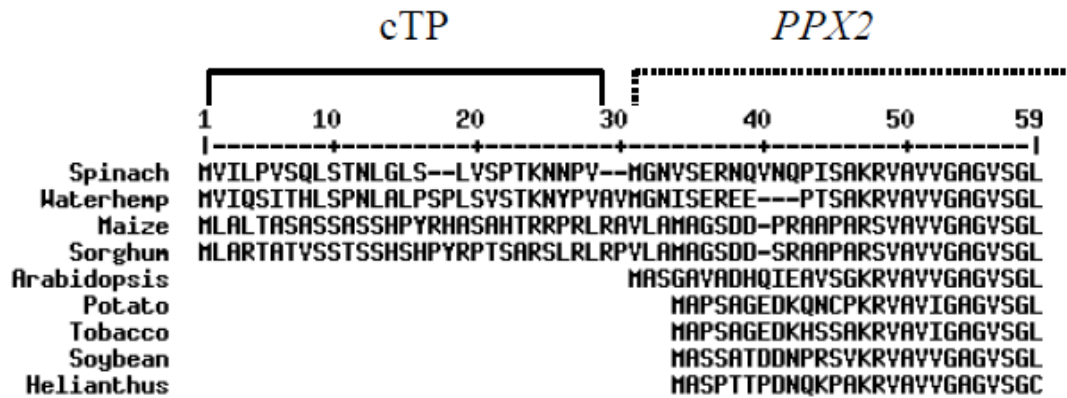
Two *A. thaliana* PPO cDNA sequences were later isolated by Li *et al.* (2003) using the *E. coli* PPO mutant strain SASX38, which is also unable to grow without an exogenous supply of heme or an alternate source of PPO activity (Sasarman *et al.*, 1993). The first sequence, corresponded to the sequence described by Narita *et al.*, (1996) was designated *PPO-1* (NCBI accession number: AX084732). The *Arabidopsis PPO-1* contained a putative peptide sequence targeted to the chloroplast organelle (Li *et al.*, 2003). The second sequence was designated *PPO-2* (NCBI accession number: AX084734), which was 1738 bp long, and encoded a putative transit peptide targeted to the mitochondria. The *PPO-2* cDNA sequence was 53% similar and 28% identical to the *PPO-1* cDNA sequence (Ward and Volrath, 1998; Li *et al.*, 2003). Following the isolation of the *A. thaliana PPO-1* gene and its use as hybridization probe, *PPO-1* genes were also successfully identified in *Zea mays*, *Triticum aestivum*, *Oryza sativa*, *Gossypium hirsutum*, *Beta vulgaris*, *Saccharum officinarum* and *Brassica napus* (Narita *et al.*, 1996; Ward and Volrath, 1998; Li *et al.*, 2003).

From the analysis of isolated plant *PPO* genes, two classes of PPO enzymes can be seen to exist, namely PPO-1 enzymes, which are targeted to the chloroplast and PPO-2 enzymes,

which are targeted to the mitochondria (Ward and Volrath, 1998; Li *et al.*, 2003; Li and Nicholl, 2005; Patzoldt *et al.*, 2006). Additionally, certain PPO enzymes contain in frame transit peptides, which enable dual targeting to the mitochondria or chloroplast (Watanabe *et al.*, 2001; Patzoldt *et al.*, 2006; Rousonelos, 2010).

### 1.2.5 Natural DPE resistance in plants

The mechanism of resistance in *A. tuberculatus* was investigated by Patzoldt *et al.* (2006). Results showed that resistance to lactofen, a DPE class herbicide, was due to an incomplete dominant trait conferred by a single gene. *PPO-1* and *PPO-2* cDNA sequences from both resistant and susceptible *A. tuberculatus* biotypes were isolated, *PPX1* (NCBI accession number: DQ386112), *PPX2* (NCBI accession number: DQ386113) and *PPX2L* (NCBI accession number: DQ386114). The *PPX2L* sequence isolated from resistant *A. tuberculatus* was shown to contain a codon deletion that conferred resistance to lactofen herbicide. Specifically, the codon deletion is a glycine residue deletion at amino acid position 210. Following complementation studies, it was confirmed that the 3 bp deletion for the glycine amino acid was necessary for conferring lactofen resistance to the enzyme encoded by *PPX2L* (Sasarman *et al.*, 1993; Li *et al.*, 2003; Patzoldt *et al.*, 2006). The *PPX2L* was predicted to encode two proteins that contain transit peptides that enable into either the mitochondria or chloroplast, similar to the dual targeting seen in the spinach *PPO-2* (Figure 1.5) (Watanabe *et al.*, 2001; Patzoldt *et al.*, 2006; Rousonelos, 2010).



**Figure 1.5:** Alignment of N terminal extension of plant PPO-2 genes. Amino acid alignment of translated PPO-2 sequences from various plant species, showing the in frame N terminal extension that enables dual targeting to either the mitochondria or chloroplast. Sequences are from spinach (BAB60710.1), waterhemp (NCBI accession number: ABD52329.1), maize (NCBI accession number: NP\_001105004), sorghum (NCBI accession number: XP\_002446710), *Arabidopsis* (NCBI accession number: NP\_196926.2), potato (CAA12401), tobacco (CAA73866.1), soybean (BAA76348), and Helianthus (HELI\_7CDS.CSA1.5882) (<http://www.ncbi.nlm.nih.gov/>) (Rousonelos, 2010).

### 1.2.6 Development of DPE resistant crop plants and *PPO* genes

Two goals are desired with the development of DPE herbicide resistance in crop plants. The first would be the development of a DPE herbicide resistance selection system for the creation of cisgenic and transgenic plants. This would entail the isolation of a gene that encodes for a DPE herbicide resistant PPO enzyme, which can function as an *in vitro* selection gene situated within a plant expression plasmid vector. The second goal would be the creation of transgenic crop plants that would be resistant to DPE herbicides when applied in crop fields. This would essentially enable herbicide treatment in fields so as to kill unwanted weeds, while leaving crop plants unaffected (Li and Nicholl, 2005).

Various strategies have been utilized in order to create DPE herbicide resistant plants. These have involved the improvement of plant *PPO* genes via directed evolution, where these genes were subjected to random mutagenesis and then screened for novel mutations that are resistant to DPE herbicides (Li *et al.*, 2003; Li and Nicholl, 2005). Efforts have also been made to create DPE resistant plant lines, through traditional tissue culture methods and selection on media containing DPE herbicides (Ichinose *et al.*, 1995; Watanabe *et al.*, 2001; Li and Nicholl, 2005). Overexpression of plant and certain microbial *PPO* transgenes have also been used in attempts to create DPE resistant plants (Li and Nicholl, 2005).

Different results have been observed when *PPO* gene expression is altered in either the mitochondria or chloroplast (Narita *et al.*, 1996; Lermontova *et al.*, 1997; Li and Nicholl, 2005; Patzoldt *et al.*, 2006). For example, DPE resistant tobacco and soybean cell lines have been developed by Ichinose *et al.*, (1995). The cell line was initially derived from a photomixotrophic suspension culture and selected for with medium containing S23142, otherwise known as *N*-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide, to decrease chlorophyll levels (Ichinose *et al.*, 1995; Li and Nicholl, 2005). Determination of *PPO* mRNA levels showed that mitochondrial *PPO-2* mRNA levels were ten times higher in YZ1-1S in comparison with wild type cells, while plastid *PPO-1* mRNA levels were the same in both lines. They concluded that the tolerance of YZ1-1S cells to S23142 was due to the up-regulation of the tobacco *PPO-2* gene within these mutant cells (Watanabe *et al.*, 1998; Li and Nicholl, 2005). Likewise, a soybean cell line that is resistant to the DPE herbicide oxyfluorfen was developed by Warabi *et al.*, (2001). They used a stepwise selection method with oxyfluorfen, expression analysis showed that the resistant cell line had higher levels of mitochondrial *PPO-2* mRNA in comparison with wild type cells (Warabi *et al.*, 2001; Li and Nicholl, 2005).

Furthermore, when the *A. thaliana PPO-1* gene was overexpressed in tobacco plants, the resulting transgenic plants were observed to be five-fold more resistant to treatment with acifluorfen (Lermontova and Grimm, 2000). The *B. subtilis PPO* gene was also constitutively expressed and tested in rice. Lee and colleagues (2000) noted that transgenic rice lines with the *B. subtilis PPO* targeted to the chloroplast had higher resistance to DPE herbicides in comparison with lines with the transgene targeted to the mitochondria. Expression of the *B. subtilis PPO* gene in tobacco plants also resulted in transgenic plants being tolerant to treatment with oxyfluorfen, where the transgene was stably transmitted into T<sub>3</sub> rice plants (Choi *et al.*, 1998).

Li *et al.*, (2003) reported on the induction of random mutations in the *Arabidopsis PPO-1* gene, which in turn was used to create a PPO selectable marker system. Random *in vivo* mutagenesis was induced in the cDNA and screened in SASX38 *E.coli* cells grown on medium containing butafenacil. This initial screening identified a *PPO-1* insert containing a single amino acid change near the N-terminus that conferred tolerance to butafenacil by increasing the growth rate of SASX38 cells (Li *et al.*, 2003). A second round of random mutagenesis was performed and 90% of the resulting *PPO-1* inserts contained single amino acid changes. Some of the amino acid changes identified that conferred significant resistance to butafenacil were A220V, Y426C and G221S (Li *et al.*, 2003; Li and Nicholl, 2005). Clones

containing either the A220V or Y426C mutations had delayed growth in the absence of butafenacil. A third round of mutagenesis was performed on these two mutant clones in order to mitigate the growth delay. Of the resulting mutants, one amino acid change in particular, S305L increased growth rate significantly for both A220V and Y426C clones. Site directed mutagenesis of wild type *PPO-1* gene sequences further identified butafenacil resistance without growth defects, such as alanine at position 220 that could be changed to valine, threonine, leucine, cysteine or isoleucine and tyrosine at position 426 that could be changed to cysteine, isoleucine, leucine, threonine or methionine. Further research done by Li and colleagues successfully developed DPE resistant *Arabidopsis* and maize, utilizing constructs derived from the double mutant Y426M + S305L (Li *et al.*, 2003, Li and Nicholl, 2005).

Transgenic plants showed a fifty fold increase in resistance to butafenacil when compared with wild type seedlings (Li *et al.*, 2003; Li and Nicholl, 2005). Transgenic *Arabidopsis* seedlings were also tested for cross tolerance to other DPE herbicides. Transgenic seedlings showed up to tenfold tolerance to fomesafen or lactofen treatment and more than tenfold tolerance to acifluorfen treatment, amongst several other DPE herbicides tested (Li *et al.*, 2003).

In transgenic maize, the Y426M + S305L PPO double mutation conferred butafenacil resistance, resulting in plants resistant to field rates of butafenacil, namely 50  $\mu\text{m}$  butafenacil or higher (Li *et al.*, 2003). Li *et al.*, (2003) also developed this Y426M + S305L PPO double mutant gene as an *in vitro* selectable marker gene for maize transformations. Putative transformed calli were selected on 750 nM butafenacil and resulted in a 19.2% transformation efficiency.

### 1.2.7 PPO as an alternative *in vitro* selection system

Selectable marker systems are available for use in transgenic work for a large number of plant species. However, development of multiple selectable marker systems would allow for trait stacking through sequential transformations (Armstrong, 1999; Li *et al.*, 2003). Furthermore, new selections systems are desired which might reduce the time required for selection of transformants. An example of this was shown in results from Li *et al.*, (2003), where they noted that the time to detect transformants was reduced by 6 to 8 weeks when using light treatment along with the PPO selectable marker system. Furthermore, new selectable marker systems could allow more flexibility in detecting transformed callus when other selective

pressures such as light and temperature are introduced, in addition to a herbicide. The possibility of still utilizing the selectable system after the selection of transformed callus, has also been mentioned. An example of this would be the ability to spray the herbicide used during tissue culture in a field of crops to separate transgenic plants from non-transgenic plants in a field. Another aspect to consider when creating a new selectable marker system is to use a cisgenic strategy, by isolating endogenous genes and to develop these for use in a selectable marker system.

#### 1.2.8 PPO research aims and objectives

- The first objective is to start developing a selectable marker system for sugarcane callus, using a plant *PPO* gene and a DPE herbicide as a selectable marker. Results from Li *et al.*, (2003) were considered, specifically the Y426M + S305L *PPO* double mutated gene, which was used successfully as a selectable marker gene in maize callus grown on medium containing DPE herbicide. To test if this strategy would work in sugarcane, the tobacco *PPO-1* gene will be isolated and targeted mutagenesis performed, to induce double mutations in the gene that correspond to the double mutation used by Li *et al.*, (2003). This double mutated tobacco *PPO-1* gene will then be transformed into sugarcane callus and be constitutively expressed under the maize ubiquitin promoter (*UBI*). Transformed sugarcane callus will then be selected on medium containing DPE herbicide to test whether this double mutated gene could be used as a selectable marker.
- The second objective is to identify additional mutations that could render the isolated tobacco *PPO-1* resistant to DPE herbicides. Once the tobacco *PPO-1* gene is isolated, it will be subjected to random mutation, where the resulting random mutations can be tested for resistance to DPE herbicides. For random mutagenesis, inserts will be transformed into XL1-Red *E.coli* cells. Randomly mutated gene inserts will be tested by transforming into the mutant VSR-800 *E. coli* strain. Mutations that confer DPE herbicide resistance can be identified by their ability to complement the growth defect in VSR-800 cells (Narita *et al.*, 1996).
- The third objective is to isolate a sugarcane *PPO-1* gene to use as a base gene instead of the tobacco *PPO-1*. To isolate the sugarcane *PPO-1*, both 5' Rapid amplification of cDNA ends (5' RACE) and PCR with sorghum based primers will be used, where sorghum based primers will be designed using the putative sorghum *PPO-1* sequence. Once a full length sugarcane *PPO-1* gene is isolated this could be altered and established as a workable *in vitro* selectable marker gene for sugarcane. The use of a sugarcane *PPO-1* gene would be favoured as to us a cisgenic strategy in creating a new selectable marker system for sugarcane callus selection.

### 1.3 Trehalose-6-phosphate Synthase

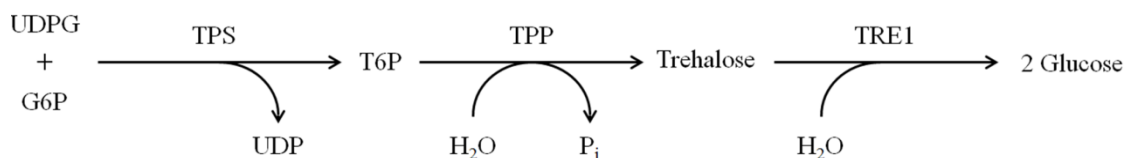
Trehalose is a non-reducing disaccharide, which consists of two  $\alpha$ -glucose molecules joined by an  $\alpha,\alpha$ -1,1-glucoside bond (Schluepmann *et al.*, 2012). This disaccharide is found in various organisms, such as algae, archaea, bacteria, fungi, insects and plants (Schluepmann *et al.*, 2012). It was initially thought that trehalose did not occur in plants, except for resurrection plants, since trehalose accumulated in previously undetectable levels in plants (Muller *et al.*, 1995; Wingler, 2002). Later, trehalose synthesis genes were discovered in various plant species with multiple isoforms (Schluepmann *et al.*, 2012).

Since its detection in plants, various roles have been revealed for trehalose and its intermediate, trehalose-6-phosphate (T6P). One of the functions of trehalose is that of a stress protectant during abiotic stress conditions, where it has been shown to be capable of stabilizing membranes and protein structures during abiotic stresses. It is suggested that during these abiotic stresses, trehalose stabilizes proteins by forming hydrogen bonds with polar residues, replacing the role of water (Crowe *et al.*, 1998; Wingler, 2002; Schluepmann *et al.*, 2012). Much research has been focused on manipulating T6P synthesis in order to create transgenic plants that are resistant to desiccation and high salinity stresses (Jang *et al.*, 2003; Penna, 2003; Zang *et al.*, 2011). Research into the function of T6P has also indicated that it could act as a signal of sucrose availability in plants (Kolbe *et al.*, 2005; Lunn *et al.*, 2006; Schluepmann *et al.*, 2012). T6P has been shown to influence the regulation of enzymes involved in carbon utilization, in turn effecting enzymes such as sucrose non-fermenting 1-related protein kinase (SnRK1) and ADP-glucose pyrophosphorylase (AGPase) (Kolbe *et al.*, 2005; Lunn *et al.*, 2006; Zhang *et al.*, 2009; Schluepmann *et al.*, 2012; Martins *et al.*, 2013). T6P levels have been shown to increase in *Arabidopsis* seedlings that were supplied with sucrose exogenously (Lunn *et al.*, 2006). However, the exact relation between T6P levels and sucrose levels is not fully understood yet. One theory put forward so far is that T6P acts as a signal of carbon availability in plants, where T6P is synthesized when sucrose is readily available and so T6P activates enzymes or processes that are involved in anabolic processes within the plant (Lunn *et al.*, 2006; Schluepmann *et al.*, 2012; O'Hara *et al.*, 2012).



### 1.3.1 Trehalose synthesis pathways

Many alternative pathways for trehalose synthesis have been identified in bacterial and fungal species, but one pathway, namely the TPS/TPP pathway, both synthesizes and uses T6P (Koen *et al.*, 2000). This pathway is well characterised and is present in bacteria, fungi and plants (Figure 1.6). This pathway uses uridine diphospho-glucose (UDPG) and glucose-6-phosphate (G6P) as substrates to synthesize trehalose and consists of two reactions. The first is catalysed by the trehalose-6-phosphate synthase enzyme (TPS) (EC 2.4.1.15) and joins UDPG to G6P via an  $\alpha,\alpha$ -1,1-glucoside bond, resulting in the formation of T6P. In the second reaction, T6P is dephosphorylated, which is catalysed by trehalose-6-phosphate phosphatase (TPP) (EC 3.1.3.12) to form trehalose. Trehalose can be further hydrolysed by trehalase (EC 3.2.1.28) to produce two molecules of glucose (Elbein *et al.*, 2003; O'Hara *et al.*, 2012).



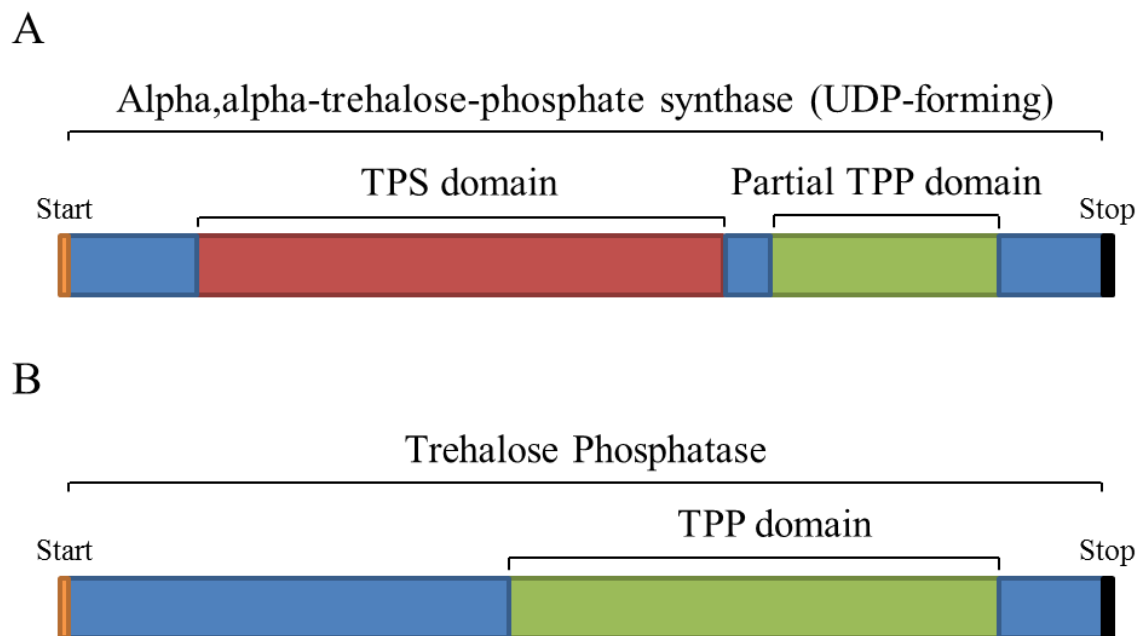
**Figure 1.6:** Basic outline of the TPS/TPP pathway. Abbreviations, trehalose-6-phosphate synthase (TPS); uridine diphospho-glucose (UDPG); glucose-6-phosphate (G6P); trehalose-6-phosphate phosphatase (TPP); and trehalase (TRE1).

In literature concerning bacteria, *TPS* genes are named *otsA* and *TPP*, *otsB*, where the *otsAB* operon can code for trehalose synthesis in the TPS/TPP pathway (Giaever *et al.*, 1988). In literature concerning yeast, *TPS* genes are sometimes referred to as *TPS1* and *TPP* as *TPS2* (Koen *et al.*, 2000; Schluepmann *et al.*, 2012).

### 1.3.2 Domains and general protein structure

All bacterial TPS enzymes so far characterised contain a single TPS domain, which is sometimes called a GT1 TPS domain. This domain is part of the glycosyltransferase GTB type superfamily (Marchler-Bauer *et al.*, 2013). Bacterial TPP enzymes usually contain a single TPP domain, which is also called PRK10187 and forms part of the PRK101817 superfamily (Marchler-Bauer *et al.*, 2013). Fungal TPS enzymes, such as the *S. cerevisiae* TPS enzyme and the *Schizosaccharomyces pombe* TPS enzyme, possess a single TPS domain (Schluepmann *et al.*, 2012). Most functional plant TPS enzymes characterized so far possess a

TPS domain situated near the N terminus and a partial TPP domain near the C terminus (Figure 1.7) (Schluepmann *et al.*, 2012). More specifically the TPS domain in plant TPS sequences is the GT1 TPS domain and the partial TPP domain is called trehalose\_PPase (Marchler-Bauer *et al.*, 2013). In plant *TPS* sequences, both these domains form part of a larger alpha,alpha-trehalose-phosphate synthase (UDP-forming) domain (Figure 1.7). The amino acid sequences in these domains are longer than similar domains found in yeast, but are similar in that the TPS domain is active and the TPP domain is incomplete and unable to catalyse the phosphorylation of T6P. Plant TPP enzymes, however, possess a single catalytically active TPP domain, situated within a larger trehalose phosphatase domain (Figure 1.7) (Schluepmann *et al.*, 2012; Marchler-Bauer *et al.*, 2013).



**Figure 1.7:** General conserved domain structure of plant TPS and TPP proteins. **(A)** a functional plant trehalose-6-phosphate (TPS) protein, which contains a functional TPS domain on the N terminus of the protein and a partial TPP domain on the C terminus; **(B)** a plant trehalose-6-phosphate phosphatase (TPP).

### 1.3.3 Isolation and characterization of trehalose synthesis genes

T6P and trehalose synthesis genes have been isolated or characterised in various eukaryotes, where the function of these genes can vary across the different eukaryotic kingdoms and phylums.

### 1.3.3.1 Characterization of trehalose synthesis in yeast

Trehalose synthesis genes have been extensively researched in yeast, where a working model has been put forward for its function in regulating carbon metabolism and glycolysis (Blazquez *et al.*, 1993; Schluepmann *et al.*, 2012). Research in yeast had also resulted in the discovery of mutants with defects in these trehalose synthesis pathways. These greatly aided in the identification of *TPS* and *TPP* genes in plants (Gonzalez *et al.*, 1992; O'Hara *et al.*, 2012; Schluepmann *et al.*, 2012)

In yeast, trehalose has been suggested to act as a storage carbohydrate along with glycogen, but mainly it has been proposed to act as a stress protectant (Bell *et al.*, 1992). *TPS* and *TPP* genes have been isolated and characterized in *S. cerevisiae* and *Schizosaccharomyces pombe* (Bell *et al.*, 1992; Blazquez *et al.*, 1994). Studies on *S. cerevisiae* mutants suggested that the yeast *TPS1* gene and T6P regulate the flow of glucose into the glycolysis reaction (Van Aelst *et al.*, 1993; Blazquez *et al.*, 1993; Neves *et al.*, 1995; Zentella *et al.*, 1999), while studies using *Schizosaccharomyces pombe* suggest that T6P is needed for spore germination (Blazquez *et al.*, 1994).

Initially it was observed that the *S. cerevisiae* mutants, *fdp1* and *cif1* were unable to grow on media containing glucose as the only carbon source. Both these mutants were used during studies conducted to understand the regulation of glycolysis in yeast and the role of fructose-1,6-diphosphate (Van de Poll *et al.*, 1974; Navon *et al.*, 1979; Blazquez *et al.*, 1994). The *fdp1* and *cif1* mutants were renamed to *tps1Δ* mutants with the discovery that the yeast *TPS1* gene shared a high similarity to the *cif1 fdp1* genes. Similar to *fdp1* and *cif1* mutants, *tps1Δ* mutants were unable to grow on media containing glucose (Bell *et al.*, 1992). The addition of glucose to *tps1Δ* mutants did not cause an increase in cyclic adenosine monophosphate (cAMP) concentrations but, instead caused a depletion of ATP levels and an increase in glycolytic intermediates (Van de Poll *et al.*, 1974; Navon *et al.*, 1979; Gonzalez *et al.*, 1992; Blazquez *et al.*, 1994). These results suggested that T6P regulated the rate at which the initial steps of glycolysis occurred (Blazquez *et al.*, 1993). Later the yeast *TPS1* enzyme complex, along with its product T6P were shown to inhibit the catalytic activity of hexokinase II which regulates the entrance of glucose into glycolysis (Blazquez *et al.*, 1993; Schluepmann *et al.*, 2012)

Loundesborough and Vuorio, (1991) reported the purification of a proteolytically modified enzyme complex with an approximate size of 800 kDa from *S. cerevisiae*. This was identified

to have both TPS and TPP activity and contained three polypeptides of 57, 86 and 93 kDa. Additionally they also purified a protein dimer composed of 58 kDa subunits, which increased the activity of TPS but not TPP activity (Loundesborough and Vuorio, 1991; Bell *et al.*, 1992). Bell *et al.*, (1992) added to these results by isolating the *TPS1* gene that encoded the smallest subunit of the TPS/TPP complex. That contained an open reading frame of 1485 bp, and coded for a protein of 495 amino acids (Bell *et al.*, 1992). Upon sequencing the *TPS1* gene it was found to be almost identical to the *CIF1* gene, which was known to be involved in de-activating carbon catabolites. In their report, Bell and colleagues generated a yeast mutant containing disrupted *TPS1*. This mutant was confirmed to have low TPS activity and was unable to grow on media containing glucose, similar to *fdp1* and *cif1* mutants (Bell *et al.*, 1992).

The effects of T6P on inhibiting enzymes involved in the initial stages of glycolysis were investigated by Blazquez *et al.*, (1993). Specifically, they looked at the effects of T6P on phosphorylation of glucose and fructose by hexokinase isoforms in a number of organisms (Blazquez *et al.*, 1993; Blazquez *et al.*, 1994). In *S. cerevisiae* they reported that T6P competitively inhibited the phosphorylation of glucose and fructose by hexokinase II and weakly inhibited hexokinase I (Blazquez *et al.*, 1993). When yeast *tps1Δ* mutants were fed glucose, a depletion of ATP levels and an increase in glycolytic intermediates was observed (Blazquez *et al.*, 1994). Disruption of the hexokinase II encoding gene, *HXK2* in *tps1Δ* mutants, restored their ability to grow on media with glucose as a carbon source. It was concluded that T6P acts as a feed-back inhibitor of hexokinase II in yeast (Blazquez *et al.*, 1994). This would mean that a disruption in the yeast *TPS1* gene causes uncontrolled phosphorylation of the fermentable sugars glucose and fructose which leads to sequestration of the phosphate pool as G6P and F6P. This is accompanied by an inability to produce ATP because of the lack of phosphate (Blazquez *et al.*, 1993; Blazquez *et al.*, 1994).

Functional *TPS* genes can be identified by complementation of *tps1Δ* mutants grown on media containing glucose. These have been used successfully to isolate functional *TPS* genes from many eukaryotic organisms (Gonzalez *et al.*, 1992; O'Hara *et al.*, 2012; Schlupepmann *et al.*, 2012).

### 1.3.3.2 Characterization of trehalose synthesis in plants

Early research had identified trehalose accumulation in desiccation resistant plants, such as *Selaginella lepidophylla*, *Myrothamnus flabellifolius* and *Craterostigma planitagineum*

(Adams *et al.*, 1990; Drennan *et al.*, 1993; Crowe, 2002; O'Hara *et al.*, 2012). It was hypothesised that in these plants trehalose protected against desiccation. In crop plants, trehalose synthesis could only be confirmed by trehalase activity, which was found in sugarcane (Glasziou and Gayler, 1969; Alexander, 1972; Bosch, 2005). Trehalose was eventually detected in crop plants through methods utilizing gas chromatography mass spectrometry (GC-MS), first in potato tuber extracts and secondly in *Arabidopsis* (Roessner *et al.*, 2000; Vogel *et al.*, 2001). Characterization of the *Arabidopsis* TPS encoding gene, *AtTPS1* (NCBI accession number: NM\_106505.4), was accompanied by detection of its activity in protein extracts (Blazquez *et al.*, 1998). With the sequencing of the *Arabidopsis* genome in 2000, eleven TPS isoforms were identified through alignment searches (Leyman *et al.*, 2001).

In plants, functional TPS genes have been isolated and characterized in *A. thaliana*, *S. lepidophylla*, *Oryza sativa* and *Zea mays* (Blazquez *et al.*, 1998; Zentella *et al.*, 1999; Jiang *et al.*, 2010; Zang *et al.*, 2011). Although many TPS isoforms can exist in a single plant species, not all are functional. For example in *Arabidopsis*, of the eleven TPS genes known to exist only *AtTPS1* (Blazquez *et al.*, 1998) and *AtTPS6* (Chary *et al.*, 2008) have been shown to encode functional enzymes through complementation assays with yeast *tps1Δ* mutants (Blazquez *et al.*, 1998; Leyman *et al.*, 2001). It has been proposed that these isoforms could have an unknown regulatory role(s) (Vandesteene *et al.*, 2010; Schlupepmann *et al.*, 2012).

Plant TPS genes have been divided into two classes based on their similarity to the *S. cerevisiae* *ScTPS1* and *ScTPS2* genes (Leyman *et al.*, 2001). Current models suggest that functional TPS genes are part of the class I TPS gene subfamily, while the inactive TPS isoforms belong to the class II subfamily of TPS genes. An exception to this seems to be *AtTPS6*, which was classed as a class II TPS (Leyman *et al.*, 2001), but has been shown to possess both TPS and TPP activity (Chary *et al.*, 2008).

### 1.3.4 Possible functions of trehalose synthesis in plants

#### 1.3.4.1 Effects of trehalose synthesis on plant growth

Results from experiments done on *S. cerevisiae* were initially considered when determining the role of trehalose and T6P in plant species. However, these would be shown to have dissimilar functions in plant species compared with yeast as well as between diverse plant

species. A primary difference is that the hexokinase activity in *Arabidopsis* is not inhibited by T6P (Eastmond *et al.*, 2002; Schluepmann *et al.*, 2012). Instead results suggest that it plays a role as a signal molecule in carbon metabolism, growth regulation and plant embryo development. Also, trehalose accumulation has been observed in certain desiccation resistant plants but trehalose accumulation in other plants is too low to serve as a primary metabolite or transport carbohydrate (Muller *et al.*, 1995; O'Hara *et al.*, 2012). Overexpression of *TPS* genes from either *E. coli* or *S. cerevisiae* in plants has resulted in increased resistance to abiotic stresses in plants. Some of these transgenic plants have also displayed stunted growth or other aberrations, suggesting that T6P plays a role in growth development in plants (Schluepmann *et al.*, 2012; O'Hara *et al.*, 2012).

Early experiments conducted on *Cuscuta reflexa* indicated that exogenously fed trehalose was toxic to plants and that it acted as an inhibitor of plant growth (Veluthambi *et al.*, 1981). Species that exhibited high trehalase activity however, seemed to be unaffected by trehalose feeding, displaying no growth inhibition (Veluthambi *et al.*, 1981). Furthermore, *Arabidopsis* seedlings were growth arrested when grown on medium containing trehalose. Specifically root growth was inhibited, while shoots accumulated starch and showed decrease levels of sucrose (Wingler *et al.*, 2000; Schluepmann *et al.*, 2004). However, transgenic plantlets expressing the *E. coli* trehalase gene *treF* were capable of normal growth (Schluepmann *et al.*, 2004). Later research attributed the growth inhibition to be due to increased T6P levels (Schluepmann *et al.*, 2004).

Schluepmann *et al.*, (2004) reported a rapid increased of endogenous T6P levels in *Arabidopsis* plantlets that were fed trehalose exogenously. Transgenic *Arabidopsis* expressing either an *E.coli* T6P hydrolase or trehalase genes were capable of uninhibited growth on trehalose media, but growth was noted to be slower (Schluepmann *et al.*, 2004). Schluepmann *et al.*, (2004) also reported microarray analysis data that showed that increased T6P levels correlated with the expression of genes involved in responses to abiotic stresses. What has not been determined yet is how endogenous T6P levels rise in response to high trehalose supplied exogenously. Another undetermined factor is how exactly these increased T6P levels cause growth aberrations and growth inhibition. It has been proposed that increases in T6P levels could influence the expression of genes involved in responses to abiotic stress, where this expression in turn affects plant growth (Schluepmann *et al.*, 2004; Schluepmann *et al.*, 2012; O'Hara *et al.*, 2012).

These data indicate that the presence of increased trehalose or T6P can be detrimental to plant growth. Exceptions to this were shown with the overexpression of TPS activity in rice and sugarcane. Li *et al.*, (2011) overexpressed *OsTPS1* in rice and observed increased tolerance to abiotic stresses, without growth aberrations. Zhang *et al.*, (2006) reported on the overexpression of a fungal *Grifola frondosa* TPS in sugarcane, which improved drought tolerance without any growth aberrations. This would also suggest that monocotyledonous plant species may not be sensitive to fluctuations in T6P and trehalose levels (Zhang *et al.*, 2006; Li *et al.*, 2011).

Other experiments have also shown that T6P is also necessary for normal growth and embryo development. Eastmond *et al.* (2002) reported that *Arabidopsis AtTPS1* deletion mutant seeds failed to germinate, with embryo development becoming terminated at the torpedo stage. These seeds still failed to germinate even after trehalose was supplied in the medium. *Arabidopsis AtTPS1* deletion mutant seeds expressing the *E. coli otsA* gene were, however, capable of germination (Schluepmann *et al.*, 2003). These results indicate that T6P is required for embryo development in *Arabidopsis*, but its exact role is still unclear (Schluepmann *et al.*, 2012; O'Hara *et al.*, 2012).

Later experiments suggested that T6P acts as a signal of carbon availability. Transgenic *Arabidopsis* seedlings overexpressing the *otsB* and T6P-hydrolase encoding *treC* gene were generated by Schluepmann *et al.*, (2003), to create plants with lowered T6P levels. *Arabidopsis* overexpressing *otsA* were also generated. Seedlings with lowered T6P levels displayed slow growth when grown on glucose, fructose or sucrose (Schluepmann *et al.*, 2003). In contrast, seedlings overexpressing *otsA* grew faster than wild type *Arabidopsis* when grown on medium containing sucrose. Seedlings overexpressing the *E. coli* trehalase *treF* gene were observed to have similar growth to wild type seedlings. These results indicate that T6P is required for normal carbon utilization in *Arabidopsis* seedlings, since T6P overexpression seems to improve growth when sugars are supplied in high amounts. Increased T6P as a result of trehalose feeding has been shown to inhibit plant growth, but this growth inhibition can be overcome by the addition of sucrose to medium (Wingler *et al.*, 2000; Schluepmann *et al.*, 2004). Thus, T6P is inhibitory to growth in plants, unless it is increased in balance with carbon supply where it would improve growth. Thus, it has been proposed that T6P acts as a signal of carbon availability in plants (Schluepmann *et al.*, 2003; Schluepmann *et al.*, 2004). Results by Lunn *et al.*, (2006) also supported this theory, as they observed an increase in *Arabidopsis* endogenous T6P levels in response to exogenously fed sucrose.

#### 1.3.4.2 Effects of T6P on AGPase and SnRK1

Some of the previously mentioned studies indicated that the toxicity of trehalose was due to the increase in starch content (Wingler *et al.*, 2000). The *Arabidopsis* starch-less mutant *adg1-1* has also been observed to have partial resistance to growth inhibition when grown on trehalose (Fritzius *et al.*, 2001). These results indicate that trehalose may lead to the accumulation of starch and a decrease of transport carbohydrates, such as sucrose. This shift in carbon metabolism would cause growth inhibition, due to sucrose not being available for transport throughout the plant (Wingler *et al.*, 2000; O'Hara *et al.*, 2012). However, this theory is not supported by the observation that trehalose inhibits growth in the starch-less *pgm-1 Arabidopsis* mutant (O'Hara *et al.*, 2012; Schluepmann *et al.*, 2012).

A study by Kolbe *et al.*, (2005) reported that *Arabidopsis* overexpressing *otsA* had increased redox activation of AGPase activity and starch synthesis, while *otsB* overexpression prevented the redox activation of AGPase in response to sucrose feeding or trehalose feeding. Intact chloroplasts incubated in T6P were also shown to increase the reductive activation of AGPase activity. Kolbe *et al.*, (2005) concluded that T6P increased AGPase activity by increasing post translational redox activation of AGPase. The precise mechanism of how T6P led to the activation of AGPase was not shown. Results from Lunn *et al.*, (2006) supported the theory that T6P acts as a signal of carbon availability in plants. *Arabidopsis* seedlings were first starved of sucrose, after which sucrose was fed exogenously. In these seedlings, T6P levels were observed to increase 26 fold in response to sucrose re-supply (Lunn *et al.*, 2006). These increases in T6P were also accompanied by redox activation of AGPase activity and increased starch synthesis. Lunn *et al.*, (2006) did conclude that T6P could act as a signal of carbon availability in plants, mediating sucrose induced changes in starch synthesis. Martins *et al.*, (2013) investigated this proposed theory further by controlling the expression of the *otsA* gene in *Arabidopsis*, via an ethanol inducible promoter. During the day with ethanol induction they observed an 11 fold increase in T6P levels, followed by an increase in starch accumulation, but they showed that this increase in starch was not due to redox activation of AGPase. Martins *et al.*, (2013) concluded that T6P is probably part of a signalling pathway, which mediates the feedback regulation of starch breakdown by sucrose. They also theorised then that T6P links starch degradation to the sucrose demand by growing organs during night time.

SnRK1 is a plant protein kinase that shares significant homology with the yeast sucrose non-fermenting 1 kinase (SNF-1) and is involved in plant energy signalling. SnRK1 has been



shown to activate genes involved in catabolic processes and photosynthesis, while also deactivating anabolic processes (Zhang *et al.*, 2009; Schlupepmann *et al.*, 2012; O'Hara *et al.*, 2012). Interestingly, SnRK1 has also been shown to phosphorylate class II TPS proteins and regulate their transcription (Glinski and Weckwerth, 2005; Harthill *et al.*, 2006; Baena-Gonzalez *et al.*, 2007; Zhang *et al.*, 2009). Zhang *et al.*, (2009) showed that SnRK1 activity in *Arabidopsis* seedlings is inhibited by T6P. This was shown to be non-competitive and required a yet unknown intermediate (Zhang *et al.*, 2009). Further analysis showed that SnRK1 activity was inhibited in all tissue of *Arabidopsis* seedlings except mature leaves. Microarray analysis also showed that T6P up-regulated genes that are usually down-regulated by SnRK1 (Zhang *et al.*, 2009). Delatte *et al.*, (2011) reported a down regulation of marker genes in *Arabidopsis* seedlings grown on trehalose media, where those marker genes were known to be controlled by SnRK1 activity and bZIP11. A more complex system of regulation exists with SnRK1 where the exact relations with T6P levels are not yet clear. Regardless, T6P has been shown at a basic level to inhibit SnRK1 activity (Zhang *et al.*, 2009; Delatte *et al.*, 2011).

### 1.3.5 Expression of *TPS* in plants

Attempts have been made to overexpress trehalose synthesis genes from *S. cerevisiae* and *E. coli* to create transgenic plants with increased abiotic stress tolerance. Overexpression of *TPS* genes in transgenic *Arabidopsis* and tobacco plants resulted in increased abiotic stress tolerance and photosynthetic capabilities, but also resulted in stunted growth and lancet leaf formation (Penna, 2003). Similar experiments using *TPP* genes led to reduced photosynthetic rates, yet no growth aberrations (Holmstrom *et al.*, 1996; Goddijn *et al.*, 1997; Romero *et al.*, 1997; Goddijn and Van Dun, 1999; Paul *et al.*, 2001; Penna, 2003). Tobacco plants constitutively expressing *ScTPS1* displayed growth stunting, lancet shaped leaves and reduced sucrose, along with increased drought tolerance (Romero *et al.*, 1997). These growth defect phenotypes would later be eliminated through either controlled expression of *TPS* or by expressing bi-functional fusions of *TPS* and *TPP* genes, while still maintaining increased abiotic stress tolerance. It had also been noted that reports of growth defects due to trehalose accumulation, were mostly observed in dicotyledonous plant species (Garg *et al.*, 2002; Penna, 2003; Li *et al.*, 2011). A bi-functional fusion of the *otsA* and *otsB* genes was characterised by Seo *et al.*, (2000), where the encoded fusion TPSP protein was shown to have a higher efficiency due to the increased proximity of the TPS and TPP protein domains. This TPSP protein was transformed into rice and expressed under the control of a tissue specific and stress inducible promoter. Transgenic rice plants displayed increased tolerance to

abiotic stresses, with no growth aberrations (Garg *et al.*, 2002). Li *et al.*, (2011) reported the overexpression of an N terminal truncated *OsTPS1* gene in rice that was capable of conferring increased tolerance to cold, salinity and drought stresses with no growth aberrations. This showed that the increase of TPS activity alone in rice did not cause growth defects as it did in *Arabidopsis* and tobacco. Stress related genes *WS118*, *RAB16C*, *HSP70* and *ELIP* were found to be up-regulated in in transgenic rice lines overexpressing *OsTPS1* (Li *et al.*, 2011).

### 1.3.6 Trehalose in sugarcane

The earliest indication of trehalose synthesis being present in plants was the detection of trehalase activity in sugarcane in 1969 (Glasziou and Gayler, 1969; Alexander, 1972; Fleischmacher *et al.*, 1980; Bosch, 2005). Current methods are capable of detecting the small amounts of trehalose that occur in plants, but reports of trehalose accumulation in sugarcane are sparse (Bosch, 2005). Trehalose accumulation in five sugarcane cultivars was reported by Bosch (2005), using quadropole GC-MS to measure sucrose and trehalose in sugarcane internode tissue. Trehalose was found to accumulate at levels ranging from 0.42 to 078 nmol/g internode tissue, in comparison with sucrose levels which range from 132 to 293  $\mu$ mol/g internode tissue (Bosch, 2005). The *Grifola frondosa* trehalose synthase gene was constitutively expressed in sugarcane by Zhang *et al.*, (2006), which resulted in transgenic sugarcane that displayed increased abiotic stress resistance. Zhang *et al.*, (2006) reported that trehalose accumulated in transgenic sugarcane leaves up to 8.805 and 12.863 mg/g fresh weight, whereas trehalose levels in non transgenic sugarcane was undetectable.

*TPS* expression has been studied in sugarcane placed under water or temperature stresses, using partial sugarcane *TPS* and *TPP* sequences (McCormick *et al.*, 2009; Nicolau *et al.*, 2013). McCormick *et al.*, (2009) reported a decrease in *TPS* and an increase in *TPP* expression as sucrose levels increased in sugarcane leaves subjected to cold girdling. These results again suggest a role for T6P as a sugar signal in plants (McCormick *et al.*, 2009). A better understanding of the influence T6P has on sucrose levels could potentially lead to increasing sucrose content in transgenic sugarcane (McCormick *et al.*, 2009; Schluemann *et al.*, 2012).

### 1.3.7 Trehalose research aim and objectives

The reported research concerned with T6P and trehalose synthesis in plants, aimed to understand the role of T6P and trehalose in sugarcane carbon metabolism. Various signalling roles have been proposed so far but limited information is available on the influence that trehalose and T6P can have on sucrose levels, where these shifts in carbon metabolism can also affect plant growth. Sugarcane is a plant known to naturally accumulate high amounts of sucrose, compared with other plants. Understanding sucrose synthesis in sugarcane could aid in increasing sucrose content and so lead to better yields. Further understanding of the effect T6P has on sucrose could aid in this objective. .

- The first research objective is to determine whether the manipulation of T6P levels in transgenic plants could potentially aid in manipulating sucrose levels in plants. This would be achieved by overexpressing the *E. coli otsA* and *otsB* genes separately in sugarcane, where these transgenes were constitutively expressed under the control of the maize ubiquitin promoter. Once transgenic plants are grown, expression studies can be performed and sugar levels measured, in order to correlate these and understand the effects T6P has on sucrose content in sugarcane.
- The second objective is to isolate a functional *SoTPS* gene from sugarcane. A putative *SoTPS* will have to be isolated, where this will then have to be shown to be functional by complementing *S. cerevisiae tps1Δ* mutants grown on medium containing glucose. Once a functional *SoTPS* gene is isolated further characterisation can be performed on the encoded enzyme and its biochemical properties. A functional *SoTPS* gene could also be transformed into sugarcane with the goal of overexpression or gene silencing.

## 1.4 References

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## **Chapter 2**

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# **Trehalose-6-phosphate Synthesis in Sugarcane**

## 2.1 Introduction

Trehalose is a non-reducing disaccharide found in various prokaryotic and eukaryotic organisms such as algae, bacteria, fungi, insects and some plant species. In trehalose synthesis both uridine diphospho-glucose (UDPG) and glucose-6-phosphate (G6P) are used as substrates. Trehalose-6-phosphate synthase (TPS) catalyses the bonding of UDPG to G6P via an  $\alpha,\alpha$ -1,1-glucoside bond, while trehalose-6-phosphate phosphatase (TPP) catalyses the dephosphorylation of T6P to form trehalose. Since its detection in plants, various roles have been revealed for trehalose and its intermediate, trehalose-6-phosphate (T6P). Trehalose has been shown to act as an abiotic stress protectant in fungi, bacteria and certain resurrection plants (Crowe *et al.*, 1998; Crowe, 2002; Wingler, 2002). However, initial genetic manipulation of trehalose synthesis in transgenic plants, to enhance stress tolerance, led to plant growth aberrations such as detrimental development and embryogenesis (Penna, 2003). In monocot plant species the altering of trehalose synthesis however does not seem to result in growth aberrations (Zhang *et al.*, 2006; Li *et al.*, 2011).

Further research into the function of T6P has also shown possible involvement in carbon utilization in plants. T6P, along with an unknown intermediate, has been shown to inhibit sucrose non-fermenting 1 related protein kinase (SnRK1) activity in *Arabidopsis* seedlings (Zhang *et al.*, 2009). However, T6P is unable to inhibit SnRK1 in mature leaves of *Arabidopsis* (Zhang *et al.*, 2009). Martins *et al.*, (2013) had shown that increasing T6P levels via an ethanol promoter resulted in starch accumulation, but this was not caused by redox activation of ADP-glucose pyrophosphorylase (AGPase). It was also shown that at night, increasing T6P inhibited starch degradation. These results together with results of T6P effects on plant development indicate a possible role for T6P as a signal of sugar status in plants, although the exact role of T6P and its effects on sugar levels in plants is still being elucidated (Schluepmann *et al.*, 2004; Lunn *et al.*, 2006; Schluepmann *et al.*, 2012; Martins *et al.*, 2013).

So far, a number of functional *TPS* genes have been isolated from plants, including *Arabidopsis AtTPS1* (NCBI accession number: NM\_106505.4) and *AtTPS6* (NCBI accession number: NM\_202376.2), *Selaginella lepidophylla SITPS1* (NCBI accession number: U96736.1), maize *ZmTPS1* (NCBI accession number: EU659122.2) and rice *OsTPS1* (NCBI accession number: HM050424) (Blazquez *et al.*, 1998; Zentella *et al.*, 1999; Jiang *et al.*, 2010; Zang *et al.*, 2011). In sugarcane, trehalase activity was detected as early as 1969, which indicated that trehalose may be present in sugarcane as well (Glasziou and Gayler, 1969; Alexander, 1973; Fleischmacher *et al.*, 1980; Bosch, 2005). Reports of the presence of



trehalose in sugarcane have been limited due to difficulties in measuring trace amounts of it in plants (Bosch, 2005). Accumulation of trehalose in five sugarcane cultivars was reported by Bosch in 2005. Specifically, trehalose and sucrose levels were measured in different sugarcane internode tissue, using quadrupole GC-MS. Bosch, (2005) reported trehalose accumulating at levels ranging from 0.42 to 078 nmol/g internode tissue, in comparison with sucrose levels ranging from 132 to 293  $\mu\text{mol/g}$  internode tissues. When the *Grifola frondosa* (mushroom) trehalose synthase gene was constitutively expressed in sugarcane an increase in abiotic stress resistance was seen (Zhang *et al.*, 2006). This higher level of resistance was accompanied with an increase in trehalose levels of around 8-12 mg/g leaf tissue in contrast to undetectable levels in the untransformed leaves (Zhang *et al.*, 2006). Additionally, *TPS* expression has been studied in sugarcane using partial sugarcane *TPS* and *TPP* sequences (McCormick *et al.*, 2009; Nicolau *et al.*, 2013). McCormick *et al.*, (2009) reported a decrease in *TPS* expression and an increase in *TPP* expression as sucrose levels increased in sugarcane leaves subjected to cold girdling. These results again suggest a role for T6P as a sugar signal in plants (McCormick *et al.*, 2009).

The overall goal of this study is to understand the effects of T6P on sucrose levels in sugarcane. To make progress towards this, a functional sugarcane *TPS* gene has to be isolated and characterised to commence further research. T6P levels can also be altered in sugarcane, so as to make initial observations on the effects of increasing and decreasing T6P levels on sucrose in sugarcane. The first objective is to isolate a functional full length sugarcane *TPS* cDNA and show that it encodes a functional TPS enzyme. 5' rapid amplification of cDNA ends (RACE) will be used to isolate a full length putative *TPS* gene sequence. Once that is identified, complementation of yeast *tps1Δ* mutants grown on glucose will show whether a functional TPS enzyme is encoded by the isolated sequence. The second objective is the overexpression of the *E. coli otsA* and *otsB* genes in sugarcane, in order to see the effects of their overexpression on sucrose accumulation in transgenic sugarcane. With *otsA* overexpression it is assumed that T6P would accumulate and result in decreased sucrose levels, while when *otsB* is overexpressed it should decrease T6P levels and, therefore, possibly increase sucrose accumulation (Lunn *et al.*, 2006; Schlupepmann *et al.*, 2012; O'Ha *et al.*, 2012).

## 2.2 Materials and Methods

### 2.2.1 Chemicals, kits and reagents

Acids and chemicals used were obtained from Bio-Rad (Hercules, CA, USA), Sigma-Aldrich (St. Louis, MO, USA), Merck Chemicals (Gauteng, RSA) and Whatman (Maidstone, United Kingdom). Kits and enzymes used were obtained from Clontech (Mountain View, CA, USA), Fermentas (Hanover, MD, USA), Invitrogen (Carlsbad, CA, USA), Promega (Madison, WI, USA), New England Biolabs (Ipswich, MA, USA), Qiagen (Dusseldorf, Germany), Separations (Johannesburg, RSA), Sigma-Aldrich (St. Louis, MO, USA), Takara (Otsu, Shiga, Japan), Thermo Scientific (Waltham, MA, USA) and Zymo Research (Orange, CA, USA).

Competent cells used were obtained from Agilent Technologies (Santa Clara, CA, USA), Lucigen (Middleton, WI, USA), Clontech (Mountain View, CA, USA), Invitrogen (Carlsbad, CA, USA) and Takara (Otsu, Shiga, Japan). Yeast strains used in yeast complementation assays were provided by the Laboratory of Molecular Cell Biology (Katholieke Universiteit Leuven, Leuven, Belgium). Plasmids that were not previously available within the IPB were obtained from Addgene (Cambridge, MA, USA).

### 2.2.2 Primer design and synthesis

Primers were designed using either Primer 3 (<http://primer3.ut.ee/>) or Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>). Primers were synthesized by Inqaba Biotech (Pretoria, South Africa).

### 2.2.3 General applied molecular techniques

#### 2.2.3.1 Bacterial growth

Luria Bertani (LB) medium (10 g/l (w/v) tryptone, 5 g/l (w/v) yeast extract and 10 g/l (w/v) NaCl) was used for routine growth and culturing of bacterial strains. LBA solid medium was LB medium with 12 g/l (w/v) bacterial agar added. All media was autoclaved prior to use. Antibiotics and other additions were added to media after cooling to approximately 60°C prior to pouring of plates in a laminar flow cabinet.

### 2.2.3.2 Bacterial transformation

Standard heat shock protocols were used for routine transformation of DH5 $\alpha$  competent cells (Sambrook and Russel, 2001). Briefly, competent cells were mixed with plasmid DNA (50 ng) and left on ice for 30 min prior to heating cells to 42°C for 45 seconds. After heat shock, cells were left on ice for 2 min and LB medium added to a final volume of 1 ml. Tubes containing transformed *E.coli* DH5 $\alpha$  were incubated at 37°C for 1 hour with shaking at 200 rpm. After incubation, 150  $\mu$ l of transformed cells were plated out on LBA plates containing appropriate antibiotics and incubated overnight at 37°C. Colonies were inoculated in liquid LB medium in preparation for plasmid purification.

### 2.2.3.3 Production of chemically competent cells

Chemically competent DH5 $\alpha$  cells were manufactured using a modified protocol from Sambrook and Russel, (2001), which utilizes calcium chloride. DH5 $\alpha$  cells were initially streaked out on a LBA plates and incubated overnight at 37°C. A single colony was inoculated into 5 ml of LB and incubated overnight at 37°C with shaking at 200 rpm. The overnight starter culture was then inoculated into 200 ml of fresh LB and grown to an OD<sub>600</sub> of 0.6. Cells were transferred to pre-cooled 50 ml polypropylene tubes and kept on ice for 40 min. Cells were harvested by centrifuging at 5000 g for 10 min at 4°C. The resulting cell pellet was re-suspended twice in ice cold 0.1 M CaCl<sub>2</sub> and recovered by centrifugation at 5000 g for 10 min at 4°C. Finally, the cells were re-suspended in 3 ml of ice cold 0.1 M CaCl<sub>2</sub> and 100  $\mu$ l of re-suspended cells were aliquoted into microcentrifuge tubes for storage at -80°C until use.

### 2.2.3.4 Extraction of plasmid DNA

For standard plasmid isolation, a modified protocol from Sambrook and Russel, (2001) was used. Solutions prepared and used were Alkaline Solution 1 (AS1) (50 mM glucose, 25 mM Tris-HCL pH 8, 10 mM EDTA, pH 8 and 20  $\mu$ g/ml DNase-free RNase A), Alkaline Lysis Solution 2 (AS2: 0.2 M NaOH, 1% (w/v) SDS) made fresh each time and Alkaline Lysis Solution 3 (AS3) (5 M potassium acetate, pH 5.2). Cells from 5 ml overnight cultures were harvested by centrifugation at 8000 g for 5 min, these centrifugation settings were the same throughout the extraction procedure. Bacterial pellets were re-suspended in 100  $\mu$ l of cold AS1 followed by the addition of 200  $\mu$ l of freshly prepared AS2 and mixing by inverting the

tubes 5 times. AS3 (150  $\mu$ l) was added and samples were left on ice for 5 min followed by centrifugation. The resulting supernatant was transferred to new tubes. An equal volume of PCIA solution [phenol:chloroform:isoamyl-alcohol (25:24:1)] was added, mixed and centrifuged. DNA was precipitated by adding 2 volumes of ethanol and pelleted by centrifugation. DNA pellets were washed twice with 700  $\mu$ l 70% (v/v) ethanol, after which the supernatant was aspirated and plasmid DNA re-suspended in sterile water for storage. Plasmids isolated for sequencing were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Scientific; Waltham, MA, USA), following the specifications of the manufacturer.

#### 2.2.3.5 RNA isolation

For RNA isolation, performed without the use of an RNA isolation kit, a modified protocol from Malnoy *et al.*, (2001) and Hu *et al.*, (2002) was followed. RNA extraction buffer (2% (w/v) cetyl trimethyl ammonium bromide (CTAB), 2% (w/v) polyvinylpyrrolidone (PVP), 100 mM Tris-hydroxymethyl aminomethane (Tris-HCL) pH8, 25 mM ethylenediamine tetra acetic acid (EDTA) and 2 M NaCl dissolved in diethyl pyrocarbonate (DEPC) treated water was autoclaved prior to each RNA extraction. Plant material was ground to a fine powder using liquid nitrogen. Prior to adding to ground material, 30  $\mu$ l of  $\beta$ -mercaptoethanol was added to 1470  $\mu$ l of extraction buffer and heated at 65°C. Frozen ground plant material (200 mg) was then added to pre-heated extraction buffer and vortexed. Samples were heated at 65°C for 1 hour and vortexed every 5 min. Samples were centrifuged for 15 min at 8000 g and the supernatant was transferred to new tubes. Chloroform/isoamylalcohol extraction was performed by adding 1 volume CI solution [chloroform/isoamylalcohol (24:1)] to the resulting supernatant and centrifuging for 10 min at 8000 g. The chloroform/isoamylalcohol extraction was performed twice. For RNA precipitation, 8 M LiCl was added to the resulting supernatant to a final concentration of 2 M, overnight at 4°C. The following day, samples were centrifuged at 4°C for 1 hour at 8000 g. The resulting pellets were washed twice by adding 500  $\mu$ l of 70% (v/v) ethanol and centrifuging at 8000 g for 10 min. RNA pellets were air dried and re-suspended in appropriate volumes of DEPC treated water.

#### 2.2.3.6 cDNA synthesis

Prior to cDNA synthesis, isolated RNA was treated with DNase I, RNase-free (Thermo Scientific; Waltham, MA, USA). Once RNA was cleaned of genomic DNA, cDNA was

synthesized, using the RevertAid H Minus Reverse Transcriptase kit (Thermo Scientific; Waltham, MA, USA).

#### 2.2.3.7 *Polymerase chain reactions (PCR)*

For general diagnostic PCR and colony PCR, GoTaq DNA Polymerase (Promega; Madison, WI, USA) was used. Master mixes were assembled on ice for individual 50 µl PCR reactions. Reactions contained 10 µl GoTaq 5X Green Reaction Buffer (contains 7.5 mM MgCl<sub>2</sub>), 1 µl dNTP mix (10 mM of each dNTP), 5 µl of each primer (10 µM), template DNA, 0.25 µl GoTaq enzyme (5 U/µl) and sterile water to a final volume of 25 or 50 µl. The general PCR program followed for GoTaq PCR reactions was an initial denaturation at 95°C for 2 min followed by 32 cycles of denaturation at 95°C for 20 sec, annealing for 20 sec and elongation at 72°C for 1 min per 1 kb to be amplified followed by a final elongation at 72°C for 5 min. For colony PCR's, small amounts of bacterial colonies were used as template DNA.

Phusion® High-Fidelity DNA Polymerase (Thermo Scientific; Waltham, MA, USA) was used for cloning of inserts that required a proof-reading high fidelity taq (Waltham, MA, USA). Master mixes were assembled for 50 µl reactions according to the manufactures instructions. The general PCR program followed was an initial denaturation at 98°C for 10 sec followed by 30 cycles of denaturation at 98°C for 1 sec, annealing for 20 sec, elongation at 72°C for 15 sec per 1 kb followed by a final elongation at 72°C for 2 min. For high GC content templates such as sugarcane cDNA, PCR reactions contained 5x Phusion GC Buffer and the addition of 6 to 10% DMSO with an extended denaturing time of 30 sec.

#### 2.2.3.8 *Separation of DNA and RNA fragments by gel electrophoresis*

PCR amplification products and restriction digests were usually separated on 1.2% (w/v) agarose gels containing 5% (v/v) pronosafe (Separations; Johannesburg, RSA). TBE buffer consisting of 5.4 g/l Tris base, 2.75 g/l boric acid, 0.465 g/l EDTA (pH 8.0) was used for the making and running of electrophoresis gels. Gels were submerged in TBE buffer and DNA fragments separated within the gel at 120 mV for an hour. Separated fragments were viewed under a UV-light. Depending on the separation of DNA fragments, the corresponding correct PCR products were purified using the GeneJET Gel Extraction Kit or PCR Purification Kit, both from Thermo Scientific (Waltham, MA, USA).

#### 2.2.3.9 Cloning of inserts into a vector

For cloning of inserts via ligation into a destination vector the following strategy was followed. The purified gene inserts and vector DNA were digested using restriction digestion enzymes from Thermo Scientific (Waltham, MA, USA). Restriction ends of the digested gene inserts were phosphorylated using T4 Polynucleotide Kinase (PNK) and vector ends were dephosphorylated using Fast Alkaline Phosphatase (FastAp), both from Thermo Scientific (Waltham, MA, USA). Inserts were ligated into the vector overnight at room temperature using T4 DNA Ligase Thermo Scientific (Waltham, MA, USA). Resulting constructs were transformed into competent DH5 $\alpha$  cells via heat shock (see section 2.2.3.2) and transformants were selected on LBA plates containing an appropriate antibiotic. Colony PCR was performed using GoTaq DNA Polymerase (Promega; Madison, WI, USA) to identify positive transformants. A gene-specific primer, along with a plasmid specific primer was used in colony PCR's to confirm the gene insert was cloned in the correct orientation. When sequencing was required, plasmid DNA was purified using the GeneJET Plasmid Miniprep Kit (Thermo Scientific; Waltham, MA, USA) and plasmids were sent for sequencing at the Central Analytic Facility, Stellenbosch University (CAF).

#### 2.2.4 Genetic transformation of sugarcane with *E.coli otsA* and *otsB*

##### 2.2.4.1 Initiation and maintenance of embryogenic sugarcane callus

Wild-type sugarcane plants (*Saccharum* species hybrid cultivar NCo310), grown on Welgevallen farm, Stellenbosch were used for callus initiation. Mature stalks were harvested and washed continuously with ethanol while leaves and old stem tissue were removed with a sterilized secateurs (Felco). The removal of the final leaf sheath was done in a laminar flow cabinet while spraying with 70% (v/v) ethanol. After the removal of the last leaf, the soft inner meristematic leaf tissue was cut into 2 mm thick disks. The disks were transferred to callus induction MSC<sub>3</sub> medium (4.4 g/l MS salts and vitamins) (Murashige and Skoog, 1962; Highveld Biological, South Africa), 20 g/l sucrose, 0.5 g/l casein, 3 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 2.2 g/l gelrite was added after the pH was adjusted to pH 6.8, using 1 M NaOH) and incubated for six weeks in the dark at 26°C. Every 2 weeks, actively growing calli were sub-cultured onto fresh sterile MSC<sub>3</sub> medium.

#### 2.2.4.2 Isolation of *otsA* and *otsB* genes

In order to study the effects of altering trehalose synthesis on sucrose accumulation in sugarcane, the *otsA* (NCBI accession number: EG11751) and *otsB* (NCBI accession number: EG11752) genes were isolated from BL21(DE3) *E.coli* for eventual expression in sugarcane. For isolation, the BL21 *E. coli* strain was streaked out on LBA medium (see section 2.2.3.1) containing 34 ug/ml chloramphenicol and incubated overnight at 37°C. These colonies served as template for PCR isolation reactions. Primers were designed for the full length gene with the addition of a *NotI* restriction site at the 5' end and an *EcoRI* restriction site at the 3' end of the amplified product (Table 2.1).

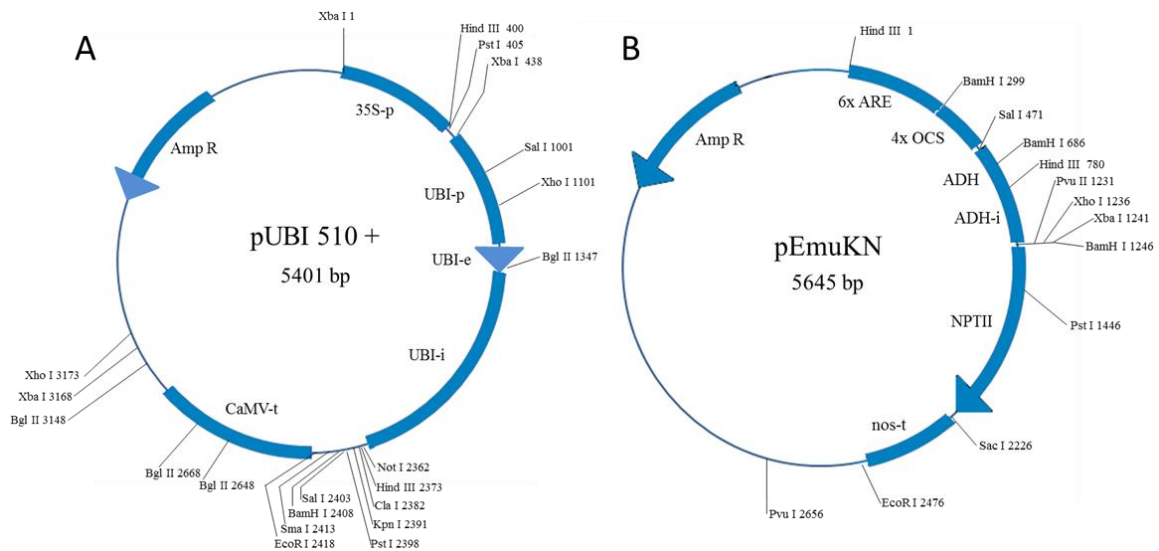
**Table 2.1:** Primers used to isolate the *otsA* and *otsB* gene fragments from *E. coli*.

Gene	Primer name	Sequence
<i>otsA</i>	otsA Fw <i>NotI</i>	5'- TGCGGCCGCGACTATGAGTCGTTTAGTC -'3
	otsA Rev <i>EcoRI</i>	5'- CGAATTCTCGGCGAAAAGAGTTATT -'3
	otsA int Fw	5'- TTTGCCAGAGCGTTTTCTCG -'3
<i>otsB</i>	otsB Fw <i>NotI</i>	5'- AGCGGCCGCACCGGATGACAGAACCGT -'3
	otsB Rev <i>EcoRI</i>	5'-AGAATTCATCCGGTTAGATACTACGACTA-'3
	otsB int Rev	5'- ACTACAGCTACTGGCAACC -'3

PCR amplification of these genes were performed using Phusion® High-Fidelity DNA Polymerase (Thermo Scientific; Waltham, MA, USA) and master mixes were assembled according to the manufacturer specifications and PCR program settings as described in section (2.2.3.7).

#### 2.2.4.3 Ligation of transgenes into the pUBI 510+ plant expression vector

Purified *otsA* and *otsB* gene fragments were ligated separately into the pUBI 510+ vector (Figure 2.1) to create the pUBI 510::*otsA* and pUBI 510::*otsB* constructs, using methods as described in section 2.2.3.9 (Groenewald and Botha, 2001).



**Figure 2.1:** Vectors utilized in sugarcane transformation. (A) pUBI 510+ plant expression vector (5401 bp). (B) pEmuKN selection vector (5465 bp).

*NotI* and *EcoRI* restriction sites within both the inserts and the pUBI 510+ vector was used for directional cloning. Transformant clones containing constructs were selected on LBA plates containing 100 µg/ml ampicillin and colony PCR was performed, using GoTaq DNA Polymerase (Promega; Madison, WI, USA). Plasmid DNA was purified using the GeneJET Plasmid Miniprep Kit (Thermo Scientific; Waltham, MA, USA) and gene insertion was confirmed by sequencing at the Central Analytic Facility, Stellenbosch University (CAF).

To isolate pUBI 510::*otsA* and pUBI 510::*otsB* plasmid DNA the Zyppy Plasmid Maxiprep Kit (Zymo Research; Orange, CA, USA) was used (Orange, CA, USA). Large scale plasmid isolation was done according to the manufacturer instruction and final plasmid concentrations of 1 µg/ul were used for particle bombardment experiments.

#### 2.2.4.4 Biolistic transfer of pUBI 510::*otsA* and pUBI 510::*otsB* constructs into sugarcane callus

Plasmids pUBI 510::*otsA* and pUBI 510::*otsB* were transformed into sugarcane callus using biolistic bombardment based on the method described by Bower and Birch (1992) and Bower *et al.*, (1996) with modifications. Briefly, small actively growing sugarcane embryogenic calli were collected from the initiation plates and transferred to MSC<sub>3</sub>Osm medium (MSC<sub>3</sub> containing 0.2 M sorbitol and 0.2 M mannitol), 3 hours prior to bombardment. Calli were



arranged in a 3 cm diameter circle in the center of the plates and incubated at 26°C in the dark. Construct DNA was co-bombarded along with pEmuKN to enable selection on MSC<sub>3</sub> medium containing 50 µg/mL geneticin (Figure 2.1B) (Bower *et al.*, 1996). For DNA preparation, 5 mg tungsten powder (Bio-Rad; Hercules, CA, USA) (0.7 micron per particle) was sterilized with 400 µl ethanol and rinsed three times with sterile dH<sub>2</sub>O. The tungsten was re-suspended in 50 µl sterile water. After this, 5 µL each of a 1 µg/µl constructed plasmid DNA and pEmuKN vector DNA were precipitated on the tungsten by the addition of 50 µL 2.5 M CaCl<sub>2</sub> and 20 µl 0.1 M spermidine (Sigma-Aldrich; St. Louis, MO, USA). For particle bombardment, outlet pressure at the helium cylinder was adjusted to 1000 kPa and the solenoid timer to 0.05 seconds. Excess supernatant (100 µl) was removed from the precipitation mixture and the remaining mixture was re-suspended. The precipitation mixture (5 µl) was loaded onto the center of the macro carrier. The MSC<sub>3</sub>Osm media plates containing the calli, were placed in center of the vacuum chamber of the gene gun, 16 cm from the macro carrier. At a pressure of 80 kPa, the tungsten containing the DNA was fired into the calli. The bombarded calli were kept on MSC<sub>3</sub>Osm medium for the following 4 hours after which it was transferred onto fresh MSC<sub>3</sub> medium. After two days in the dark at 26 °C the calli were transferred to MSC<sub>3</sub> medium containing 50 µg/mL geneticin (Sigma-Aldrich; St. Louis, MO, USA). The calli, kept in the dark, was sub-cultured every 2 weeks onto fresh MSC<sub>3</sub> geneticin medium until putative transgenic callus, surviving selection, could be identified after about 8 weeks.

#### 2.2.4.5 *Regeneration and hardening off of putative transformed sugarcane plantlets*

Putative transformed calli were regenerated on MS medium (4.4 g/l MS, 20 g/l sucrose, 0.5 g/l casein, 2.2 g/l gelrite, pH 6) and incubated at 16h/8h day/night cycles at 24°C. Positive clones were left to grow for 6 weeks, with sub-culturing every two weeks onto fresh media. Regenerated shoots were transferred into small pots containing MS media for another 8 weeks to form roots. Plantlets were transferred to pots containing a mixture of vermiculite (Rosarium, South Africa), potting soil and sand at a ratio of 1:1:1 and acclimatized for 5 days in plastic bags. Plants were watered three times a week and grown under natural day/night cycles at 25°C in the greenhouse.

#### 2.2.4.6 *Confirmation of transgene insertion*

Leaf material from putative transgenic sugarcane plants were harvested and used for genomic DNA (gDNA) extraction. gDNA was extracted using the DNeasy Plant Mini Kit (Qiagen;

Dusseldorf, Germany), according to the manufacturer's instructions. For testing putative *otsA* lines the UBI\_prom-F (Table 2.2) and *otsA* Rev *EcoRI* (Table 2.1) primers were used in PCR reactions. For testing putative *otsB* lines the UBI\_prom-F (Table 2.2) and *otsB* int Rev (Table 2.1) primers were used. PCR reactions were performed using GoTaq DNA Polymerase (Promega; Madison, WI, USA). PCR master mixes and program settings were setup as described in section 2.2.3.7. PCR products were separated and visualized on 1.2% agarose TBE gels (see section 2.2.3.8).

**Table 2.2:** General PCR and vector primers.

Gene or vector	Primer name	Sequence
Actin	Actin forw1	5'-TCACACTTTCTACAATGAGCT-'3
	Actin rev1	5'-GATATCCACATCACACTTCAT-'3
pUBI 510+	UBI_prom-F	5'-AATTTGATATCCTGCAGTGCAGCGTG-'3
	CaMV-R	5'-AGGGTTTCTTATATGCTCAAC-'3

#### 2.2.4.7 Confirmation of transgene expression – sq-RT-PCR

Semi quantitative RT-PCR (sq-RT-PCR) was performed on *otsA* and *otsB* transgenic lines to determine transgene expression levels. The actin housekeeping gene was used as internal standard to evaluate the quality and equalize the amount of cDNA from each sample in a PCR trial using the primer pair, actin forw1 and actin rev 1 (Table 2.2). The *otsA* primer pair consists of *otsA* int Fw and *otsA* Rev *EcoRI* primers and the *otsB* primer pair consists of *otsB* Fw *NotI* and *otsB* int Rev (Table 2.1). The *otsA* int Fw and *otsB* int Rev primers are primers designed to bind internally to their intended target gene. PCR reactions were performed using GoTaq DNA Polymerase (Promega; Madison, WI, USA) for 50 µl reactions according to the manufacturer's instructions. 1 µg of total RNA (see section 2.2.3.5) from each line was used to synthesise cDNA (see section 2.2.3.6) and a total of 50 ng of cDNA was used as template in the sq-RT-PCR s. The linear amplification range was determined with actin primers by removing 10 uL from the PCR reaction after 15, 20, 25, 30 and 35 cycles, respectively. These 10 µl volumes were separated on an agarose gel and visualised under UV light. The quantity of actin PCR fragments were visually estimated according to band intensity and the linear amplification range determined. A minimal signal density of 30 cycles was chosen as the baseline and the cDNAs adjusted to give the same signal strength for actin when amplifying the cDNA stocks.

#### 2.2.4.8 Confirmation of transgene expression – Western blotting

##### *Protein extraction and visualization*

Protein extraction buffer [1 mM MgCl<sub>2</sub>, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 1% bovine serum albumin (BSA) and 10% (v/v) glycerol] was added to 50 mg grounded sugarcane leaf material. The samples were centrifuged at 8000 g for 5 minutes at 4°C and the supernatant containing crude protein extract was transferred to new tubes and stored at -20°C until use. Protein concentrations were determined using assay protocols developed by Bradford, (1976). The OD<sub>595</sub> was measured on a spectrophotometer (Fluostar Optima Spectorphotomer; BMG Lab Tech) using Bradford reagent (Bio-Rad; Hercules, CA, USA). 0.1 µg to 0.6 µg BSA were used to create a standard curve of protein concentration vs absorption, and used to estimate protein concentration of extracts.

For separation of proteins, crude protein extracts were denatured and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The 10% (v/v) SDS-PAGE resolving gel [0.1% (w/v) sodium dodecyl sulphate (SDS), 30% (v/v) acrylamide/Bis solution diluted to 10% (v/v), 375 mM Tris-HCL pH 8.8, 0.1% (w/v) ammonium persulfate (APS) and 0.04% (w/v) Temed (tetramethylethylenediamine)] was cast first and left to solidify for 40 min, after which a stacking gel [0.1% (w/v) SDS, 30% (v/v) acrylamide/Bis solution diluted to 5% (v/v), 130 mM Tris-HCL pH 6.8, 0.1% (w/v) APS and 0.01% (w/v) Temed] was cast on top of the resolving gel with plastic loading wells mold and left to solidify for 40 min. The SDS-PAGE gel was then connected and fastened into a protein gel electrophoresis tank and submerged in PAGE running buffer [0.1% (w/v) SDS, 0.25% (w/v) glycine and 25 mM Tris-HCL, pH 8.3]. Prior to loading in SDS-PAGE gels, protein samples were denatured with protein loading dye. 4X Protein loading dye [0.2 M Tris-HCl pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 588 mM β-mercaptoethanol, 50 mM EDTA and 0.08% (w/v) bromophenol blue] was added to 10 µg of crude protein extract and protein samples were boiled at 95°C for 15 min. Denatured protein samples were then loaded onto the cast SDS-PAGE gel and separated at a voltage of 140 mV for approximately 1 hour, until the samples were separated completely.

Coomassie blue staining was used to visualize proteins on SDS-PAGE gels. SDS-PAGE gels were stained by covering with coomassie blue staining solution [0.1% (w/v) Coomassie Blue Stain R-250, 50% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) H<sub>2</sub>O] and shaking

gently overnight. The next day, stained gels were washed by covering with de-staining solution [50% (v/v) methanol and 10% (v/v) acetic acid in H<sub>2</sub>O] and shaking gently for approximately 30 min and pouring off excess stain solution. Wash steps were repeated at least 3 times until individual protein bands could be distinguished.

### *Immunoblotting*

10 µg crude protein extracts were separated on 10% (v/v) SDS-PAGE gels as previously described. SDS-PAGE gels containing the separated proteins were soaked in 1X transfer buffer [190 mM glycine, 25 mM Tris-HCL pH 8.3 and 20% (v/v) methanol] for 30 min with gentle agitation. A piece of nitrocellulose membrane (Whatman; Maidstone, United Kingdom) was cut to the same dimensions as the gels and soaked in 1X transfer buffer for 30 min with gentle agitation. For semi-dry transfer of proteins to the nitrocellulose membrane the Trans-Blot SD system (Bio-Rad; Hercules, CA, USA) was used. Three filter paper pieces were briefly soaked in 1X transfer buffer and layered onto the transfer pad followed by the SDS-PAGE gel on top of the filter paper layer. The nitrocellulose membrane was then placed on top of the SDS-PAGE gel and covered with 3 filter paper pieces, also soaked in 1X transfer buffer. The transfer setup was then fastened within the Trans-Blot SD and proteins were transferred to the nylon membrane at 30 mV for 2 hours.

Once proteins were transferred, the nitrocellulose membrane was briefly rinsed with 1X Tris buffered Saline Tween (TBST) [50 mM Tris-HCL pH 7.3, 150 mM NaCl and 0.1% (v/v) Tween 20]. The membrane was then incubated with 40 ml of blocking buffer [2% (w/v) BSA and 3% (w/v) non-fat milk in 1X TBST] with shaking for 1 hour at room temperature. The membrane was washed with TBST for 5 min and incubated with a 1:500 dilution of primary antibody in TBST with gentle shaking at 4°C, overnight. The primary antibodies used were either anti *otsA* or anti *otsB* (both raised in rabbit) and were a kind gift of Dr John Lunn (Max Planck Institute of Molecular Plant Physiology, Germany). The next day the membrane was washed twice with TBST for 5 min each. The membrane was then incubated with a secondary antibody (1:7500 dilution; Anti Rabbit IgG from Sigma-Aldrich) in TBST, with gentle shaking for 1 hour. The membrane was washed twice with TBST and finally rinsed with 1X Tris buffered Saline buffer (50 mM Tris-HCL pH 7.3 and 150 mM NaCl). For the colour reaction, SIGMA-FAST RCIP/NBT (Sigma-Aldrich; St. Louis, MO, USA) was dissolved in 10 ml of sterile H<sub>2</sub>O. The membrane was incubated with the dissolved colour reagent for 1 hour with gentle shaking, until bands could be visualized.

#### 2.2.4.9 Determination of sugar accumulation in transgenic lines

##### *Sugar extraction*

Sugarcane leaf material from each transgenic line and control wild type plants were grounded to a fine powder with liquid nitrogen. Approximately 20 mg of ground leaf tissue was weighed in separate tubes and exact weights were recorded. Sugar extraction was done twice with the addition of 250  $\mu$ l of 80% (v/v) ethanol, incubation at 95°C for 30 min with shaking. After each incubation, samples were centrifuged at 8000 g for 10 min and the resulting supernatant was collected and kept on ice. For the last extraction the ethanol concentration was lowered to 50% (v/v). Supernatant was stored at -20°C until measurements were performed.

##### *Sugar measurement*

Prior to sugar level determinations the following stock solutions were assembled; sugar assay buffer (100 mM KOH, 3 mM MgCl<sub>2</sub>, pH 7), in which 2 U/ $\mu$ l hexokinase (HK), 2 U/ $\mu$ l phosphoglucosomerase (PGI) and 6 U/ $\mu$ l invertase (Inv) were dissolved. For measurement of sugar levels in samples with spectrophotometry a buffer mix consisting of 15.5 ml sugar assay buffer, 480  $\mu$ l 100 mM ATP, 480  $\mu$ l 45 mM nicotinamide adenine dinucleotide (NAD) and 60 U of glucose-6-phosphate dehydrogenase (G6PDH) was assembled. In each 96-well assay plate well, 160  $\mu$ l of buffer mix was added to 10  $\mu$ l of sample supernatant and the absorbance continuously measured at OD<sub>340</sub>. Sugars were measured through the consecutive addition of 1  $\mu$ l of HK (for glucose), 1  $\mu$ l of PGI (for fructose) and 1  $\mu$ l of Inv (for sucrose). Only after the endpoint of each reaction was achieved was the next enzyme added. Sugar concentrations were determined based on the  $\Delta$ OD<sub>340</sub> after addition of each enzyme.

## 2.2.5 Isolation of a sugarcane *TPS* gene

### 2.2.5.1 Amplification of a partial *SoTPS1* cDNA

A partial sugarcane *TPS* cDNA sequence (*SoTPS1*) (NCBI accession number: EU761244.1) was available on the National Centre for Biotechnology Information (NCBI) database (Nicolau *et al.*, 2013). This sequence was used as a search reference in nucleotide BLAST (blastn) homology searches (<http://www.ncbi.nlm.nih.gov/>) and for designing primers. The TA49 and CA13 primer pairs were designed using the partial *SoTPS1* sequence to amplify 558 bp and 1066 bp segments respectively (Table 2.3). The OsCons primer pair was designed to amplify the GT1 TPS conserved domain found in the rice *OsTPS1* sequence (HM050424), amplifying an 804 bp segment (Table 2.3). Furthermore, due to the high similarity between the sorghum hypothetical *TPS* (NCBI accession number: XM\_002440041.1) and the isolated sugarcane partial *TPS1* sequence, the sorghum hypothetical *TPS1* sequence fragment was used as a reference to design *SoTPS1* internal primers (Table 2.3). The *SoTPS1* inter primers were designed to amplify a 544 bp segment in the 5' region of the *SoTPS1* gene.

**Table 2.3:** Primers designed for *TPS* gene fragment amplification.

Primer pair	Primer name	Sequence
TA49	Ta49 Forward	5'-TGAGAGCCCTCTGTGAGGAT-3
	Ta49 Reverse	5'-CAGAAGGGTACTCGGGATCA-3
CA13	CA13 Forward	5'-GCCTCCCTCTTGTCATGTCC-3
	CA13 Reverse	5'-GGGGTAGTCGAAGGTGTGAA-3
OsCons	OsCons Forward	5'-CAGTGGTCCCTGGAGATCAG-3
	OsCons Reverse	5'-TCTTTTGCGGAATTCCTTG-3

PCR amplification was performed using Phusion® High-Fidelity DNA Polymerase (Thermo Scientific; Waltham, MA, USA), with reaction reagents being assembled according to manufacturer's instructions and program settings as outlined in section 2.2.3.7. Template DNA used was cDNA synthesized (see section 2.2.3.6) from sugarcane leaf-roll RNA (see section 2.2.3.5). PCR products were visualised on TBE gels (see section 2.2.3.8) and DNA bands of interest were excised and purified. Purified PCR products were cloned into the pJET 1.2 vector for sequencing purposes, using the CloneJET PCR Cloning Kit (Thermo Scientific; Waltham, MA, USA).

### 2.2.5.2 Rapid Amplification of cDNA Ends (RACE)

RNA for 5' RACE was isolated using the RNeasy Plant Mini Kit (Qiagen; Dusseldorf, Germany), according to the manufacturer's specifications. RNA was isolated from mature *Saccharum officinarum* cv. NCo310 leaf-roll tissue. RiboLock RNase Inhibitor (Thermo Scientific; Waltham, MA, USA) was used to halt any potential digestion of extracted RNA. RNA was DNase treated with DNase I, RNase free (Thermo Scientific; Waltham, MA, USA). DNase I reactions were set up according to the manufacturer's instructions. RNA was separated on a 1.2% agarose gel at 100V for 20 min, to confirm the quality of RNA as previously described in section 2.2.3.8.

**Table 2.4:** SoTPS 5' RACE primers.

Gene	Primer name	Sequence
<i>SoTPS1</i>	SoTPS1 GSP 1	5'-CAAGTGATTGTGCTGCCCCAGCAAAGTC-3'
	SoTPS1 GSP 2	5'-AGTGGAACAGCGGCCACAGGATG-3'
RACE universal primers	Long primer	5'-CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT-3'
	Short primer	5'-CTAATACGACTCACTATAGGGC-3'
	Nested primer	5'-AAGCAGTGGTATCAACGCAGAGT-3'

The technique of rapid amplification of cDNA ends (Sambrook and Russel, 2001) was used in order to isolate unknown fragments of the putative sugarcane *TPS* genes (*SoTPS*). 5' RACE was performed using the SMARTer™ RACE cDNA Amplification Kit (Clontech; Mountain View, CA, USA). Briefly, 5' RACE cDNA was synthesized using the SMARTScribe™ Reverse Transcriptase enzyme, which creates a unique 5 bp overhang. An oligonucleotide containing this 5 bp sequence as well as an additional 25 bp was added to the cDNA synthesis reaction. PCR reactions were performed on 5' RACE cDNA using a forward universal primer that contains the 25 bp sequence on the 5' ends and a reverse gene-specific primer designed to anneal to the partial *SoTPS1* gene (Table 2.4). Specifically, a universal primer mix containing 0.4 μM long primer and 2 μM short primer was used with the gene-specific reverse primer (Table 2.4). The first gene-specific reverse primer SoTPS1 GSP 1 was designed based on results from amplifying a partial *SoTPS1* segment. The second gene-specific reverse primer was designed based on 5' RACE results. 5' RACE PCR was performed using Phusion® High-Fidelity DNA Polymerase (Thermo Scientific; Waltham, MA, USA), with master mixes assembled as described in section 2.2.3.7. 400 ng of 5' RACE

cDNA was used as template in each RACE PCR reaction. The PCR program used was as follows: initial denaturation at 98°C for 30 sec, 38 cycles of 98°C for 5 sec, 68°C for 20 sec and 72°C for 1 min followed by a final elongation at 72°C for 3 min. The elongation time of 1 min allowed for the possible amplification of PCR products of 4 kb. RACE PCR products were visualised on agarose TBE gels and amplicons of expected size were isolated and cloned into the pJET 1.2 vector and sequenced.

### 2.2.5.3 Assembling and cloning the full length putative *SoTPS* gene

*SoTPS* full length primers were designed, using the 5' RACE data and the hypothetical sorghum *TPS* sequence (Table 2.5). PCR amplification was performed using Phusion® High-Fidelity DNA Polymerase with an optimal annealing temperature of 67.8 °C and an elongation time of 45 sec.

**Table 2.5:** Putative *SoTPS* cloning primers.

Primer pair	Primer name	Sequence
SoTPS inter	SoTPS int Forward 1	5'-TACGACATCTTCGCGTCGGA-3
	SoTPS int Reverse 1	5'-TTGCCAGCCTGTCCTCCTGTGGT-3
SoTPS full length	SoTPS Forward 1	5'-CACCGCGGCGCATGAGCTCTGACG-3
	SoTPS Forward 2	5'-CACCCGGCGATGCCAACCTCATCGC-3
	SoTPS Reverse 1	5'-AAAGCCCGGAGGCTCCACCAG-3

## 2.2.6 Investigation of the activity of the isolated sugarcane *TPS* gene

### 2.2.6.1 Yeast media

Medium used for regular growth of yeast strains consisted of 10 g/l yeast extract and 20 g/l peptone with an additional appropriate sugar carbon source (YP). When solid growth medium was required, 20 g/l bacteriological agar was added (YPA). As a sugar carbon source, either galactose or glucose was added to a final concentration of 3% or 2% (w/v), respectively as recommended by Zentella *et al.*, (1999).

For selection of transformed autotrophic yeast mutants, synthetic complete (SC) medium was used. For use in SC medium, yeast synthetic drop-out medium (Drop-out mix) and yeast



nitrogen base, without amino acids, (YNBa) was purchased (Sigma-Aldrich; St. Louis, MO, USA). The drop-out mix included all amino acids, except histidine, leucine, tryptophan and uracil. SC medium [0.67% (w/v) YNBa, 2% (w/v) Drop-out mix and 2% (w/v) Bacto agar] was autoclaved and a filter sterilized sugar carbon source was added after SC medium was cooled to 60°C. Additional filter sterilized amino acids were also added after autoclaving of media to a final concentration of 0.3 mM histidine, 2 mM leucine, 0.4 mM tryptophan and 0.2 mM uracil.

#### 2.2.6.2 *Yeast strains and growth*

The following yeast strains were used for TPS activity determinations: W303 a *leu2-3*, 112 *ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100 GAL SUC2* (wild-type yeast), W303-1A *tps1Δ::TRP1* (*tps1Δ* yeast), W303-1A *tps2Δ::LEU2* (*tps2Δ* yeast) and W303-1A MATa *tps1Δ::TRP1 tps2Δ::LEU2* (*tps1Δ + tps2Δ* yeast). Yeast strains were provided by the Laboratory of Molecular Cell Biology, Katholieke Universiteit Leuven, Leuven, Belgium.

To initiate growth of yeast for transformation, yeast strains were inoculated from glycerol stocks on YPA + 3% (w/v) galactose medium and incubated for 3 days at 28°C.

#### 2.2.6.3 *Yeast transformation*

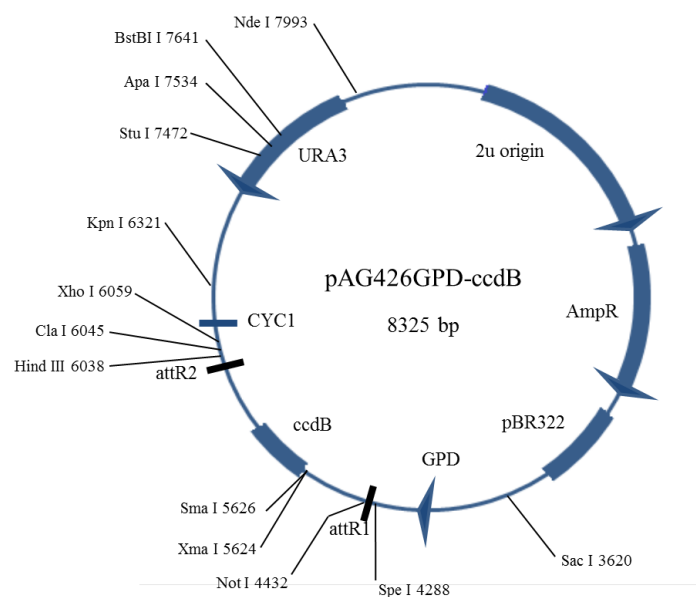
For yeast transformation, a modified protocol by Gietz and Woods (2002) was followed. A single yeast colony was inoculated into 5 ml YP + 3% (w/v) galactose medium (YPgal) and incubated overnight at 28°C with shaking at 200 rpm. 700 µl was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of YP + 3% (w/v) galactose medium. The culture was incubated at 28°C with shaking for 3 hours, after which cells were harvested by centrifuging at 3000 g for 1 min. The supernatant was removed and pelleted cells were re-suspended in 25 ml of sterile water. Cells were pelleted at 3000 g, the supernatant removed and re-suspended first in 1 ml and after a second harvest in 400 µl of 100 mM lithium acetate (LiAc). 50 µl of cells were aliquoted into tubes and the following transformation mix added: 240 µl of 50% (w/v) PEG 4000, 35 µl of 1 M LiAc, 25 µl of boiled carrier ssDNA (10 mg/ml), 50 µl of sterile water and lastly 5 µl of construct DNA (100 – 200 ng/µl).

Cells were re-suspended in transformation mix by vortexing and incubated at 30°C for 30 min, after which they were heat shocked at 42°C for 25 min. Cells were then pelleted by

centrifuging at 3000 g for 1 min and the supernatant was discarded. Pelleted cells were re-suspended in 200  $\mu$ l sterile water and plated out on appropriate selection medium. Plates with transformed yeast were incubated at 28°C for 3 days.

#### 2.2.6.4 Yeast expression vector

For expression in yeast, the pAG426GPD vector was purchased (Addgene; Cambridge, MA, USA). pAG426GPD is based on PRS yeast shuttle vectors that were modified to be Gateway compatible by Alberti *et al.*, (2007) (Figure 2.2).



**Figure 2.2:** Map of the pAG426GPD-ccdB vector. 2u origin = 2 micron origin, AmpR = ampicillin resistance gene, pBR322 = pBR322 promoter, GPD = GPD promoter, attR = attR recombination sites, ccdB = cytotoxic ccdB protein gene, CYC1 = CYC1 terminator and URA3 = uracil selection marker.

#### 2.2.6.5 Creation of yeast complementation assay control constructs

The pAG426GPD-ccdB vector was purchased because it is Gateway compatible, but complications occurred with clonase reactions and further cloning reactions were done using standard ligation instead. For yeast complementation assays, two control plasmids were made for use alongside experimental constructs. The *E.coli otsA* (EG11751) was cloned into the pAG426GPD-ccdB vector to create the pAG426GPD::*otsA* construct to act as a positive control. An “empty” plasmid construct was also created, where a segment of the MCS in

pAG426GPD-*ccdB*, which included the *ccdB* gene, was removed to create the empty pAG426GPD vector.

The *otsA* gene was isolated with PCR from a single DH5 $\alpha$  colony using the following primer pair: 5' GCGGCCGCGACTATGAGTCGTTTAGTC '3 and 5' CTGCTCGAGCGAAAAGAGTTA '3. PCR amplification was performed using Phusion® High-Fidelity DNA Polymerase (Thermo Scientific; Waltham, MA, USA). The *otsA* gene was cloned into the pAG426GPD-*ccdB* vector via ligation, using *NotI* and *XhoI* restriction sites.

To create the empty vector control, the pAG426GPD-*ccdB* vector was subjected to a restriction digestion with *NotI* and *XhoI*. Both *NotI* and *XhoI* sites border a 1600 bp nucleotide segment that includes the *ccdB* gene. Restriction digested DNA was separated on a 1.2% agarose TBE gel and the linearized vector was isolated and gel purified using the GeneJET Gel Extraction Kit (Thermo Scientific; Waltham, MA, USA). Digested ends of the linearized pAG426GPD vector were blunted using T4 DNA Polymerase I and self-circularised using T4 DNA Ligase, both from Thermo Scientific. Plasmid DNA was then transformed into competent *E.coli* DH5 $\alpha$  cells using standard heat shock (see section 2.2.3.2) and transformants were selected for on LB plates containing 100  $\mu$ g/ml ampicillin. Plasmid DNA was purified using the Wizard® Plus Minipreps DNA Purification System (Thermo Scientific; Waltham, MA, USA) and stored at -20°C until its transformation into yeast and use in complementation assays.

#### 2.2.6.6 Cloning of putative *SoTPS* sequences into pAG426GPD-*ccdb*

Putative *SoTPS* sequences were re-amplified from the pJET cloning vector using the following primers; *SoTPS SpeI* fwd: 5' CGCACTAGTGCATGAGCTCTGACG 3' and *SoTPS XhoI* rev: 5' AAAGCCCGGAGGCTCGAGCAG 3' using Phusion® High-Fidelity DNA Polymerase in GC buffer with 6% DMSO (see section 2.2.3.7). Putative sequences were then ligated (see section 2.2.3.9) into pAG426GPD-*ccdb* using *SpeI* and *XhoI* sites for directional cloning.

#### 2.2.6.7 Yeast complementation assay

For selection of autotrophic yeast mutants transformed with gene constructs, synthetic complete (SC) medium was made that included all amino acids, except uracil. Additionally,

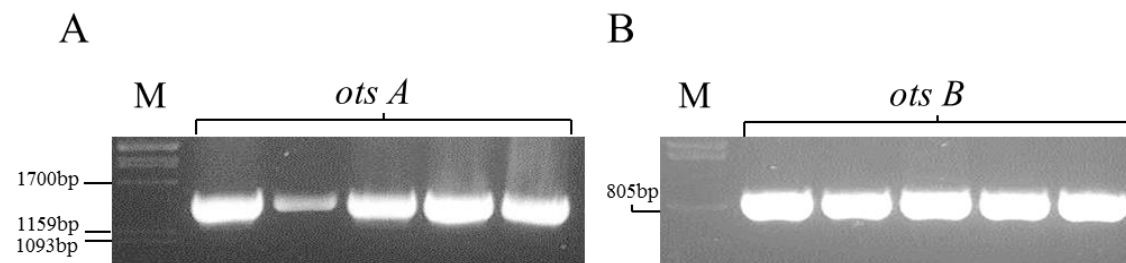
either 3% (w/v) galactose was added to SC medium (SCgal) or 2% (w/v) glucose was added to SC medium (SCglu) as carbon sources. Thus, either SCgal medium excluding uracil (SCgal –ura) or SCglu medium, excluding uracil (SCglu –ura) was used in yeast complementation assays. Yeast cells, transformed with both test and control constructs, were plated on SCgal –ura and SCglu –ura media and incubated at 28°C for 3 days.

## 2.3 Results

### 2.3.1 Overexpression of *otsA* and *otsB* in transgenic sugarcane

#### 2.3.1.1 Isolation, cloning and transformation of *otsA* and *otsB* into sugarcane callus

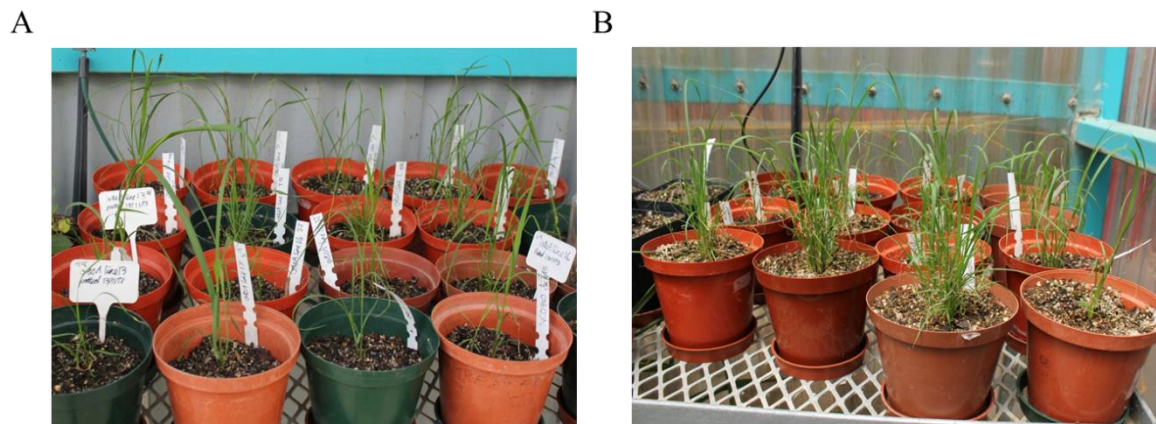
The *otsA* and *otsB* genes were successfully isolated from BL21 (DE3) *E.coli* (Figure 2.3) and cloned into the pUBI 510+ vector. Sequencing of the final pUBI 510::*otsA* and pUBI 510::*otsB* constructs confirmed intact transgene sequences (Annexures, Figures A2.1 and A2.2).



**Figure 2.3:** PCR amplification of *otsA* and *otsB* genes from *E. coli*, visualised on 1.2% agarose gels. Lanes M = *Pst*I digested lambda DNA. (A) *otsA* gene with an expected size of 1425 bp, (B) *otsB* gene with an expected size of 800 bp. Lanes 2 to 6 represent multiple *E.coli* stocks used as template for PCR amplification.

The pUBI 510::*otsA* and pUBI 510::*otsB* constructs were separately co-bombarded with the selection plasmid pEmuKN (Figure 2.1) into sugarcane embryogenic callus. Eighteen putative transgenic *otsA* callus clones and 7 putative transgenic *otsB* callus clones survived after 8 weeks of *in vitro* selection on geneticin. For *otsA*, 9 of the 18 putative clones regenerated shoots and roots *in vitro*. These *otsA* lines (1, 7, 8, 10, 11, 12, 13, 14 and 15) were hardened off in the greenhouse (Figure 2.4A). For *otsB* calli, 3 of the 7 putative clones were capable of

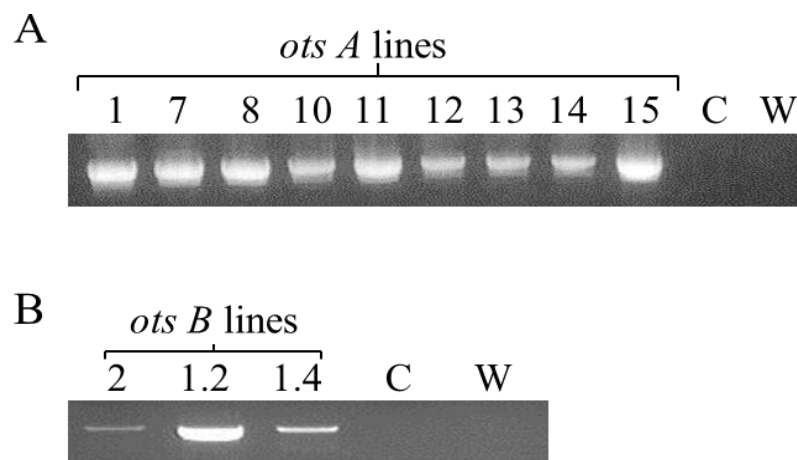
forming shoots and were further regenerated. *otsB* lines 2, 1.2 and 1.4 were hardened off in the greenhouse (Figure 2.4B).



**Figure 2.4:** Four week old transgenic sugarcane plantlets in the greenhouse after 1 week of acclimatization. (A) putative *otsA* expressing sugarcane; (B) putative *otsB* expressing sugarcane .

### 2.3.1.2 Analysis of *otsA* and *otsB* transgenic sugarcane lines

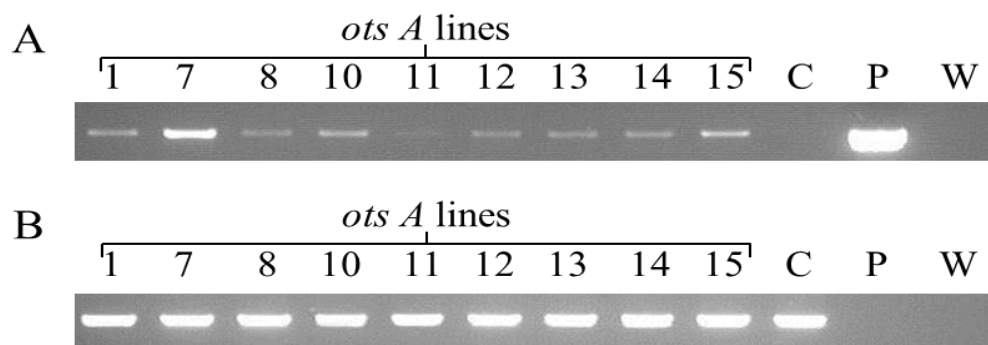
Genomic DNA was extracted from the leaves of hardened off putative transgenic *otsA* and *otsB* plants to confirm transgene insertion. This was confirmed in all 9 of the putative *otsA* transgenic lines and all 3 of the putative *otsB* transgenic lines with gene-specific *otsA* Rev *EcoRI* and *otsB* int Rev primers in combination with the UBI\_prom-F promoter specific primer in a PCR reaction (Figure 2.5).



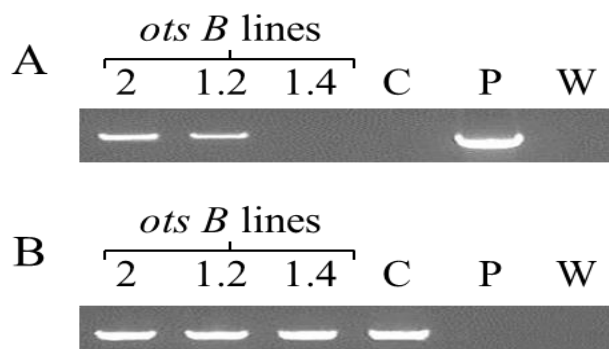
**Figure 2.5:** PCR amplification of transgenes from genomic DNA extracted from putative transgenic *otsA* and *otsB* leaf material, visualized on 1.2% agarose gels. (A) *otsA* lines = *otsA* transgenic lines. (B) *otsB* lines = *otsB* transgenic lines. C =NCo310 wild type control. W = Water control.

Sq-RT-PCR was used to investigate the relative transgene expression levels in the transgenic sugarcane plants. The actin gene expression signal was used as internal standard denominator to measure relative gene transcript quantity (Annexures, Figure A2.3).

Sq-RT-PCR results showed that all nine *otsA* transgenic sugarcane lines expressed the *otsA* transgene (Figure 2.6). *otsA* expression varied between lines, with the weakest expression occurring in *otsA* line 11 (Figure 2.6). Only two of the three *otsB* transgenic lines expressed the *otsB* transgene (Figure 2.7). *otsB* expression in lines 2 and 1.2 vary slightly (Figure 2.7).



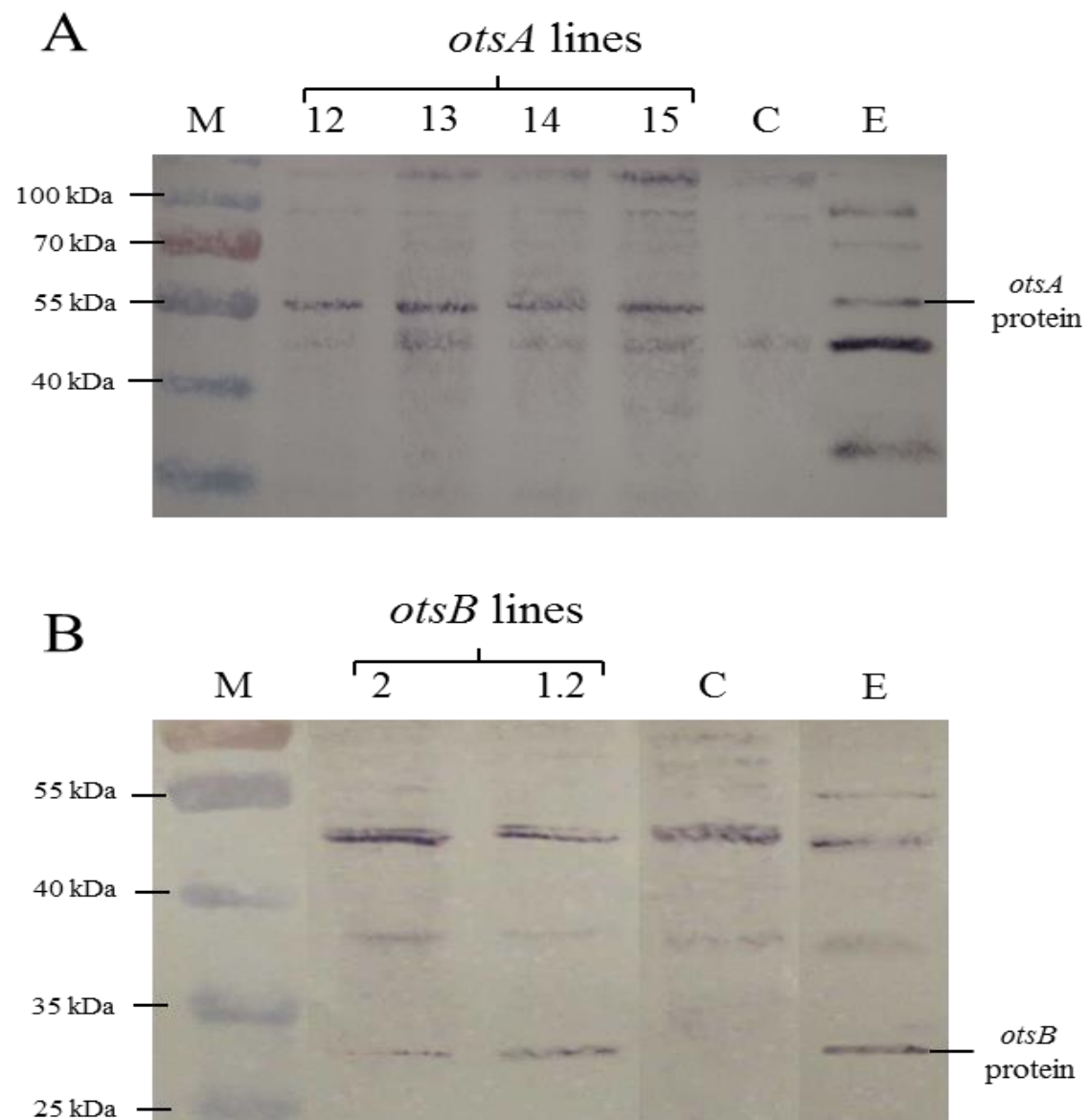
**Figure 2.6:** Sq-RT-PCR on *otsA* transgenic sugarcane lines as visualized on a 1.2% agarose gel. (A) = *otsA* primers. (B) = Actin primers. C = NCo310 control. P = pUBI 510::*otsA* plasmid DNA. W = Water control.



**Figure 2.7:** Sq-RT-PCR on *otsB* transgenic sugarcane lines as visualized on 1.2% agarose gel. (A) = *otsB* primers. (B) = Actin primers, C = NCo310 control. P = pUBI 510::*otsB* construct DNA. W = Water control.

### 2.3.1.3 *otsA* and *otsB* immunoblots

Immunoblotting was performed as described in (section 2.2.4.8) on *otsA* and *otsB* overexpressing lines (Figure 2.8). Immuno detection showed expected signals at around 55 kDa in the *otsA* lines (Figure 2.8A) and expected signals between 35 kDa and 25 kDa in the *otsB* lines (Figure 2.8B).



**Figure 2.8:** Immunoblots of *otsA* and *otsB*. (A) immunoblot of *otsA* transgenic lines, (B) immunoblot of *otsB* transgenic lines. M = PageRuler Prestained Protein Ladder 10 kDa to 170 kDa. C = NCo310 control. E = crude protein from *E. coli*.

#### 2.3.1.4 *Transgenic sugarcane trials with lines containing otsA and otsB transgenes*

Non-transgenic control and transgenic lines expressing transgenes were planted (July 2014) in a randomised pot trial in a growth tunnel on the Welgevallen experimental farm, Stellenbosch (Figure 2.9). The phenotype of all the transgenic plants is similar to that of the non-transgenic control plants. Analysis of these plants will commence when they reach maturity in approximately 8 to 12 months' time.

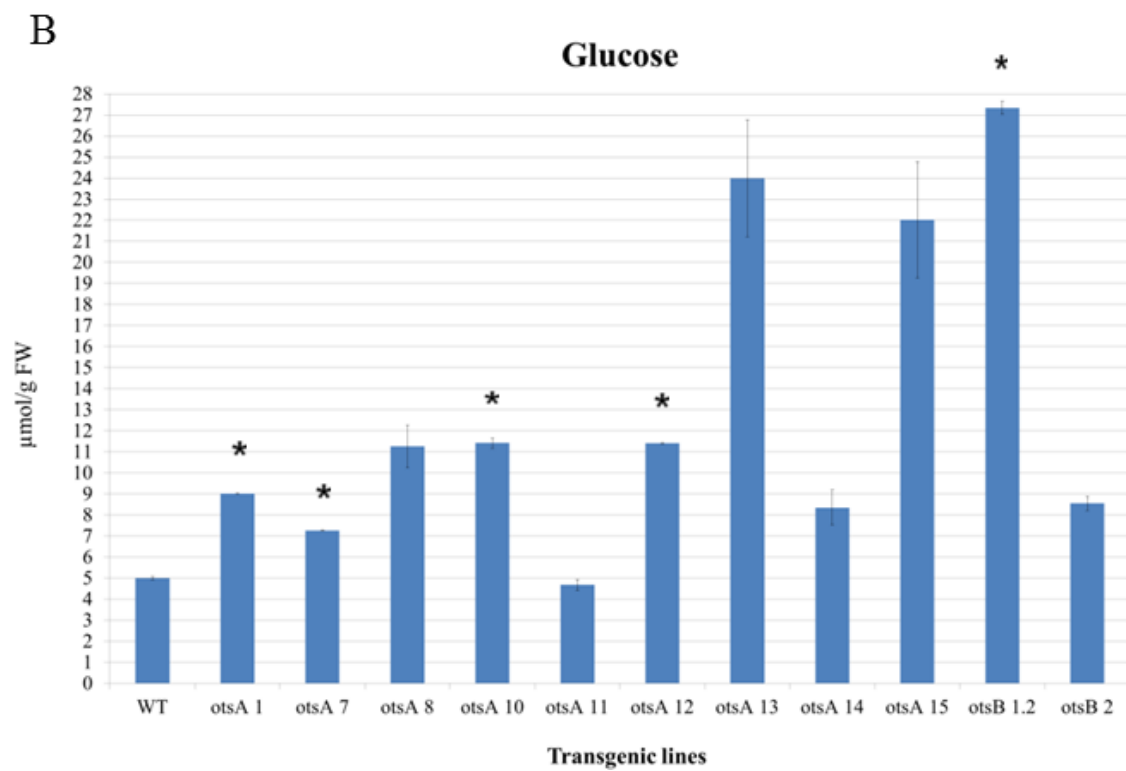
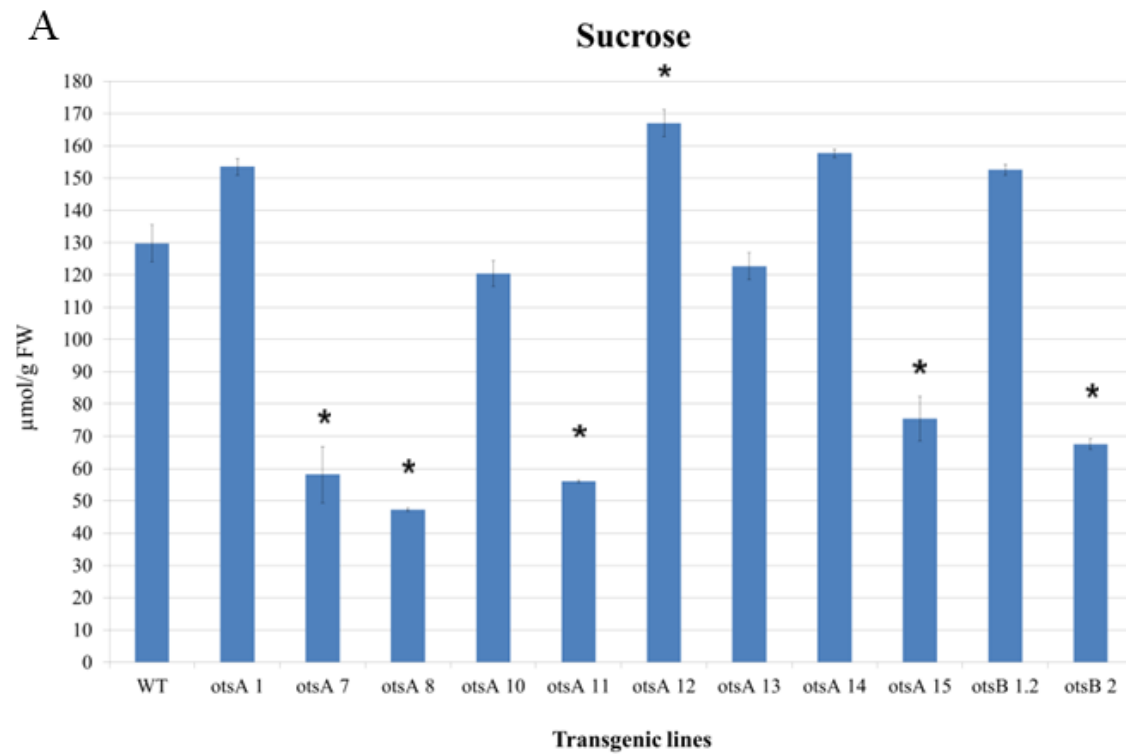


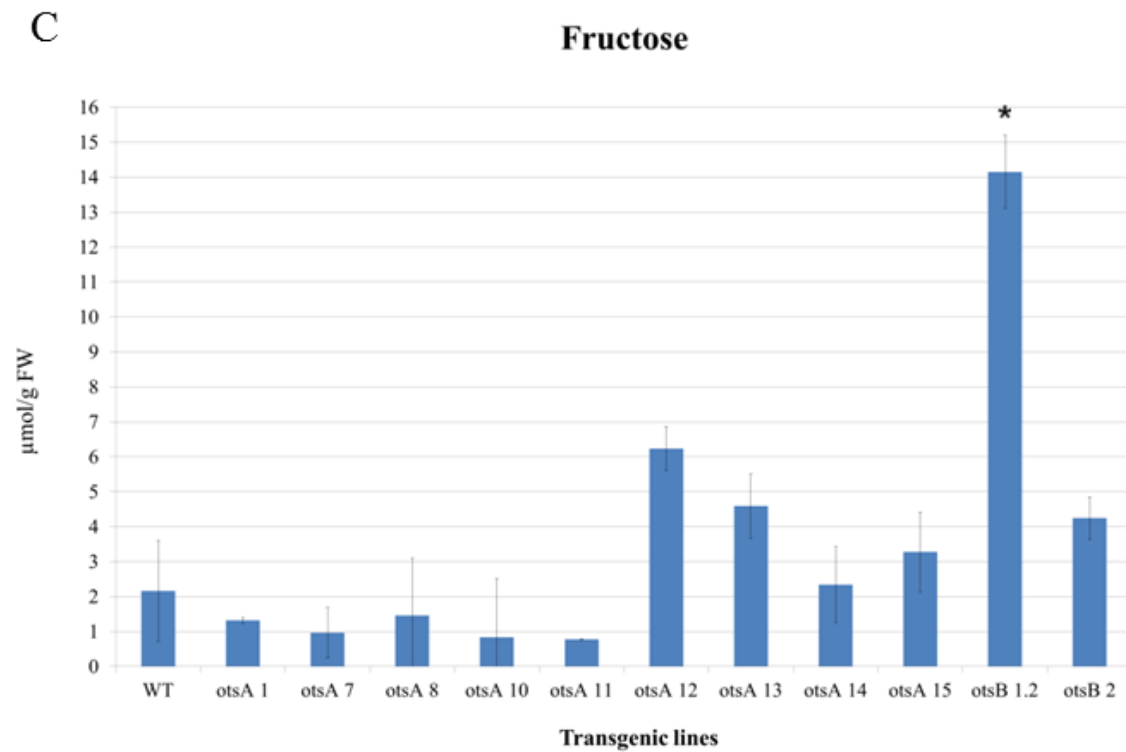
**Figure 2.9:** *otsA* and *otsB* transgenic plants in a growth tunnel on the Welgevallen experimental farm, Stellenbosch.

#### 2.3.1.5 *Sugar level determination*

Sugar extraction was performed on ground leaf material from both *otsA* and *otsB* transgenic lines using the method described in section 2.2.4.9. Changes in  $OD_{340}$  after the addition of enzymes were used in calculating the preliminary sucrose (Figure 2.10A), glucose (Figure 2.10B) and fructose (Figure 2.10C) levels in young leaves. The mean value for concentrations was calculated from 3 biological repeats from one individual plant per transgenic line. Standard error of the mean was calculated from the standard error calculated for each transgenic line. An asterisk marks the significantly different sugar concentrations ( $p < 0.05$ ) when compared with sugar concentrations in wild type NCo310 (WT). Significant differences ( $p < 0.05$ ) were calculated using a two tailed t-test that assumed unequal variance, with the null hypothesis being that there is no difference in the mean sugar levels measured in wild type NCo310 (WT) leaves and transgenic leaves.





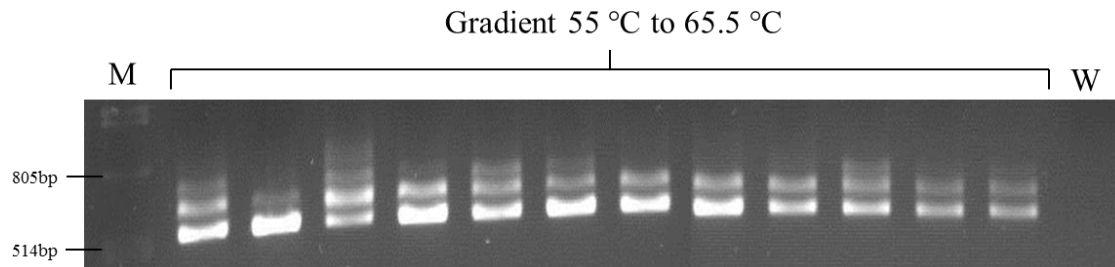


**Figure 2.10:** Soluble sugar concentrations ( $\mu\text{mol/g FW}$ ) in young leaves of *otsA* and *otsB* overexpressing lines. (WT) = wild type NCo310 and average sucrose (A), glucose (B) and fructose (C) concentrations. Asterisks mark the significantly different values ( $p < 0.05$ ) from wild type NCo310 (WT).

### 2.3.2 Isolating a full length sugarcane *TPS* gene

#### 2.3.2.1 Initial isolation of a partial *SoTPS1* coding sequence

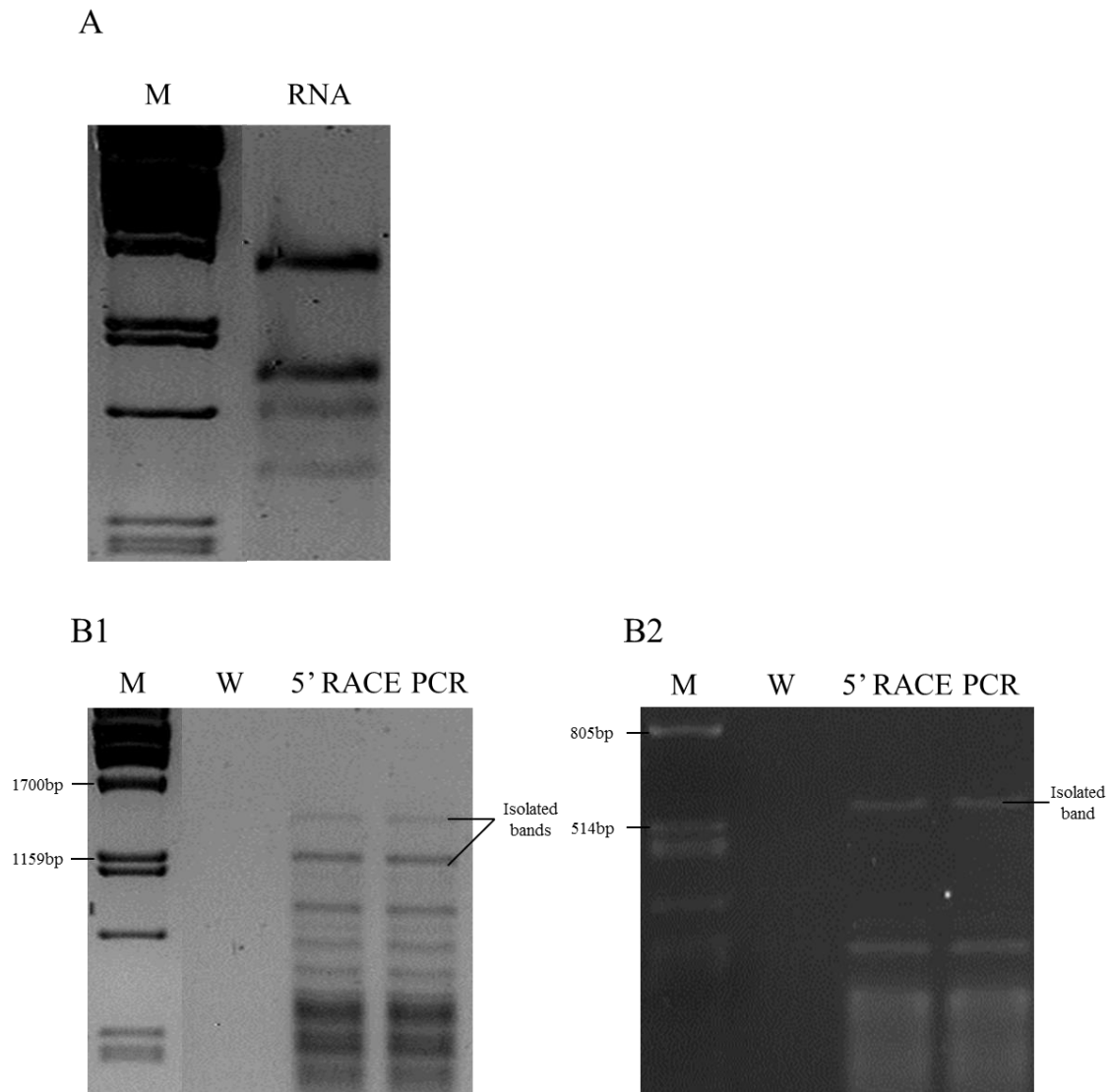
Gradient PCRs were performed with the sugarcane Ca13, Ta49 and the OsCons rice primer pairs (Table 2.3), using cDNA from sugarcane leaf-roll tissue as a template. No amplification was achieved with Ca13 and OsCons primers. Gradient PCR using Ta49 primers yielded three bands of around 500 bp, 600 bp and 700 bp (Figure 2.11), where the expected amplicon size was 558 bp. The 600 bp and 700 bp bands were subsequently isolated, cloned and sequenced. Sequencing results showed that the smaller band was 558 bp long and aligned with the known database *SoTPS1* partial sequence. The larger band was shown to be 631 bp long and contained a 75 bp insertion fragment that was not present in other monocotyledonous *TPS* sequences. These two isolated sugarcane *TPS* sequences were then used as template to design a gene-specific primer (SoTPS1 GSP1 primer; Table 2.4) for use in 5' RACE PCR.



**Figure 2.11:** Gradient PCR using sugarcane leaf roll cDNA template over an annealing gradient range from 55°C to 66.5°C with Ta49 primers, visualized on a 1.2% agarose gel. M = *Pst*I digested lambda DNA. W = water control.

### 2.3.2.2 5' RACE

High quality RNA was isolated from sugarcane leaf tissue (Figure 2.12A) to perform RACE. An initial 5' RACE PCR reaction was performed with the *SoTPS1* GSP1 primer (Table 2.4), using 5' RACE cDNA synthesized from sugarcane leaf-roll RNA. Various size PCR amplicons were generated and only the two largest bands were excised from the gel, cloned and sequenced (Figure 2.12B1). Sequencing results indicated that the two amplicons were homologous and only differed in size. Thus 5' RACE PCR resulted in the addition of 758 bp of new sequence to the known *SoTPS1* gene fragment. However, alignments with *TPS* sequences from other monocotyledonous plant species showed that a potential 497 bp of sequence still had to be amplified. 5' RACE was repeated using the *SoTPS1* GSP2 primer (Figure 2.12B2), however this only revealed a further 33 bp of sequence and still lacked the 5' ATG codon (Annexures, Figure A2.4). The new partial *SoTPS* revealed through 5' RACE, shared 97% identity with the sorghum hypothetical *TPS* sequence (XM\_002440041.1), 96% identity with the functional maize *ZmTPS1* (EU659122.2) and 88% identity with the functional rice *OsTPS1* (HM050424).



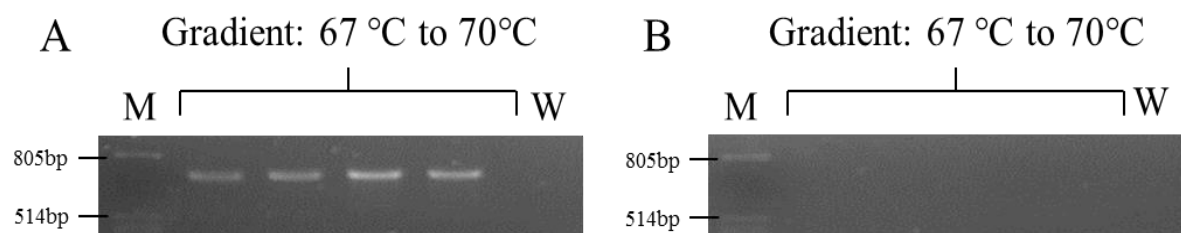
**Figure 2.12:** *SoTPS1* 5' RACE as visualized on a 1.2% agarose gel. M = *Pst*I digested lambda DNA, W = water control. (A) = Isolated RNA for 5' RACE cDNA synthesis; (B1) 5' RACE with primer *SoTPS1* GSP1; (B2) 5' RACE with primer *SoTPS1* GSP2. Isolated bands indicated on the figure were gel purified for further analysis.

### 2.3.2.3 *SoTPS* full length amplification

The newly isolated partial *SoTPS* RACE sequence showed high homology to the predicted sorghum *TPS* gene. New primers based on the homologous regions with the sorghum *TPS* sequence were designed. These primers included two forward primers, designed to bind at separate potential start codons present at nucleotide positions 1 and 70 of the sorghum *TPS* sequence (Table 2.5: *SoTPS* Forward 1 and *SoTPS* Forward 2). Both these start codons are in

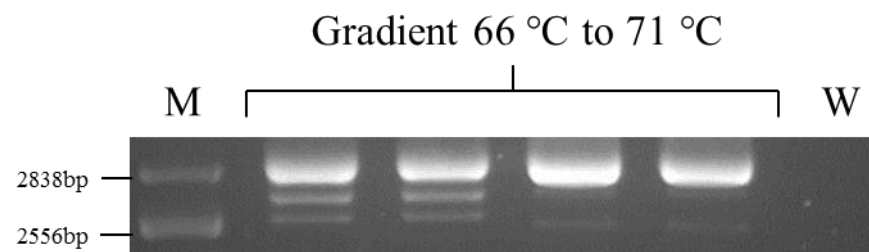
frame with one another and the second start codon at nucleotide position 70 aligns with the functional maize *TPS* start codon (EU659122.2).

Gradient PCR's were performed using the SoTPS Forward 1 and SoTPS Forward 2 primers in combination with the SoTPS int Reverse primer 1 with annealing temperatures ranging from 67°C to 70°C in combination with a high GC and 6% DMSO PCR buffer mix (see section 2.2.3.7). A PCR fragment of the expected size 700 bp was amplified with primers binding at the first codon (Figure 2.13A), but no amplification occurred with the second start codon forward primer (Figure 2.13B).



**Figure 2.13:** Gradient PCR using sugarcane leave roll cDNA template over an annealing gradient range from 67°C to 70°C. The SoTPS Forward 1 and SoTPS Forward 2 primers were used in combination with the SoTPS int Reverse 1 primer and visualized on a 1.2% (w/v) agarose gel. (A) SoTPS Forward 1. (B) SoTPS Forward 2. M = *Pst*I digested lambda DNA, W = water control.

Finally, putative full length sugarcane *TPS* gene fragments were amplified using the first ATG codon primer in combination with the 3' SoTPS Reverse 1 primer (Table 2.5). Three DNA amplicons of approximately 2800 bp, 2700 bp and 2600 bp were amplified (Figure 2.14).



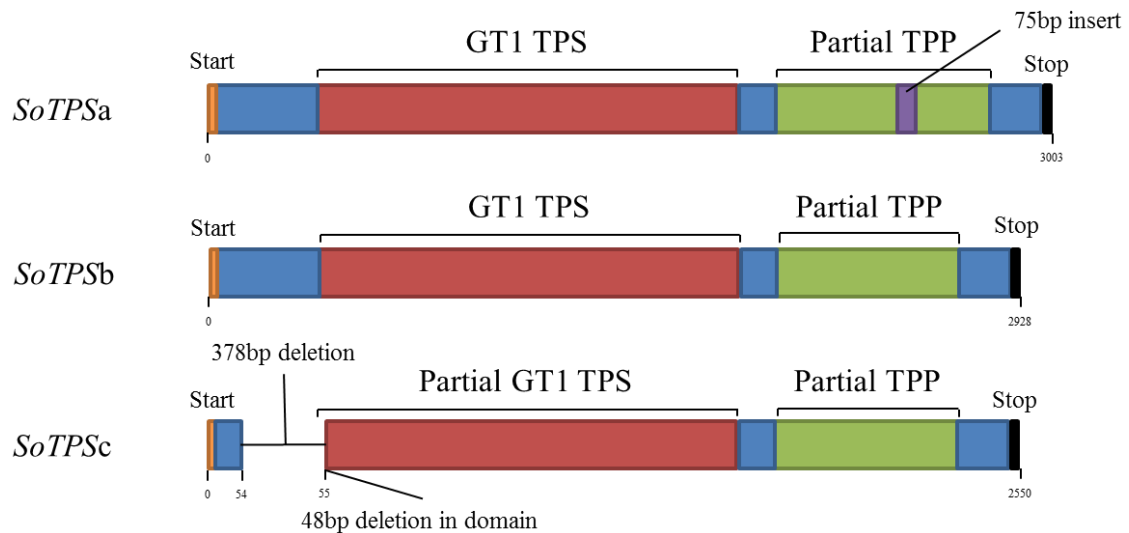
**Figure 2.14:** Full length *SoTPS* Gradient PCR. Gradient PCR using sugarcane leave roll cDNA template over an annealing gradient range from 66° to 71°C with SoTPS Forward 1 and SoTPS Reverse 1, visualized on a 1.2% agarose gel. M = *Pst*I digested lambda DNA. W = water control.

The amplicons were purified from the gel, cloned separately into the pJET 1.2 cloning vector and sequenced. Sequencing results analysed with blastx (<http://www.ncbi.nlm.nih.gov/>), showed that these amplicons represent three possible isoforms of putative sugarcane *TPS* sequences. Two of the isolated isoforms encode a 24 amino acid N-terminal sequence also found in the sorghum *TPS*, while the third isoform encodes an 18 amino acid N-terminal segment also matching the sorghum *TPS*. (Figure 2.15).

<b>SbTPS</b>	MSSDAAGGQR	SISNSTRGD~	AAAAMPTSSP	FVVGDSGGGA	GSPIRVDRMV
<b>ZmTPS</b>	-----	-----	---MPTSSP	FVG--DSGGA	GSPIRVERMV
<b>SoTPSa</b>	MSSDAAGGQR	SISNCTRDDA	AAAAMPTSSP	FVG-DSSSGA	GSPIRVDRMV
<b>SoTPSb</b>	MSSDAAGGQR	SISNCTRDDA	AAAAMPTSSP	FVGGDSGGGA	GSPIRVDRMV
<b>SoTPSc</b>	MSSDAAGGQR	SISNCTRG--	-----	-----	-----

**Figure 2.15:** Amino acid alignment of the N-termini of predicted *TPS* genes from sorghum, maize and sugarcane. SbTPS = hypothetical sorghum *TPS* (NCBI accession number: XP\_002440086.1), ZmTPS = functional maize *TPS* (NCBI accession number: ACD35326.2), SoTPSa = putative sugarcane *TPS* isoform a, SoTPSb = *TPS* isoform b and SoTPSc = *TPS* isoform c.

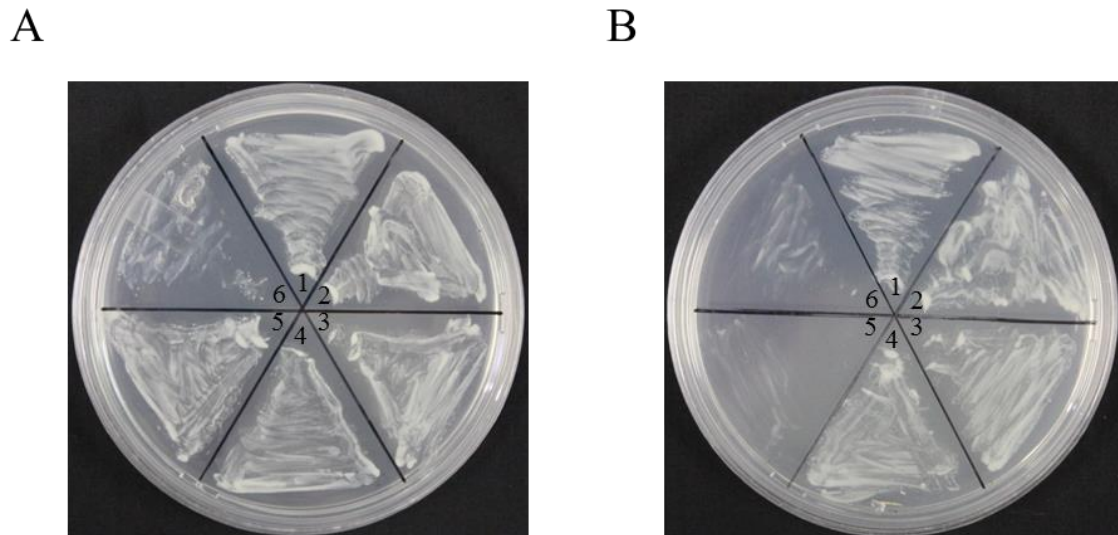
The largest amplicon (3003 bp), termed *SoTPSa*, contains a 75 bp in-frame insertion in the 3' end of the sequence that matches the insertion found in the original 631 bp Ta49 sequence (Figure 2.16). The middle sized amplicon (2928 bp) termed *SoTPSb*, does not contain any significant insertions or deletions, when compared with the *SbTPS*, *ZmTPS1* and *OsTPS1* sequences in alignments (Figure 2.16). The smallest amplicon (2550 bp) termed *SoTPSc*, contains a 378 bp deletion at the 5' end (Figure 2.16). This deletion is situated 54 bp downstream from the start of the gene and ends 48 bp in the GT1 *TPS* conserved domain, resulting in a partial GT1 *TPS* domain. *SoTPSa* shares 98%, 95% and 89% sequence identity with *SbTPS*, *ZmTPS1* and *OsTPS1* respectively. *SoTPSb* shares 97%, 95% 88% sequence identity with *SbTPS*, *ZmTPS1* and *OsTPS1* respectively. *SoTPSc* shares 98%, 96% and 88% sequence identity with *SbTPS*, *ZmTPS1* and *OsTPS1* respectively. A visual representation of the construction of these three sequences can be seen in figure 2.16. The *SoTPSa*, *SoTPSb* and *SoTPSc* nucleotide sequences are shown in Annexures figures A2.4, A2.5 and A2.6, respectively.



**Figure 2.16:** A graphical representation of the three putative *SoTPS* sequences isolated from sugarcane cDNA. *SoTPSa*, *SoTPSb* and *SoTPSc* differ in size due to both an insertion and deletions as described. The red block represents the GT1 TPS domain and the green block represents the partial TPP domain, which are both found in plant *TPS* sequences.

#### 2.3.2.4 Functional analysis of putative *SoTPS* sequences

The pAG426GPD::*SoTPSa*, pAG426GPD::*SoTPSb* and pAG426GPD::*SoTPSc* constructs were transformed into *tps1Δ* yeast cells to assess the complementation of the growth defect of *tps1Δ* yeast cells on SCglu-ura medium. All three putative *SoTPS* sequences were able to complement the growth defect of *tps1Δ* yeast cells grown on glucose medium (Figure 2.17).



**Figure 2.17:** Complementation of *tps1Δ* yeast cells transformed with putative *SoTPS* genes. **(A)** SCgal-ura medium containing 3% galactose and all amino acids except uracil; **(B)** SCglu-ura medium containing 2% glucose and all amino acids except uracil. *tps1Δ* yeast transformed with constructs were streaked out in the following order 1: *tps1Δ*pAG426GPD::*SoTPSa*, 2: *tps1Δ*pAG426GPD::*SoTPSb*, 3: *tps1Δ*pAG 426GPD ::*SoTPSc*, 4: *tps1Δ* pAG426GPD::*otsA*, as positive control 5: *tps1Δ* pAG426GPD, 6: *tps1Δ* with no plasmid.

## 2.4 Discussion

### 2.4.1 Overexpression of *otsA* and *otsB* in transgenic sugarcane

To progress towards understanding how T6P affects the sucrose content in sugarcane, two trehalose synthesis genes from *E. coli*, *otsA* and *otsB*, were successfully introduced into sugarcane. These overexpressing transgenic sugarcane lines were successfully generated and transgene insertion and expression confirmed with PCR, sq-RT-PCR and immuno-blotting with *otsA* and *otsB* antibodies.

#### 2.4.1.1 Phenotype of transgenic plants

In past research, when yeast or *E. coli* *TPS* genes were constitutively expressed in dicotyledonous plant species such as *Arabidopsis*, tobacco, tomato and potato, stunted growth had been observed as a resulting phenotype (Romero *et al.*, 1997; Goddijn and Van Dun, 1999; Schluemann *et al.*, 2003; Cortina and Culianez-Macia, 2005). These growth



aberrations were most likely due to accumulation of T6P and trehalose levels. In contrast, monocotyledonous plant species do not seem to be sensitive to fluctuations in T6P and trehalose levels (Zhang *et al.*, 2006; Li *et al.*, 2011). In this study no phenotypic differences were observed in either *otsA* or *otsB* transgenic lines when compared with wild type sugarcane plants. Thus, the constitutive expression of an *E. coli* *TPS* or *TPP* gene in sugarcane, a monocotyledonous species, did not result in any pleiotropic phenotypes. Similar results was obtained when Li *et al.*, (2011) and Zhang *et al.*, (2006) overexpressed the functional *OsTPS1* or fungal *Grifola frondosa* *TPS* in rice and sugarcane, respectively, without any growth defects. In these plants, the overexpression of *TPS* resulted in the up-regulation of *TPP* and trehalase which most likely caused the catabolization of T6P and so decreased the toxic effects caused by excessive T6P accumulation. Future enzymatic analysis of our mature transgenic sugarcane plants will determine whether a similar scenario occurs within the *otsA* and *otsB* overexpressing sugarcane lines.

#### 2.4.1.2 Soluble sugar accumulation in transgenic plants

Early studies involving *TPS* overexpression in plants was concerned with increasing drought tolerance and not much data is available on sucrose levels in these plants (Holmstrom *et al.*, 1996; Romero *et al.*, 1997; Goddijn and Van Dun, 1999; Garg *et al.*, 2002; Penna, 2003; Cortina and Culianez-Macia, 2005; Li *et al.*, 2011; Lyu *et al.*, 2013). Only one study by Romero *et al.*, (1997), noted a decrease in sucrose levels as a result of accumulating T6P levels in transgenic tobacco plants. Later studies reported the effect of T6P on starch accumulation in *Arabidopsis*, where increases in T6P levels caused an accumulation of starch in transgenic plants when sucrose was exogenously supplied to plants (Kolbe *et al.*, 2005; Lunn *et al.*, 2006; Martins *et al.*, 2013). Martins *et al.*, (2013) did observe that induced *TPS* expression resulted in a decrease in sucrose concentrations in the leaves of transgenic *Arabidopsis*, during night time. However, these studies did not focus on sucrose accumulation due to changes in T6P levels. This current study is the first attempt to better understand the influence of *TPS* and *TPP* overexpression on the internal sucrose levels in plants with special emphasis on sugarcane, a plant species which normally accumulates high amounts of sucrose.

Initially it was hypothesised that *otsA* overexpression would result in reduced sucrose levels, as certain studies had shown that T6P increases result in starch accumulation, accompanied by a decrease in sucrose (Romero *et al.*, 1997; Wingler *et al.*, 2000; Schluemann *et al.*, 2004; Martins *et al.*, 2013). Preliminary analysis of soluble carbohydrate concentrations in the *otsA* transgenic lines showed six out of the nine lines had reduced sucrose levels. Four of these

lines, namely *otsA* 7, 8, 11, and 15 had significantly reduced sucrose levels and only *otsA* 12 had significantly increased sucrose levels in comparison with the non-transgenic control sugarcane plants (Figure 2.10). When looking at glucose, eight of the nine *otsA* lines showed increase glucose levels with four lines, namely *otsA* 1, 7, 10 and 12 with significantly increased glucose in comparison with wild type NCo310. Only *otsA* 7 and *otsA* 12 showed significant changes in both sucrose and glucose. Although glucose levels were increased in both the *otsA* 7 and *otsA* 12 lines, and contrast was seen in sucrose accumulation, where *otsA* 7 had decreased sucrose levels and *otsA* 12 had increased sucrose levels.

When looking at the relative *otsA* gene expression levels using sq-RT-PCR (Figure 2.6) all the lines with the higher gene expression, *otsA* 7 and to a lesser extend, *otsA* 10 and 15 seem to have lower sucrose and higher glucose levels, with the change in sucrose only being significant for *otsA* 7 and 15. It is possible that a high level of *otsA* gene expression results in a decrease in sucrose. However, analysis of the carbohydrate levels in mature cane is needed before any conclusion can be made. Including measurements of T6P levels alongside measurements of other sugars would also aid in future analysis. Along with quantitative gene expression studies, more biological and technical sample replicates should be included in future experiments to improve the experimental setup.

With *otsB* overexpression it was assumed that T6P levels would be decreased, but it was considered that a flux might occur driving more T6P synthesis (Lunn *et al.*, 2006; Schlupepmann *et al.*, 2012; O'Hara *et al.*, 2012; Martins *et al.*, 2013). When looking at sucrose accumulation in the *otsB* lines, lines 2 and 1.2 acted contradictory to one another, but only the decrease in sucrose in *otsB* 2 was considered to be significant. Glucose and fructose levels seemed to increase in both lines when compared with the non-transgenic control, with the changes in *otsB* 1.2 being significant. Specifically, *otsB* 1.2 leaf extract had roughly five fold increased glucose when comparing with extract from control leaves (Figure 2.10). Semi quantitative PCR showed similar levels of relative transgene expression in both lines (Figure 2.7). Once again, final conclusions will only be made once data from mature cane can be analyzed with the proper replicates in place.

Concerning the possible effects of *otsA* and *otsB* overexpression on glucose, only assumptions can be made until trehalose levels are measured in leaf extracts. Glucose can either be a source (G6P) for trehalose production or the end product if trehalose is hydrolyzed by trehalase into glucose. It is considered unlikely that trehalose hydrolysis would affect glucose levels, since Bosch, (2005) observed that trehalose occurs at concentrations below 1

nmol/g in sugarcane internodes. Thus, trehalose would probably occur at levels at least a thousand fold less than glucose concentrations. However, Zhang *et al.*, (2006) reported trehalose accumulation in the leaves of transgenic sugarcane overexpressing a fungal TPP gene at around 8 to 12 mg/g leaf tissue, compared with undetectable levels in wild type controls. Trehalose levels of *otsA* and *otsB* sugarcane would have to be measured in future studies to determine these unknown factors.

## 2.4.2 SoTPS isolation

### 2.4.2.1 Isolation of a partial *SoTPS*

Initially, a partial *SoTPS1* sequence (EU761244.1) was available on NCBI website, which has been isolated from two Brazilian sugarcane cultivars, *Saccharum* spp. SP90-1638 and SP83-2847, during a water stress experiment (Nicolau *et al.*, 2013). Attempts to PCR amplify this known sequence from *Saccharum* cv. NCo310 sugarcane material resulted in three different length amplicons. Sequencing of the two largest amplicons showed that both were partial segments of the partial *SoTPS1* sequence on NCBI, where the larger band contained a 75 bp previously unknown insertion fragment. By applying 5' RACE, 758 bp additional sequence was added to this known partial database *TPS* sequence. Alignment of the 5' RACE fragment with the *SbTPS*, *ZmTPS1* and *OsTPS1* gene sequences showed that parts of the TPS domain and a start codon were still missing. The newly isolated 2431 bp sugarcane *TPS* sequence showed between 88 and 98% homology with other monocot *TPS* genes. Especially the known sorghum *TPS* showed very high homology (98%) to the newly isolated putative sugarcane *TPS* sequence fragment.

### 2.4.2.2 Isolation of full length putative sugarcane *TPS* gene sequences

After aligning a number of *TPS* sequences with each other and with the newly isolated partial sugarcane *TPS* sequence, two possible start codons, 69 bp apart, that are in frame with each other, were identified. It has been shown that isoforms from which the first start codon is absent, namely *ZmTPS1* (EU659122.2) and *OsTPS1* (HM050424), still can encode for a functional TPS in maize and rice, respectively (Jiang *et al.*, 2010). Both start codons can also be seen in the hypothetical sorghum *SbTPS* (XM\_002440041.1) and the predicted *Setaria italica* *TPS* (NCBI accession number: XM\_004961429.1) gene sequences. However, recently, *ZmTPS* isoforms KC788563 to KC788570 (unpublished), that do contain this first start codon

along with the second start codon, have been submitted to the NCBI database by Jiang, (2013). Initial PCR's had been performed with primers binding at both possible start codons. Only the primer binding in the first start codon in combination with an internal reverse primer yielded a single amplicon of the expected size. Using this first start codon primer, three full length *SoTPS* sequences were eventually isolated and sequenced, ranging in size from 2550 to 3003 bp. With the exception of *SoTPSc*, which had a 378 bp deletion when compared with other monocot *TPS* genes, the newly isolated full length sugarcane *TPS* isoforms were very similar to known *TPS* genes, with homology around 97%, 95% and 88% when compared with sorghum, maize and rice sequences, respectively. Of importance is the insertion of 75 bp found in sugarcane isoform *SoTPSa* and the 5' end deletion in *SoTPSc* that removes 48 base pairs of the GT1 *TPS* domain and excludes the second start codon when compared with the other available monocot *TPS* genes.

#### 2.4.2.3 The possibility of identifying more putative *SoTPS* isoforms

Past studies have shown numerous *TPS* isoforms to be present in plant species, such as the eleven putative *Arabidopsis* *TPS* isoforms identified by Leyman *et al.*, (2001). Other examples are the eleven putative rice *OsTPS* isoforms identified by Zang *et al.*, (2011), and the recently submitted eight maize isoforms on NCBI (Jiang, 2013). During this study, three potential *SoTPS* isoforms have successfully been isolated. Furthermore, during initial attempts to amplify partial sugarcane *TPS* gene fragments, primers designed to bind within the 3' region of the gene resulted in three amplicons of which the smallest were not isolated and characterized (section 2.3.2.1). Assuming that the smaller band amplified with the initial 3' end primers (Figure 2.11) was a *SoTPS* sequence, at least one other truncated potential *SoTPS* isoform, containing a deletion located within the 3' end of the *SoTPS* sequence, might be present in sugarcane. Another consideration is that during attempts to isolate a full length *SoTPS* sequence, cDNA derived from sugarcane leaf tissue was used as a template. Using cDNA derived from intermodal tissue as a template could probably yield new *SoTPS* isoforms in PCR, since different tissue might express different isoforms, but no studies have observed whether this does occur with plant *TPS* genes. Future work can consider these aspects in attempts to identify more putative *SoTPS* isoforms.

#### 2.4.2.4 Functional analysis of putative sugarcane TPS sequences

Multiple TPS isoforms have been isolated in other plants species, which share high sequence similarity, to one another and other plant TPS sequences. However, further analysis of these sequences show that not all these isoforms would code for functional enzymes. Examples of this is the eleven isoforms in *Arabidopsis* and rice, where only one in each species have been shown to code for a functional enzyme (Leyman *et al.*, 2001; Zang *et al.*, 2011). Similarly, although the *SoTPSa*, *SoTPSb* and *SoTPSc* sequences show high similarity to the functional *ZmTPS1* and *OsTPS1* sequences, yeast complementation assays needed to be performed to determine whether these sequences coded for functional TPS enzymes. Additionally, further analysis of the three isoforms showed alterations in domains and it remained to be seen whether these would still be able to complement yeast mutant strains.

Both the *tps1Δ* and *tps1Δ + tps2Δ* strains are incapable of growing on media containing fermentable sugars, such as glucose, but galactose can be used as a replacement carbon source to initiate growth. In addition, *tps1Δ* and *tps1Δ + tps2Δ* are incapable of growing on medium containing 2% glucose, unless supplemented by a functional TPS gene. The pAG426GPD-cdB vector contains a uracil synthesis gene for the selection of transformed autotrophic yeast mutants on medium that lacks uracil. The pAG426GPD::*otsA* construct acted as a positive control where the encoded *otsA* protein should synthesize T6P and enable growth of *tps1Δ* yeast mutants on medium containing glucose as a carbon source (Wang *et al.*, 2000). The pAG426GPD empty vector control should enable growth on medium lacking uracil, yet not enable growth of *tps1Δ* yeast mutants on medium containing glucose as a carbon source; additionally showing that complementation of the *tps1Δ* yeast mutation is not due to the plasmid vector sequence. All three sugarcane isoforms were shown to be able to complement the growth defect of *tps1Δ* yeast grown on glucose medium and it was concluded that all three isoforms code for functional TPS activity.

Most plant TPS genes consist of a GT1 TPS domain (TPS domain) and a partial TPP domain, where the GT1 TPS domain is around 466 residues long with 13 active sites, according to the Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2013). In the isolated *SoTPSa* isoform, a 75 bp insert is present in the TPP domain. However, since this isoform was shown to be active in the yeast mutants, it is assumed that the insertion did not disrupt the TPS activity, most likely because it is not located within the GT1 TPS domain. Future enzymatic studies could show if this insertion has any effect on the rate of T6P synthesis. The *SoTPSc* isoform contains a deleted segment that excludes the first 16 residues of the GT1 TPS domain.

According to CDD on NCBI, this deletion also excludes 1 out of the 13 active site residues for the GT1 TPS domain (Marchler-Bauer *et al.*, 2013). However, the complementation assay showed that missing 1 active site and excluding 16 out of 466 amino acids (3.43%) of the GT1 TPS domain did not disable TPS activity. Van Dijck *et al.*, (2002) showed that truncating the N terminal extensions of the *Arabidopsis AtTPS1* and *Selaginella lepidophylla SITPS1* resulted in higher TPS activity when overexpressed in yeast. Specifically, 86 residues was truncated on the N terminal extension of *AtTPS1* and 99 residues was truncated on the N terminal extension of *SITPS1* (Van Dijck *et al.*, 2002; Li *et al.*, 2011). Thus, the enzymatic activity of the encoded protein may have been altered, since the truncation stretches for 126 residues. It could be assumed that alternative splicing of *SoTPS* sequences occur in sugarcane, resulting in altered TPS activity. Future enzymatic studies will have to be conducted to study the effects on TPS activity, caused by either the N terminal truncation of *SoTPSc* or the insertion of *SoTPSa*.

## 2.5 Conclusions

*otsA* and *otsB* overexpressing transgenic sugarcane lines were successfully created with no phenotypic abnormalities. Preliminary soluble sugar determinations suggested a possible trend where *otsA* overexpression results in a decrease in sucrose content in sugarcane, however, *otsB* overexpressing lines resulted in inconsistent carbohydrate levels which will have to be determined again in the future when mature cane material becomes available. In the future, additional replicate measurements and expression analysis of mature cane material will lead to more conclusive results.

Three putative *SoTPS* isoforms, namely *SoTPSa*, *SoTPSb* and *SoTPSc* were isolated from sugarcane leaf-roll cDNA. All three isoforms were shown to encode functional TPS enzymes by complementing the growth defect of *tps1Δ* yeast grown on glucose. Differences between the three *SoTPS* isoforms suggest alternative splicing and future enzymatic studies will determine the effect of splicing on TPS activity. Results suggest that potentially more isoforms exist which can be isolated in future studies.

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## 2.7 Annexures

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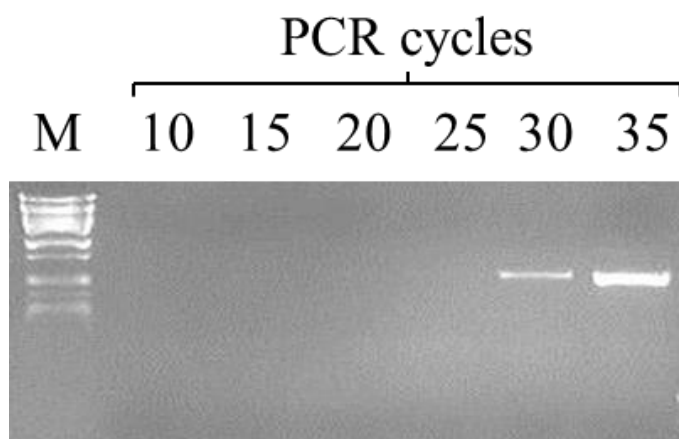
**Figure A2.1:** Nucleotide sequence of *otsA* in the pUBI 510+ vector. The start and stop codons of the *otsA* gene are highlighted in green and red respectively. The *NotI* and *EcoRI* restriction sites are highlighted in light blue.

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TGACTATGAGTCGTTTAGTCGTAGTATC TAA CCGGAT GAATTC

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**Figure A2.2:** Nucleotide sequence of *otsB* in the pUBI 510 + vector. The start and stop codons of the *otsB* gene are highlighted in green and red respectively. The *NotI* and *EcoRI* restriction sites are highlighted in light blue.



**Figure A2.3:** Sq-RT-PCR amplification of wild type NCo310 cDNA using actin primers (Actin forw1 and Actin rev1). A total of 50 ng of cDNA was used as template in PCR. PCR cycles = number of cycles.

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**Figure A2.4:** Nucleotide sequence of the isolated *SoTPS*a isoform. The start codon is highlighted in green. The GT1 TPS and partial TPP domains are highlighted in light blue and yellow, respectively. The unique 75 bp insert area is highlighted in purple.

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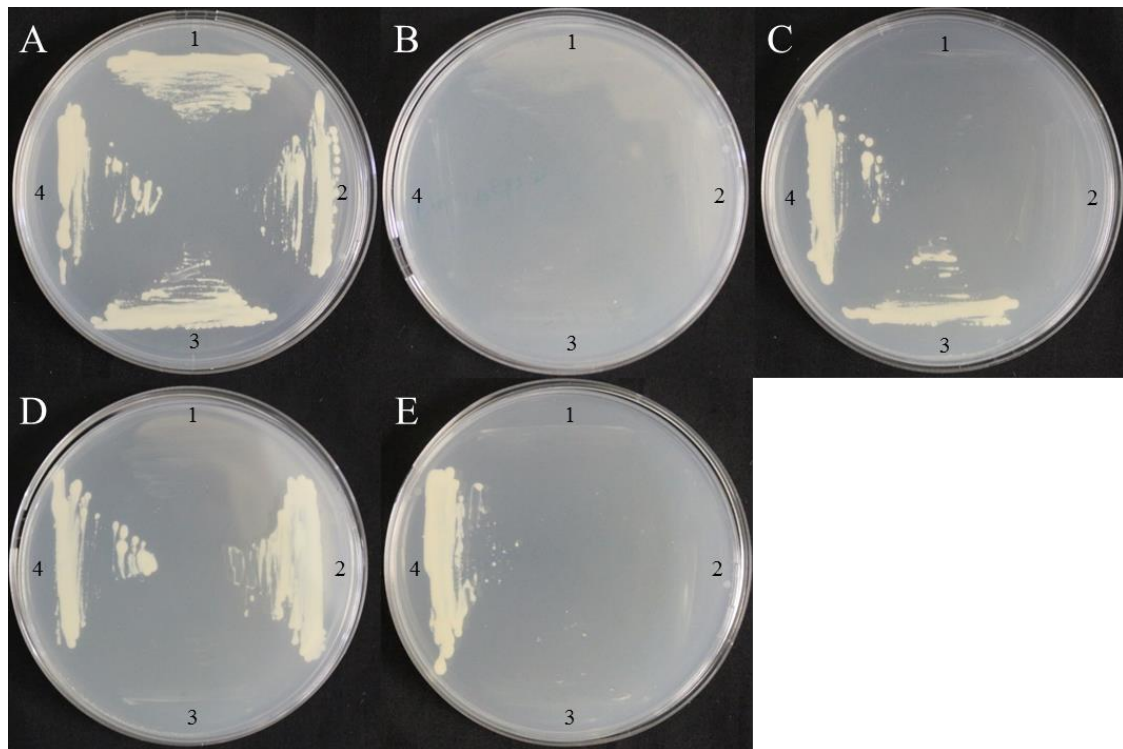
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TTTGTGCGGATTTCATCTACGACTATGCGAGGCATTTTGTGAGTGTCTGCCTAGAATCTGGACTTGAGGGTACCCCTGAGGGTGTGGAAAGTCA
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AATTGACAGAACGTTTGTGCTGAAAGGTAATGCTTGGTGTGATCGACTTGACATGATTAAGGGAATCCACAAAAGATTTGGCCTTTGAAAAG
TTTTTTGAGGAAAACCCAGATTGGAACAACAAAGTTGTTCTACTGCAGATTGGTGTGCCAACAAGAACTGACGTCCTGAGTATCAAAAGCTAACGAG
ACTCAATACCGCATGCTTAAACGATGCCATCCGATGAGAGAGAGAAAACGACAGGCACAACATATGCTCATGTCAAACTCACCGCTCAAGATTG
GGCTGAAACTTTTGTATTGAGCTAAATGACACGGTGTGAGGCACTACTGAGGACAAGACAAGTCCCTCTGGTCTTCTAGTCAAAATGGCAATCC
AGCAATATTTGCGCTCAAAAATCGTCTGCTCTATATTGGGTTTCAATTCACATTTGACTGAGCCAGTCCGAATCTCTGGGAGAAAGGGTGGTGACCAA
ATCAAGGAAATGGAACTAAGTTGCATCTGACTTAAAGGGTCTCTGAGAGCCCTCTGTGAGGATGAGCACACTACAGTTATTGTTCTCAGTGGCAG
TGACAGGAGTGTCTTGATGAAAATTTGGAGAATTTAAAATGTGGTGGCGGCAGAGCATGGGATGTTTTACGCCGACTTACGGAGAATGGATGA
CAACAATGCGCTGAGCATCTGAACATGGATTGGGTTGACAGCGTAAAGCATGTTTTGAATACTTTACAGAAAAGAACCCCAAGATCCCATTTGAAACAT
CGTGAAACATCATTTGTGTGGAACATAAAGTATGCTGATGTTGAAATTTGGAAGGCTACAAGCAAGAGATATGCTGACAGCACTTGTGGACAGGTCCGAT
CTCAATGACAGCTGTGATGTTGTTCAAGGGAGTCCGTCAGTTGAAGTTCGGTCTGTGGAGTTACAAGGGTGTGCAATTGATCGTATTTAGGGGA
GATATTCACAGCGAAAACATGGTTACTCCAATTGACTATGTCTGTATAGGGCATTTCCTGGGAAGGATGAGGACATCTATGTCTTTTTGATCC
CGAGTACCCTTCTGAATCCAAAATAAAAACAGAGGGTGGCTCAGCTTCACTTGACCGGAGGCCGAACGGAAAGTCAACATCGAATGGCAGGAGCAG
TCCAGAACCCACAGTCCAGGACACAGAAGGCGCAGCAGGCTGATCCGAGAGGTCATCTCATCAAGCCACAGCAGCGCAAGCAGCAACCGACT
GGCGGAAAGGTTCTCGGTCTTGATCTCAAGGGCGAGAACTACTTCTCTGCGCGCTCGGAAGGAAGCGGTCAACGCCGTTACCTGCTGATGTTCC
TCGGAGGAGGTTGTCTTCTCAAGGAATGGCATCGGCAACAGCTGGCTTCCAATCCAGCTGTGCTGATTACATGTTTCATGGATAGGCGAGTAA
    
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**Figure A2.5:** Nucleotide sequence of the isolated *SoTPS*b isoform. The start codon is highlighted in green. The GT1 TPS and partial TPP domains are highlighted in light blue, and yellow, respectively.

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GCGGCG ATCAGCTCTGACGCCGCGGGGGACAGCGCAGCATCAGCAACTGCACGAGGGGCGCGAGGACCAATGGTCTCTTGAGATCAGCGCGCGG
GGCCTCGTGAGCGCCTTGTCTGGCGTGAAGGACGTGATGCGAAATGGATTGGCTGGCGGGCGTCAACGTACCAGATGAGGTTGGCCAGCGAGCCC
TCACAAAAGCTCTTGCCGAGAAGAGATGCATACCAGTGTCTCTGGACGAGGAGATTGTGCACCAGTACTACAATGGGTACTGCAACAACATCTGTGG
CCGCTGTCCACTACCTAGGACTACCACAGGAGGACAGGCTGGCAACAACGAGGAACCTTGAGTACAGTTCGACGCGTACAAGCGTCTAACACAGA
TGTTCGCTGATGCTGTACCAGCACTACCAGGAGGGGATGTAATCTGGTGCATGACTACCACCTCATGTTCTGCCAAGTGCCCTCAAGGACCAT
GACATCAATATGAAGTTCGGTGGTCTGCACACGCCATTCATCATCAGAGATTACCGAACACTGCCCTCCGCTGGAGCTGCTTCGCTCGGTG
TTGTGTGCTGATTAGTCGGATTTACATCTACGACTATGCGAGGCATTTTGTGAGTGTCTGCCTAGAATCTGGACTTGAGGGTACCCCTGAGGGT
GTGGAAATCAAGGAAGACTAACAGGGTTCGAGCGTTTCTATTGGGATAGACTCTGATCGTTTCAAGCGAGCATTGGAGCTTCCAGCAGTAAAAG
GCACATCAGTGAATGACAGAACGTTTGTGGTGAAGGTAATGCTTGGTGTGATCGACTTGACATGATTAAGGGAATCCACAAAAGATTTTGG
CCTTTGAAAAGTTTCTTGAGGAAAACCCAGATTGGAACAACAAAGTTGTTCTACTGCAGATTGCTGTGCCAACAAGAACTGACGTCCTGAGTATCAA
AAGCTAACGAGCCAAGTGCATGAAATTTGTTGGCGCATAAATGGTGCATTCGGAACGTTGACTGTGTCCCTATTTCATCATCTGGACCGATCTCTGAT
TTCCATGCCTTGTGTCTTTATGCAGTCACTGATGTTGCTTGTAAATCACTGAGAGATGGGATGAACCTTGTGAGCTATGAGTACGTTGCATGC
CAAGGGTCTAAGAAGGAGTTCTGATCTTGTGAGTTTGTGGGCGACACAATCACTTGGAGCTGGCGCATTTCTAGTAAACCCTTGGAAATATTAC
AGAAGGTGACAGCTCAATACGGCATGCTTAAACGATGCCATCCGATGAGAGAGAGAAAACGACAGGCACAACATATGCTCATGTCAAACTCACAG
GCTCAAGATTGGGCTGAACTTTTGTATTGAGCTAAATGACACGGTGTGAGGCACTACTGAGGACAAGACAAGTCCCTCTGGTCTTCTAGTCA
AATGGCAATCCAGCAATATTTGCGCTCAAAAATCGTCTGCTCTATATTGGGTTTCAATTCACATTTGACTGAGCCAGTCAATCCCTGGGGAAGAGGG
GTGGTGACCAAATCAAGGAAATGGAACCTCAAGTTGCATCTGACTTAAAGGGTCTCTGAGAGCCCTCTGTGAGGATGAGCACACTACAGTTATTGTT
CTCAGTGGCAGTGACAGGAGTGTCTTGATGAAAATTTGGAGAATTTAAAATGTGGTGGCGGCAGAGCATGGGATGTTTTACGCCGACTTACGG
AGAATGGATGACAACAATGCTGAGCATCTGAACATGGATTGGGTTGACAGCGTAAAGCATGTTTTGAATACTTTACAGAAAAGAACCCCAAGATCCC
ATTTGAAACATCGTGAACATCATTTGTGTGGAACATAAAGTATGCTGATGTTGAAATTTGGAAGGCTACAAGCAAGAGATATGCTGACAGCACTTGTGG
ACAGGTCGATCTCAATGACGCTGTGATGTTGTTCAAGGGAGTCCGTCAGTTGAAGTTCGGTCTGTGGAGTTACAAGGGTGTGCAATTGATCGG
TATTTTGGGAGATATTTACAGCGAAAACATGGTTACTCCAATTGACTATGCTCTGTATAGGGCATTTCCTTGGGAAGGATGAGGACATCTATGT
CTTTTTGATCCCGAGTACCCTTCTGAATCCAAAATAAAAACAGAGGGTGGCTCAGCTTCACTTGACCGGAGGCCGAACGGAAAGTCAACATCGAATG
GCAGGAGCAGTCCAGAACCCACAGTCCAGGACACAGAAGGCGCAGCAGGCTGCATCCGAGAGGTCATCTCATCAAGCCACAGCAGCGCAAGCA
GCAACACGACTGCGCGGAAGGGTCCGGTCTTGTATCTCAAGGGCGAGAACTACTTCTGCGCGCTCGGAAGGAAGCGGTCAACGCCGTTAC
CTGCTGAGTTCGCGGAGGAGGTTGTCTTCTCAAGGAATGGCATCGGCAACAGCTGGCTTCCAATCCAGCTGTGCTGATTACATGTTTCATGGAT
AGGCGAGTAA
    
```

**Figure A2.6:** Nucleotide sequence of the isolated *SoTPS*c isoform. The start codon is highlighted in green. The partial GT1 TPS and partial TPP domains are highlighted in light blue and yellow, respectively.



**Figure A2.7:** Yeast mutants streaked out on SCgal medium. **(A)** SCgal medium. **(B)** SCgal medium excluding histidine, leucine and tryptophan. **(C)** SCgal medium excluding leucine. **(D)** SCgal medium excluding tryptophan. **(E)** SCgal medium excluding leucine and tryptophan. Yeast mutants were streaked out in the following order 1: wild type yeast, 2: *tps1* $\Delta$  yeast, 3: *tps2* $\Delta$  yeast, 4: *tps1* $\Delta$  + *tps2* $\Delta$  strain.

**Table A2.1 :** TPS sequences from members of the Poaceae family

Gene	Accession	Sequence
Sorghum bicolor hypothetical <i>SbTPS</i> gene	XM_002440041 .1	<p>CACTCGCTGCTCGAAACAAGCGTCCGAGTTCGAGGCGTGCGGCCCGTTTTGTCATGTTGG  TTTTGCATGCCGTAGCGGCTCTGCTGCCGGTGTCTCCCTCCCGAACCTTGGCCACCACCTCT  CGGCCGTCTCGTGATTGCAATCACTGCTCCCATTGCGAACGAGCCGCACAGCTGTTGCCAGGC  TCAAGGAGGGCTCGGGTCCACACGCGCGGGGGCGATCGATCGATCGGAGAGGGCGGCGACT  CGGGATCGGGTAGTCTGTTTTCATCTTCCGCTTCGTCGCGAAACCAACCCGCGCTCCCTCGC  TGCGGTATGGGCGGCTGCCTGTTGTGTCCGCTGTTGCCGCTCGTCGTCGCTCAACAAAG  CGCGTCCGCTATGAATGCTGCAGGCGGGGACGCGGGGAGTCGCGGCGGCATGAGCTC  TGACCGCGGGGGGACAGCGCAGCATCAGCACTCCACGAGGGGACGCGCGGGCGGCA  TGCCAACTCATCGCCCTTGTCTCGGCGACAGCAGCGGGCGGGCTCCCGATCCGCGT  CGACGAATGGTCCGGAGCAGGCCCGCTACGACATCTTCGCGTCGACGCGATGGATAAC  CGACGCGCGGAGCGGCTCGGCTTCCGCGGGCCCTTCGCGTGGATGGGGTCCAGTCGCT  GGCGTGTGTACCCGCCAACATGGAGGATGCCGCGGGCGGCGGCTGGGCACGCGCGCGA  CCGCGCTCGCGGCTCCCGCAGCGGTTTCCGCGCTCGGCTCCGTCGATGAAGCAGCGCT  TCTCGTGTGGCAACCGCTCCCTGTTTCCGCAACCGCGGGGAGGACCACTGGTCGCT  TGAGATCAGCGCGGCGGCTCGTGAGCGCCCTGCTTGGGGTGAAGGACGCGGCAAAATG  GATTGGTGGGCGGCGTCAACGTTCCAGACGAGGTTGGCCAGCGAGCCCTACCAAAGCTCTT  GCCGAGAAGAGATGCATACAGTGTCTCGATGAGGAGATTGTGACCAGTACTACAATGGG  TATTGCAACAACATCTGTGGCCGCTGTCCACTACCTAGGACTACCACAGGAGGACAGGCTGG  CAACAACGAGGAACCTTGAGTACAGTTCGACCGGTACAAGCGTGTAAACAGATGTTGTGTA  TGTCGTGTACCAGCACTACCAGGAGGGGATGTAATCTGGTGCCATGACTACCACCTCATGTT  TGCCCAAGTGCTCAAGACCATGACATCAATATGAAAGTCGGTGGTTCCTGCACAGCCAT  TCCCATCAGAGATTTACCGAACACTGCCATCCCGCTGGAGCTGCTTCGCTCGGTGCTGTGT  GCTGATTTAGTCGATTTACTACTACGACTATGCGAGGCATTTGTGAGTGTGCACTAGAAT  ACTTGGACTTGAGGTACCCTGAGGGTGTGGAAGATCAAGGAAGACTAACAGGGTTGCAGC  GTTTCTATTGGGATAGACTCTGATCGTTTCAAGCGAGCATTGGAGCTTCCAGCAGTAAAAGG  CACATCAGTGAATTGACACAACGTTTGTGTGCGAAAGGTAATGCTTGGTGTGATCGACTTG  ACATGATTAAGGGAATCCACAAAAGATTTGGCCTTTGAAAGTTTCTTGAGGAAAACCCAGA  CTGGAACGACAAAGTTGTTCTACTGCAGATTGCTGTGCCAACAAAGACTGACGTCCTGAGTAT  CAAAGCTAACAAGCAAGTGCATGAAATTGTTGGGCGCATAAACCGGTCGATTCGGAACGTTG  ACTGCTGCCCTATTCATCATCTGGACCGATCTCTGATTTCCATGCTTGTGTGCTCTTATGCA  GTCAGTATGTTGCTTGTAAACACTGAGAGATGGGATGAACCTTGTGAGCTATGAGTATG  TTGCATGCCAAGGGTCTAAGAAAGGAGTTTGTACTTACTGAGTGTGCTGGGCGAGCACAATC  ACTTGGAGTGGGCCATTCTAGTAAACCTTGGAAATTTACAGAAAGTGCAGACTCAATACCG  CACGCTTTCAGATGCCATCCGATGAGAGAGAGAAACGGCACAGGCAACAATATGCTCATGTC  AAACTCACACGGCTCAAGATTGGGCTGAAACTTTTGTATTGAGCTAAATGACACGGTTGCTG  AAGCACTAGGAGCAAGACAAGTTCTCTGGACTTCTAGTCAAACGTCGATTCGGAACGTAAT  ATTTGCGCTCAAAAATCGTCTGCTCATATTGGGTTTCAATTCAACATTGACTGAACAGTCGAA  TCCTTGGGAGAAGGGTGGTGACCAATCAAGGAAATGGAACCTCAAGTGTGATCTGACTTA  AAGGTCTCTGAGAGCCCTCTGTGAAGATGAGCGCACTACAGTTATTGTTCTTGTGAGGAGTG  ACAGGAGTGTCTGATGAAAATTCGGAGAATTTAAAATGTGGTGGCAGCAGAGCATGGGA  TGTTTTACGCCGACTTATGGAGAATGGATGACAACAATGCCTGAGCATGCAACATGGATTG  GGTTGACAGCGTAAAGCATGTTTTGAACTTTACAGAAAGAACCCCAAGATCCCATTTCGAA  CATCGTGAACATCATTGTGTGGAACATAAGTATGCTGATGTTGAAATTTGGAAGGCTACAAG  CAAGAGATATGCTGCAGCACTTGTGACAGGTCGATCTCAAATGACAGCTGTTGATGTTGTTCA  AGGGAGTCGGTCAGTTGAAGTTCGGTCTGTTGGAGTTACAAAGGGTGTGCAATTGATCGCATT  TTAGGGGAGATAGTTCACAGCGAAAACATGGTACTCCAATTGACTATGCTGTGTATAGGGC  ATTCCTTGGGAAGGATGAGGATATCTATGCTTTTTGATCCCGAGTACCCTTCTGAATCCAAA  ATAAAACCAGAGGGTGGCTCAGCTTCACTTGACCGGAGGCCAACGGAAGGCCACCATCGAAC  GGCAGGAGCAACTCCAGGAACCCACAGTCCAGGACACAGAAGGCGCAGCAGGCTCAGGCTGC  ATCCGAGAGTTCATCTTCAAGCCACAGCAGCGCAAGCAGCAACCATGACTGGCGCGAAGG  GTCTCGGTCCTTGTCTCAAGGGCGAGAATACTTCTCTCGCGGTTGGAAGGAAACGGTCC  AACGCCGCTACCTGCTGAGTTCGTCAGAGGAGGTTGCTCTCTCAAGGAGCTGGCAACAG  CAACAGCTGGCTTCAAATCCAGCTGTGCTGATTACATGTTCTGGATAGGAGTAAAGTAGATTG  GTGAGAGCCTCCGGGCTTACCAGAGAAGCACCTTGGCAAGAAAAAATATATTCATCC  TCATTTGCGCGACAGAGTTACACCCGTAGCTAGCCAGCGTGTGTACAATCTGTACAAAATTT  ATGCTCTGATGATAAACTGCGAGAGGGGAGCAATGGGAAAAGGATAAAGGAGTTAGAG  AGTTCGGTGTCTTGGTATGATATACAATCGTTCGCGCTTTGATTTCTC</p>





<p><i>Zea mays ZmTPS1</i></p>	<p>EU659122.2</p>	<p>ATGCCAACCTCATCGCCCTTTGTCGGCGACAGCGGGCGCGGGCTCCCCGATCCGGCTCGAGC          GAATGGTCCGCGAGCGTAGCCGCCGTACGACATCTTCGCGTCGGACGCGATGGATACCGACG          CCGAGGCGGCCTTCGCGCTGGATGGGGTCCAGTCGCTGGACGTGCGTACCCGCCAACATGG          AGGATGCTGGCGCGCGGCCGACGCGGACCGCGCTCGCGGGCTCCCGACGCGGTTTCGGCC          GCCTAGGCTCCGTTGGCATGAAGCAGCGCTCCTCGTCGTGGCCAACCGCCTGCTGTATCCGC          CAACCGCCGCGAGAGGACCACTGGTTCGTTGAGATCAGCGCCGGCGGCTTGTGAGCGCCCT          CCTTGGCGTGAAGGACGTGACGCGAAGTGGATTGGCTGGGCAGGCATCAACGTACCGGACGA          GGTGGTTCAGCGAGCCCTCAATAAGCTCTTGGCGAGAAGAGATGCATACCAGTGTCTCGGAC          GAAGAGATTGTGACCACTACTACAATGGATACTGCAACAACATCCCGTGGCCACTGTTCCTACT          ACCTAGGACTACCACAGGAGGACAGGCTGGCAACAACGAGGAACCTTTGAGTACAGTTCGACG          CGTACAAGCGTGTAAACCAGATGTTTCGCTGATGTCGTGTACGAGCACTACCAGGACGGGATG          GATCTGGTGCCATGACTACCACCTCATGTTTCTGCCAAGTGCCTCAAGGACCATGACATCAAT          ATGAAGGTCCGGTGGTTCCTGCACACGCCGTTCCCGTCATCAGAGATTTACCGGACTGCGCGT          CCCGCTTGGAGCTGCCTCGGTCGGTGTGTGCCGATTTAGTTGGATTTCACTTACGACTAT          GCGAGGCAATTTGTGAGTGTGCTGACTAGAACTTGGACTTGGGGTACCCTGAGGGCGTTG          AAGATCAAGGAAGGCTAACAGGGTTGCAGCGTTTCTATTGGGATAGACTCTGATCGTTTCAA          GCGAGCATTTGGAGCTTCCAGCAGTGAAGGACGTCAGTGAATTGACAGAACGTTTTCGCCG          TCGAAAGTAATGCTTGGTGTGACCGACTTGACATGATTAAGGAATTCGCAAAAAGATTTTG          GCCTTTGAAAAGTTTCTTGAGGAAAACCCAGACTGGAAACAACAAGTTGTTCTACTCGAGATTG          CTGTGCCAACAAGAACTGACGTCCCTGAATATCAAAAGCTAACGAGCCAAAGTGCATGAAATTG          TTGGGCGCATAAACGGTCGATTTGGAACGTTGACTGCTGTCCCTATTCATCATCTGGACCGATCT          CTTGATTTCCATGCCTTGTGTGCTTTATGCAGTCACTGATGTTGCTCTGTAAACATCACTGAG          AGATGGGATGAACCTTGTGAGCTATGAATATGTTGCATGCCAAGGGTCTAAGAAAGGAGTTCT          GATACTTAGCGAGTTTGTGGGCGAGCACAATCACTTGGAGCTGGTGCATTCTAGTAAACCCT          TGAATATTACAGAAGTTGCGGACTCAATACGGCATGCTTTAACGATGCCATCCGATGAGAGA          GAGAAACGACACAGACACAACACTACGACATGTCACAACACACGGCTCAAGATTGGGCTGAA          ACTTTGTATTTGAGCTAAATGACACGGTGTGCTGAAGCACTACTGAGGACAAGACAAGTTCCCTC          CTGGTCTTCTAGTCAAATGGCAATTCAGCAATATTTGCGCTCAAAAATCGTGTCTCATATTG          GGTTCAATTCGACATTGACTGAGCCAGTCGAATCCTCTGGGAGAAGGGGTGGTGACCAAAATCA          AGGAAATGGAACCTCAAGTTGCATCCTGACTTAAAGGGTCTCTGAGAGCCCTCTGTGAGGATGA          GCGCACTACAGTATTGTCTTAGCGGCAGTGACAGGAGTGTCTTGTATGAAAATTTGGAGAA          TTTAAAATGTGGTTGGCGGAGAGCATGGGATGTTTTACGCCGACTTACGGAGAATGGATGA          CAACAATGCCTGAGCATCTGAACATGGATTGGGTTGACAGCGTAAAGCATGTTTTTGAATACTT          TACAGAAAGGACCCCAAGATCCCATTTGAAACATCGTGAACATCATTGTGTGGAACATAAG          TATGCTGATGTTGAGTTCGGAAGGCTACAAGCAAGAGATATGCTGCAGCACTTGTGGACAGGTC          CGATCTCAAATGCAGTGTGATGTTGTTCAAGGAGTCGATCAGTTGAAGTTCGGTCTGTTGG          AGTTACCAAGGGTGTGCAATTGATCGATTTTAGGGGAGATAGTTCACAGCGAAAACATGATT          ACTCAAATTGACTATGTCTGCGCATAGGGCATTTCCTTGGGAAGGATGAGGACATCTACGTCT          TCTTTGGTCCCGAGTACCCTTCTGAATCAAAGTAAAGCCAGAGGGCGGCTCAGCATCACTTGA          CCGGAGGCCGAACGGGAGGCCACCATCGAATGGCAGGAGTAACCTCAGGAACCCACAGTCCAG          GACACAGAAAGGCGCAGCAGGCTGCATCCGAGAGGTCATCCTCATCAAGTACAGCAGCAGGAG          CAGCAACCACGACTGGCACGAAGGGTCTCGGTCTTGTATCTCAAGGGCGAGAATACTTCTCC          TGGCCGTCGGGAGGAAGCGGTCCAACGCCGCTACCTGCTGAGCTGTCGGAGGAGGTTGTCT          CCTTCTCAAAGAGTTGGCGACAGCGACAGCTGGCTTCCAGGCCACCTGTGCTGACTACATGCA          TGTCTTGGATAG</p>
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## **Chapter 3**

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# **Protoporphyrinogen Oxidase Activity in Alternative In vitro Selection Systems for Sugarcane**

### 3.1 Introduction

Two broad methods exist for the improvement of crop plants such as sugarcane, namely traditional breeding methods and targeted molecular approaches. The former usually involves the selection or cross breeding of crop plants, in order to attain new crop varieties that possess favourable agricultural traits. For example, most modern sugarcane cultivars are thought to result from a cross between *Saccharum officinarum* and *S. spontaneum*, where *S. officinarum* usually accumulates high sucrose and *S. spontaneum* possesses traits for increased adaptability to adverse conditions (Zhou *et al.*, 2013). However, the development of new crop varieties through breeding is usually hampered by the time required to develop these new varieties (Waclawovsky *et al.*, 2010; Zhou *et al.*, 2013). Targeted molecular approaches can be considered, which aim to identify and utilize specific genes that would enhance agricultural traits in crop plants. These are not as widely accepted as traditional breeding methods by consumers, but to address these concerns, cisgenic strategies have been considered (Jacobsen and Schouten, 2009; Waclawovsky *et al.*, 2010; Arruda, 2012; Zhou *et al.*, 2013). These cisgenic strategies aim to change and utilise an organism's own genetic makeup, rather than introduce a gene from another organism, such as in transgenic strategies.

Protocols for the genetic manipulation of sugarcane have been available since the early 1990's. These include those for the transformation of sugarcane callus via particle bombardment and for selecting and regenerating transformed callus into whole plants (Franks and Birch 1991; Bower and Birch 1992; Van der Vyver *et al.*, 2013). Once sugarcane is transformed with a DNA construct, selection methods are important to separate transformed cells from the rest of the cell mass. Selection would involve expression of a gene that would enable only cells containing it to grow on media which contains a selection agent, such as an antibiotic.

As mentioned in chapter 1, various strategies have been employed to develop protoporphyrinogen oxidase (PPO) based selection systems in plants. These strategies included the overexpression of plant *PPO* genes, the alteration of the PPO binding site to avoid diphenyl ether type herbicide (DPE) binding, the selection of DPE herbicide resistant *PPO* genes by random mutagenesis and the over-expression of microbial *PPO* genes with DPE resistance (Li and Nicholl, 2005). Also in these strategies, different results were observed with the use of either chloroplast targeted *PPO-1* or mitochondrial targeted *PPO-2* genes. When successfully developed, these selection genes should enable the *in vitro* selection of transformants on media containing DPE herbicide. Additionally, the selection

genes should continue functioning in the whole plant resulting in DPE herbicide tolerance in the field during crop production.

In the review by Li and Nicholl, (2005), results from various attempts to develop *PPO* genes as selectable markers are discussed. In one of these studies, Li *et al.*, (2003) was successful in using random *in vivo* mutagenesis to identify various novel mutations in the *Arabidopsis thaliana PPO-1* gene that conferred DPE herbicide resistance (Li and Nicholl, 2005). Specifically, Li *et al.* (2003) mentions three amino acid mutations that conferred resistance to DPE susceptible SASX38 *E. coli* cells. One of these mutations was a tyrosine to methionine change at amino acid position 426 (Y426M). SASX38 cells that rely on these mutations for DPE resistance showed poor growth rates, even when not grown on DPE herbicides. However, additional mutations that counteracted poor growth rates, such as a serine to leucine change at amino acid position 305 (S305L) and the double Y426M + S305L mutation was later also identified as inducing herbicide resistance in plants.

In this study the aim is to make advancements towards developing a cisgenic *in vitro* selectable marker system for sugarcane, *S. officinarum*, callus using a DPE herbicide as selection agent and a DPE resistant *PPO-1* gene as selection gene. For proof of concept, the tobacco *PPO-1* gene was isolated and targeted mutagenesis performed on it. This involved a tyrosine to methionine change at amino acid position 437 and, secondly, a serine to leucine change at amino acid position 316, which corresponds with the mutations used by Li *et al.*, (2003) in maize. This mutated tobacco *PPO-1* gene would be transformed into sugarcane callus and tested as a selectable marker for callus grown on medium containing the DPE herbicide, fomesafen. The second objective was to isolate a full length sugarcane *PPO-1* gene and identify novel mutations for herbicide resistance in sugarcane cisgenic transformation systems. As proof of concept, random mutations were induced in the tobacco *NtPPO-1* gene, using the XL-1 Red *E. coli* strain. DPE herbicide resistant mutations would be identified by screening in the mutant VSR-800 *E. coli* strain with DPE herbicides complementing the growth defect of VSR-800 cells on LB medium.

## 3.2 Materials and Methods

### 3.2.1 Isolation, cloning and mutation of a tobacco *NiPPO-1* gene

#### 3.2.1.1 Plant material and RNA isolation

RNA was isolated from tobacco leaf tissue (*Nicotiana tabacum* cv. Samsun) using a RNA extraction protocol described by Wang *et al.*, (2011), which utilizes TRIzol (Sigma-Aldrich; St. Louis, MO, USA). RNA extraction buffer (100 mM Tris-HCL, pH9 dissolved in diethyl pyrocarbonate (DEPC) treated water) was autoclaved and stored at room temperature. Prior to use, 2% (v/v)  $\beta$ -mercaptoethanol was added to the RNA extraction buffer. Other solutions prepared and used were a 20% (w/v) SDS (sodium dodecyl sulphate) solution dissolved in DEPC treated water and TRIzol.

Briefly, plant material was ground to a fine powder using liquid nitrogen and 200 mg of powdered material was added to 400  $\mu$ l of RNA extraction buffer and incubated at room temperature for 15 min. 20  $\mu$ l of a 20% (w/v) SDS solution was added to the samples, gently inverted and centrifuged at 8000 g for 10 min at 4°C. The resulting supernatant was transferred to new tubes. Two volumes (800  $\mu$ l) of TRIzol reagent was added to samples and mixed by vortexing, after which samples were incubated at room temperature for 10 min. 240  $\mu$ l of chloroform was then added to samples, mixed by vortexing and centrifuged at 8000 g for 10 min at 4 °C. Samples were kept cold for the remainder of the extraction protocol. RNA was precipitated from the supernatant with equal volumes of isopropanol and harvested by centrifugation. The RNA pellet was re-suspended gently in 400  $\mu$ l of DEPC treated water and purified by adding an equal volume of citrate buffer [saturated phenol pH 4.3/chloroform (1:1)] and mixing thoroughly. RNA samples were subjected to centrifugation at 8000g for 10 minutes. The supernatant was transferred to new tubes and an equal volume chloroform added and mixed thoroughly. The resulting supernatant was transferred to new tubes and 1/10<sup>th</sup> volume (40  $\mu$ l) of 3 M sodium acetate (pH 4.8) and 2 volumes (800  $\mu$ l) of ethanol was added to precipitate RNA at -20 °C overnight. Samples were centrifuged at 8000 g and pelleted RNA washed by adding 500  $\mu$ l of 70% (v/v) ethanol. The supernatant was discarded and pellets were air dried. RNA was re-suspended in 30  $\mu$ l of DEPC treated water after which concentrations were determined by measuring absorbance at 260nm.

RNA samples were DNase treated with DNase I, RNase free (Thermo Scientific; Waltham, MA, USA). DNase I treated RNA was visualised on 1.2% (w/v) agarose TBE gels to assess RNA quality. RNA was used as soon as possible to synthesize cDNA, using RevertAid H Minus Reverse Transcriptase (Thermo Scientific; Waltham, MA, USA).

### 3.2.1.2 *NtPPO-1* gene isolation and cloning

The tobacco *NtPPO-1* gene (NCBI accession number: Y13465.1) was isolated from tobacco leaf cDNA by PCR amplification using the following primer pair; *NtPPO-1* fwd (5' AGAATTCCGGTCTACAAGTCAGGCAGTC '3) and *NtPPO-1* rev (5' CGAATTCCTACC CCAACACAGGTTTC '3). Both primers include *EcoRI* sites for cloning into the pMOS-Blue vector (Amersham Biosciences, Amersham, UK). PCR was performed with Phusion® High-Fidelity DNA Polymerase (Thermo Scientific; Waltham, MA, USA) and master mixes were assembled as described in section 2.2.3.7. The PCR program used was as follows: initial denaturation at 98°C for 10 sec, 32 cycles of 98°C for 1 sec, annealing at 60°C for 20 sec and 72°C for 30 sec followed by a final elongation at 72°C for 2 min.

The PCR amplified *NtPPO-1* gene was gel purified and cloned into the pMOS-Blue vector, according to the manufacture's instructions. pMOS-Blue::*NtPPO-1* constructs were transformed into competent DH5α cells and transformants were selected on LBA plates containing 50 µg/ml ampicillin. Once transformants with inserts were identified, plasmid DNA was purified using the GeneJET Plasmid Miniprep Kit (Thermo Scientific; Waltham, MA, USA) and sequenced.

### 3.2.1.3 Targeted mutagenesis of *NtPPO-1* gene

Targeted nucleotide base pair mutations were introduced in the *NtPPO-1* gene inside the pMOS-Blue::*NtPPO-1* construct using primers designed to change base pairs at two desired positions in the gene (Table 3.1). These mutation sites were designed to first change the tyrosine (TAC) at amino acid position 437 to methionine (ATG) and secondly, the serine (TCT) at amino acid position 316 to leucine (CTC). Primer pairs were designed back-to-back over the desired mutation site; in order to amplify the entire vector and gene insert as one fragment.

**Table 3.1:** *NtPPO-1* targeted mutagenesis primers.

NtPPO-1 mutation	Primer name	Sequence
Ser 316 to Leu	NtPPO1 316 For	5'- ACTAAGTCAGAAAAAGGAGGATATCAC-3'
	NtPPO1 316 Rev	5'- AATGCTGAGAAGCTTCCATGATAGTTT-3'
Tyr 437 to Met	NtPPO1 437 For	5'- GCAAAAAATCCTGAAATTTTGTCTAAGAC-3'
	NtPPO1 437 Rev	5'- TCCTCCAATCATGTTCAAGAGTAGC-3'

PCR for targeted mutagenesis was performed using PrimeSTAR® GXL DNA polymerase (Takara; Otsu, Shiga, Japan). The PCR program used was 98°C for 10 sec, 30 cycles of 98°C for 10 sec, 60°C for 15 sec and 68°C for 5 min, followed by 68°C for 2 min. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Scientific; Waltham, MA, USA). The resulting linear double stranded PCR product was self-circularized in a standard T4 DNA ligase reaction (Thermo Scientific; Waltham, MA, USA) as described by the manufacturers. The tobacco *NtPPO-1* gene insert in pMOS-Blue was sequenced after each targeted mutagenesis reaction to confirm site-directed mutations and that no additional mutations were introduced during the amplification process.

### 3.2.2 Transformation of sugarcane with the mutated *NtPPO-1* gene

#### 3.2.2.1 Plant transformation vector detail

After targeted mutagenesis was performed, the mutated *NtPPO-1* (NtPPO1-DM) insert was sub-cloned into the pUBI 510+ plant expression vector (see section 2.2.4.3; Figure 1) using *EcoRI* restriction sites. The resulting pUBI 510::NtPPO1-DM construct was transformed into chemically competent DH5 $\alpha$  cells using a heat shock protocol described in section 2.2.3.2. Transformed cells were plated out on LBA plates containing 100  $\mu$ g/ml ampicillin for selection and incubated overnight at 37°C. The NtPPO1-DM insert orientation in the pUBI 510+ construct was confirmed with PCR (see section 2.2.3.7) using the gene-specific reverse primer (NtPPO-1 rev1: 5' CGAATTCCTACCCCAACACAGGTTTC 3') and a forward primer binding in the ubiquitin promoter region (PromUbi FW: 5' AATTTGATATCCTGCAGTGCAGCGTG 3'). Plasmid DNA was purified from overnight cultures, using the GeneJET PCR Purification Kit (Thermo Scientific; Waltham, MA, USA) and sequenced to re-confirm site-directed mutations.



### 3.2.2.2 Biolistic transformation of sugarcane callus with the mutated *NtPPO-1* gene

The pUBI 510::NtPPO1-DM construct was transformed into sugarcane callus using biolistic bombardment as described in section 2.2.4.4. 5 µg each of pUBI 510::NtPPO1-DM and pEmuKN was co-bombarded into embryogenic sugarcane callus. A total of 10 prepared sugarcane callus transformation plates were bombarded, where each plate contained a 2 cm diameter circle of sugarcane calli. After a two day incubation, in the dark on MSC<sub>3</sub> medium, bombarded callus was transferred to MSC<sub>3</sub> medium containing 10 µg/ml fomesafen and further incubated at 16h/8h day/night cycles at 24°C. Specifically, Fomesafen PESTANAL®, analytical standard (5-[2-Chloro-4-(trifluoromethyl)phenoxy]-N-(methylsulfonyl)-2-nitrobenzamide) (Sigma-Aldrich; St. Louis, MO, USA) was used. The optimum fomesafen herbicide concentration for sugarcane callus selection was previously determined at the IPB (Van der Vyver, personal communication). Callus was sub-cultured every 2 weeks onto fresh MSC<sub>3</sub> fomesafen medium and callus survival monitored over a 6 week period.

### 3.2.3 Random mutagenesis of the tobacco *PPO-1* gene

#### 3.2.3.1 Random mutagenesis with *E. coli* XL1-Red

The tobacco *NtPPO-1* gene was sub-cloned from pMOSBlue::NtPPO-1 into pBK-CMV (Agilent Technologies; Santa Clara, CA, USA) to create pBK-CMV::NtPPO-1 using methods as described in section 2.2.3.9. This was created in order to induce random mutations into the *NtPPO-1* gene insert. *KpnI* and *XbaI* restriction sites were used for directional cloning into pBK-CMV. The pBK-CMV::NtPPO-1 construct was then transformed into XL1-Red competent *E. coli* cells (Agilent Technologies; Santa Clara, CA, USA) for random mutagenesis according to the manufacturer's instructions. After incubation, 200 µl of transformed cells were plated out on LBA plates containing 50 µg/ml kanamycin and incubated at 37°C for 24 hours. 200 colonies were picked and inoculated into individual tubes containing 5 ml LB with 50 µg/ml kanamycin and incubated overnight at 37°C with shaking at 200 rpm. 2 ml was taken from each overnight culture and combined in 100 ml of fresh LB and further incubated at 37°C for 1 hour. This 500 ml cell mixture was used to maxi-prepare a mixture of mutated DNA constructs using the Zippy Plasmid Maxiprep Kit (Zymo Research; Orange, CA, USA).

### 3.2.3.2 *Manufacture of chemically competent VSR-800 cells*

VSR-800 cells were purchased from the National Institute of Genetics, Japan (<http://www.nig.ac.jp>). Chemically competent VSR-800 cells were produced using a modified protocol from Sambrook and Russel, (2001) as described in section 2.2.3.3. Throughout the protocol, cells were covered to block light exposure, as the mutant strain is described to be light sensitive.

### 3.2.3.3 *Testing of mutated NtPPO-1 gene in the VSR-800 E. coli strain*

The NtPPO1-DM insert was sub-cloned from pMOS-Blue into pBK-CMV to create pBK-CMV:: NtPPO1-DM using cloning methods as described in section 2.2.3.9. *KpnI* and *XbaI* restriction sites were used for directional cloning. The construct was sent for sequencing and this showed no unwanted mutations were present. It was transformed into VSR-800 *E. coli* cells to confirm the VSR-800 reverted normal phenotype on LBA medium.

### 3.2.3.4 *Growth curve determinations of the mutant VSR-800 E. coli strain*

Prior to transformation and testing of constructs, the growth of VSR-800 was compared with that of the control *E. coli* strain, DH5 $\alpha$  on both solid and liquid LB media. Fomesafen or acifluorfen herbicides were also added to liquid medium at concentrations of 25 mg/l, 50 mg/l or 100 mg/l. On solid medium, fomesafen herbicide concentrations of 10 mg/l or 40 mg/l were tested. The influence of light exposure on bacterial growth was also investigated.

Cultures were initiated from -80°C stocks on LBA medium. Single colonies from each strain were inoculated into 5 ml of LB for overnight incubation at 37°C with shaking at 200 rpm in the dark. 45 ml of sterile LB was then added and incubated until the cultures reached an OD<sub>600</sub> of 0.1. 100  $\mu$ l of this was streaked out on LB plates and incubated at 37°C overnight. LB plates containing fomesafen herbicide were also tested and replicates were made for testing in both light and darkened conditions.

For testing in liquid culture, 5 ml of an overnight cultures for both strains were inoculated into an Erlenmeyer flask containing 100 ml of sterile LB. Cultures were incubated at 37°C with shaking, while measurements of absorption at OD<sub>600</sub> was taken every hour. At least three

replicates of each culture were made for each strain. VSR-800 cultures were grown in the dark and in the light.

### 3.2.4 Isolating a full length sugarcane *PPO*-1 gene

#### 3.2.4.1 *SoPPO* 5' RACE

Initially, a partial sugarcane *PPO* sequence (NCBI accession number: BD291972) was available on the NCBI database. A reverse gene-specific primer (*SoPPO*1 GSP1) was designed based on this available partial coding sequence (Table 3.2). The second gene-specific primer was designed based on sequence revealed with the first 5' RACE PCR (Table 3.2).

**Table 3.2:** *SoPPO*1 5' RACE primers

Gene	Primer name	Sequence
<i>SoPPO</i> 1	<i>SoPPO</i> 1 GSP 1	5'-CTCAACGCATCTGCCTAGGGCAACTC-3'
	<i>SoPPO</i> 1 GSP 2	5'-GCGGCGCACGAACTCCTCCA-3
RACE universal primers	Long primer	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGT ATCAACGCAGAGT-3
	Short primer	5'-CTAATACGACTCACTATAGGGC-3
	Nested primer	5'-AAGCAGTGGTATCAACGCAGAGT-3

5'RACE ready cDNA, to be used as a template in PCR reactions, was synthesized using RNA from sugarcane leaf-roll tissue. 5' RACE ready cDNA synthesis is described in detail in chapter 2 (see section 2.2.5.2). 5' RACE PCR reactions were performed using Phusion® High-Fidelity DNA Polymerase (Thermo Scientific; Waltham, MA, USA) and master mixes were assembled according to the manufacturer's instructions using universal RACE primers in combination with either the *SoPPO*1 GSP1 or GSP2 primer (Table 3.2). 400 ng of 5' RACE ready cDNA was used as template in each PCR reaction. The PCR program used was as follows: initial denaturation at 98°C for 30 sec, 38 cycles at 98°C for 5 sec, 68°C for 20 sec and 72°C for 1 min followed by a final elongation at 72°C for 3 min. The elongation time of 1 min allowed for the possible amplification of PCR products up to 4 kb. PCR products were visualised on a 1.2% (w/v) agarose TBE gel and bands of expected size were isolated and purified. Purified PCR products were cloned into the pJET 1.2 vector and sequenced.

#### 3.2.4.2 Full length putative *SoPPO* isolation and cloning

High similarity was shared between the partial sugarcane *PPO* sequence revealed through 5' RACE and the sorghum *SbPPO-1* (NCBI accession number: XM\_002455439.1). Thus, primers *SoPPO1 fwd1* (5' GGATATGGTCGCCGCGCC'3) and *SoPPO1 rev1* (5'TTGTAGGGCAGTGAACAGAACAATCTCC'3), based on the sorghum *SbPPO-1* sequence, were used in further attempts to amplify the full length *SoPPO-1* sequence through PCR. PCR amplification was performed using Phusion® High-Fidelity DNA Polymerase (see section 2.2.3.7). The PCR program used was an initial denaturation at 98°C for 30 sec, 36 cycles of 98°C for 5 sec, anneal 68°C for 20 sec and elongation at 72°C for 30 sec followed by a final elongation at 72°C for 2 min.

### 3.3 Results

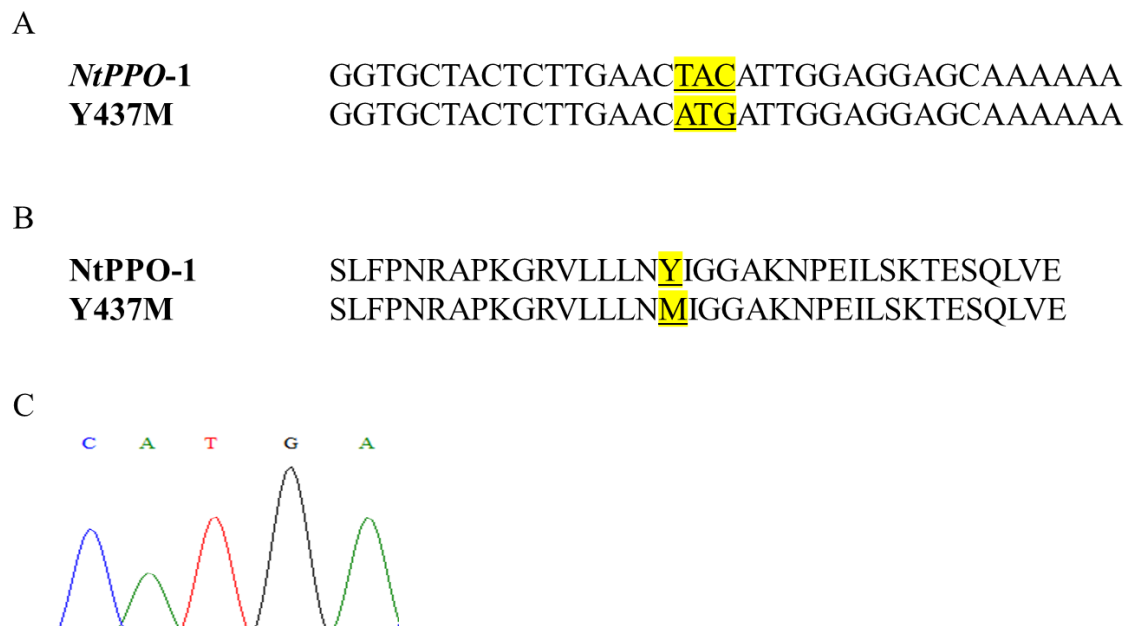
#### 3.3.1 Double mutated *NtPPO-1* as a selection marker in sugarcane

##### 3.3.1.1 Isolating of the tobacco *PPO-1* gene

The tobacco *NtPPO-1* gene was amplified from cDNA synthesised from RNA isolated from tobacco leaf material and ligated into the pMOS-Blue vector. Sequence analysis showed that it was identical to the NCBI listed sequence.

##### 3.3.1.2 Targeted mutagenesis of *NtPPO-1*

Targeted mutagenesis was performed, initially using the *NtPPO1 437* primer pair (Table 3.1). Sequence analysis revealed that the TAC sequence at nucleotide position 1278 was successfully replaced with an ATG sequence, which changes the tyrosine at amino acid position 437 to a methionine (Figure 3.1). The *NtPPO1 316* primer pair (Table 3.1) also successfully replaced the TCT sequence at the nucleotide position 975 with CTC, a switch from serine to leucine at amino acid position 316 (Figure 3.2). Sequence data showed that no unwanted mutations were present and the double mutated tobacco *PPO-1* was sub-cloned into the pUBI 510+ plant expression vector (Annexures, Figure A3.1).



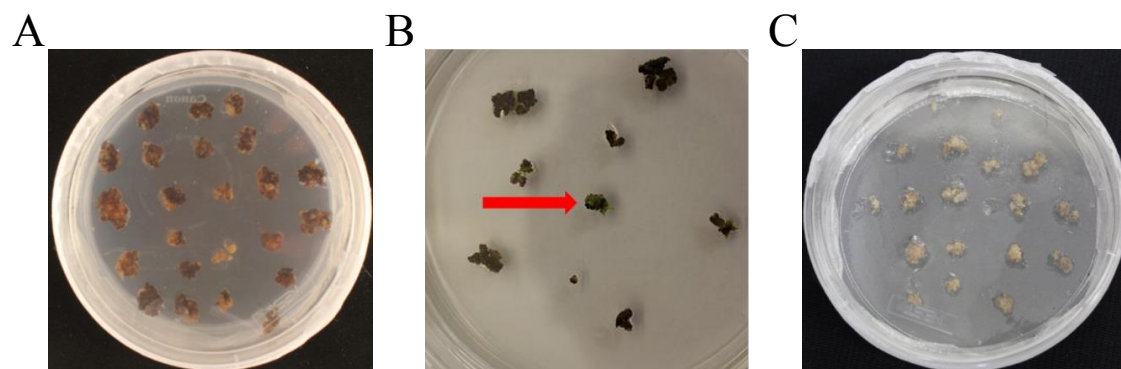
**Figure 3.1:** *NtPPO-1* Y437M mutation with changes highlighted and underlined. (A) Nucleotide change at position 1278. (B) Amino acid change at position 437. (C) Sequencing chromatogram of changed nucleotide sequence.



**Figure 3.2:** *NtPPO-1* S316L mutation with changes highlighted and underlined. (A) Nucleotide change at position 975. (B) Amino acid change at position 316. (C) Sequencing chromatogram of changed nucleotide sequence.

### 3.3.1.3 Double mutated *NtPPO-1* as a selection marker in sugarcane

The double mutated tobacco *PPO-1* construct, pUBI 510::NtPPO1-DM, was bombarded into embryogenic sugarcane callus. Putative transformed calli were selected in the light on MSC<sub>3</sub> medium containing 10 mg/l fomesafen and sub-cultured every 2 weeks. After 6 to 8 weeks of sub culturing, necrosis (browning) of callus started to occur (Figure 3A). After 8 weeks calli were transferred to 2,4-D free MS medium containing 10 mg/l fomesafen and incubated in the light. A few callus clones showed small green structures forming, but these failed to regenerate further (Figure 3B).



**Figure 3.3:** Sugarcane callus transformed with the pUBI 510::PPOI-DM construct, selected on medium containing fomesafen herbicide. (A) Necrosis of transformed callus after 6 weeks of growth on MSC<sub>3</sub> medium containing 10 mg/l fomesafen. (B) Small green structures growing on callus, further grown on MS medium containing 10 mg/l fomesafen. (C) Control Callus grown on MSC<sub>3</sub> medium without fomesafen.

## 3.3.2 Identifying novel mutations in the *NtPPO-1* gene that induce herbicide resistance

### 3.3.2.1 Random mutagenesis of *NtPPO-1* in *Xl-1 red*

The pBK-CMV::*NtPPO-1* construct was made as described in section 3.2.3.1, with the sequence being displayed in Annexures Figure A3.2. The pBK-CMV::*NtPPO-1* construct was transformed into *XL-1 red* cells to induce random mutations on the *NtPPO-1* gene insert. 200 individual transformed *XL-1 red* colonies were selected, grown and plasmids isolated from them, resulting in a mixture of randomly mutated pBK-CMV::*NtPPO-1* constructs. The final

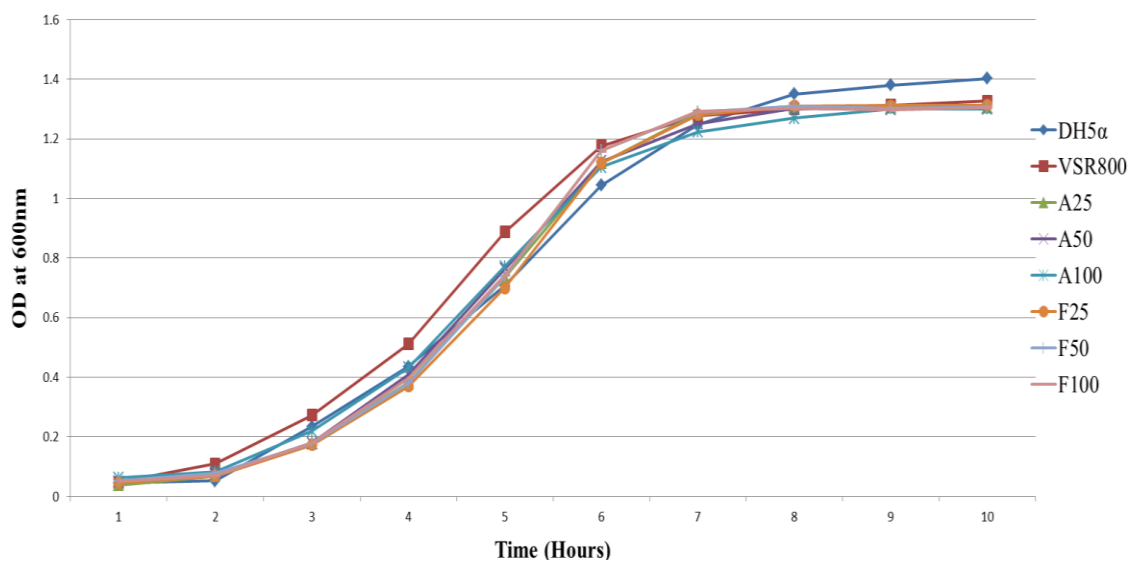
construct mixture had a concentration of 54 ng/μl in a 5 ml volume and was stored at -20°C until transformation into VSR-800.

### 3.3.2.2 *Creation of pBK-CMV::NtPPO1-DM for testing in VSR-800*

The double mutated NtPPO1-DM insert was sub-cloned from pMOS-Blue into pBK-CMV to create the pBK-CMV::NtPPO1-DM construct (Annexures, Figure A3.3).

### 3.3.2.3 *VSR-800 growth analysis*

Initial growth analysis of the un-transformed VSR-800 strain did not show the predicted phenotype of poor growth due to the *hemG* mutation. VSR-800 cells were able to grow on LBA medium plates (Annexures, Figure A3.4) both in the dark and light. Also, the growth of VSR-800 was comparable with that of normal DH5α when grown in liquid LB medium with herbicide added (Figure 3.4). The lack of a defective growth phenotype in the VSR-800 strain made it impossible to distinguish between un-transformed VSR-800 and VSR-800 complimented with a functional *PPO* gene. Thus the screening for mutations that could induce herbicide resistance and the testing of the NtPPO1-DM insert was terminated.



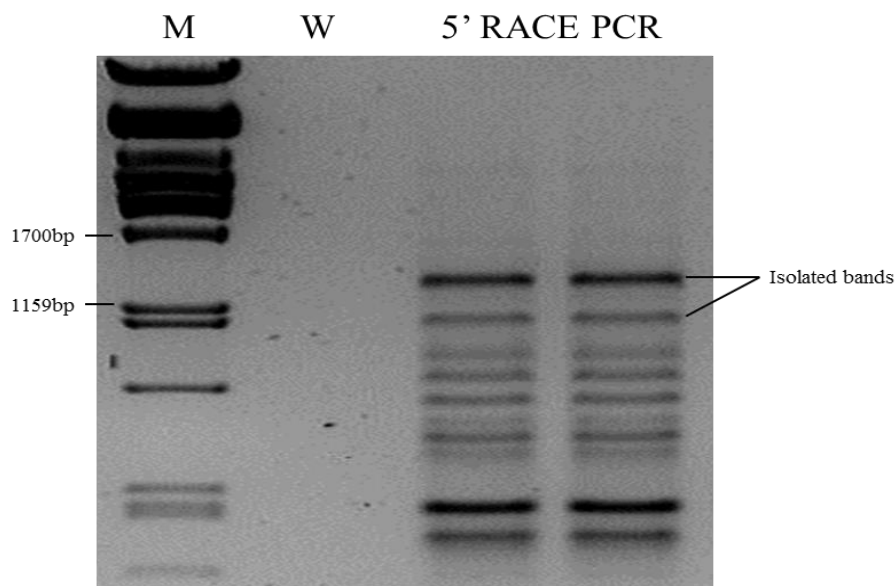
**Figure 3.4:** Comparison of growth rates of VSR-800 and DH5α in liquid culture. The OD at 600 nm for each culture was measured every hour. Each culture had 3 replicates and the average OD values were plotted against time. DH5α = DH5α in liquid LB, VSR-800 = VSR-800 in LB without herbicide, A25 = LB + 25 mg/l acifluorfen, A50 = LB + 50 mg/l acifluorfen, A100 = LB + 100 mg/l acifluorfen, F25 = LB + 25 mg/l fomesafen, F50 = LB + 50 mg/l fomesafen, F100 = LB + 100 mg/l fomesafen.

### 3.3.3 Isolating a full length sugarcane *PPO-1* gene

#### 3.3.3.1 5' RACE

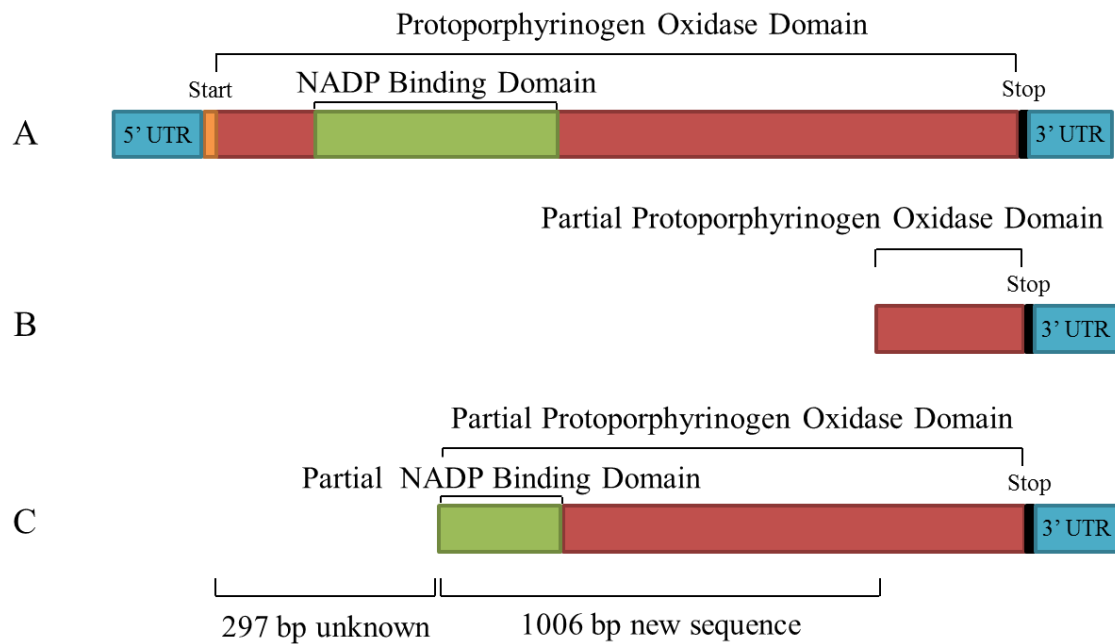
An initial 5' RACE PCR was performed with the SoPPO1 GSP1 primer (Table 3.2), using 5' RACE cDNA synthesized from sugarcane leaf-roll RNA as a template. Visualization of 5' RACE PCR products showed amplicons of various sizes, but the two largest bands were excised and cloned into pJET 1.2 for sequencing (Figure 3.5).





**Figure 3.5:** *SoPPO* 5' RACE PCR. 5' RACE PCR with *SoPPO1* GSP 1 primer and Universal primer mix using sugarcane cDNA as template, visualized on a 1.2% agarose gel. M = Lambda ladder, W = Water control. Isolated bands indicated on the figure were gel purified for further analysis.

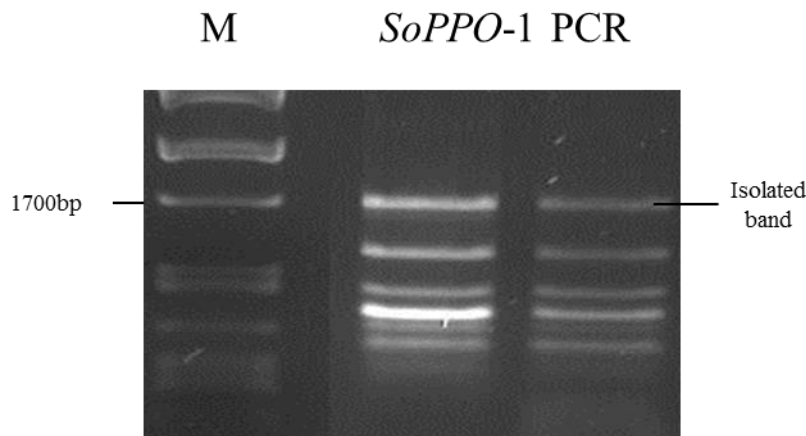
Sequencing results showed that the smaller band was a truncated version of the larger band. Further analysis showed that through 5' RACE PCR, and addition of 1006 bp was added to the initial known partial sugarcane *PPO-1* gene (BD291972). However, alignments with *PPO-1* sequences from other monocotyledonous plant species showed that a potential 297 bp of sequence still needed to be amplified (Figure 3.6). 5' RACE was repeated using the *SoPPO1* GSP 2 primer, however this did not yield any new sequence. The new partial *SoPPO-1* sequence shared 98% identity with the known sorghum hypothetical *PPO-1* sequence (XM\_002455439.1), 95% identity with the maize *PPO-1* (NCBI accession number: BT037840.1) and 88% identity with the rice *PPO-1* (NCBI accession number AB057749.1). The partial *SoPPO-1* sequence is detailed in figure A3.5 of the Annexures.



**Figure 3.6:** Graphical representation of 5' RACE PCR results. The blue regions represent un-translated regions of the *PPO* genes, the red region represents the protoporphyrinogen oxidase domain and the green region represents the NAD(P)-binding Rossmann-like domain. (A) representation of aligned maize, rice and sorghum *PPO-1* genes. (B) representation of initial partial sugarcane *PPO* gene (BD291972). (C) representation of sugarcane *PPO* revealed through 5' RACE.

### 3.3.3.2 *SoPPO-1* full length amplification

Due to the high similarity shared between the isolated sugarcane *PPO* 5' RACE fragment and the sorghum *PPO-1* (XM\_002455439.1), full length primers based on the sorghum *PPO-1* sequence were used in an attempt to amplify a full length sugarcane *PPO-1* sequence (see section 3.2.4.2). Bands of expected size were observed upon visualization alongside some other amplicons (Figure 3.7). The 1700 bp band was sequenced, however, upon analysis of the isolated sequence it was observed that the *SoPPO1* fwd1 primer alone amplified the 1700 bp fragment, which encodes an unknown sugarcane sequence that shows low similarity to a maize splicing factor U2af (NCBI accession number: EU957623.1). Numerous further attempts were made, for example, altering PCR buffer mixes, adding DMSO and using either sugarcane leaf cDNA or 5' RACE cDNA. However, no new *PPO* sequence data was generated.



**Figure 3.7:** *SoPPO-1* full length PCR using *SoPPO1 fwd1* and *SoPPO1 rev1* primers. M = Lambda ladder. Isolated bands indicated on the figure were gel purified for further analysis.

## 3.4 Discussion

### 3.4.1 Site directed mutated *NtPPO-1* as a selection marker in sugarcane

In this study the aim was to develop an *in vitro* selectable marker system for sugarcane using an herbicide resistant gene as a selection marker. The first established protocols for creating transgenic sugarcane utilized selection genes, also used in other monocotyledonous species such as the phosphinotricin acetyltransferase (*bar*), hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase (*nptII*) selection genes based mostly on antibiotic or herbicide resistance (Enriquez-Obregon *et al.*, 1998; Leibbrandt and Snyman, 2003; Van der Vyver *et al.*, 2013). Since then, new genes allowing *in vitro* selection of transgenic sugarcane have been developed, such as the acetolactate synthase (*ALS*) and phosphomannose isomerase (*PMI*) genes using sulfonylurea class herbicides and mannose as selection agents, respectively (Negrotto *et al.*, 2000; Joyce *et al.*, 2010; Van der Vyver *et al.*, 2013). Specific mutations in acetolactate synthase can confer herbicide resistance when introduced into plant species, where amino acid changes would weaken the binding of inhibitory herbicides. Endogenous plant genes can therefore be used to confer herbicide resistance by creating modified enzymes via site specific mutations (Warwick *et al.*, 2008; Powles and Yu, 2010; Van der Vyver *et al.*, 2013). An example of this was shown in the study by Li *et al.*, (2003) where a double mutation (Y426M + S305L) was identified in the *Arabidopsis PPO-1* that conferred butafenacil herbicide resistance in both *Arabidopsis* and maize. For this reason, the targeted mutagenesis performed in this chapter aimed to test the effectiveness of this specific double

mutation, introduced into the tobacco *PPO-1* gene, to convey herbicide resistance to sugarcane cells.

The choice of an endogenous herbicide resistance gene as target for the *in vitro* selection system depends on the plant species' sensitivity towards the linked herbicide selection agent. In the past the susceptibility of sugarcane callus to various herbicides was investigated (Van der Vyver *et al.*, 2013; Koch *et al.*, 2012). Sugarcane callus was shown to be sensitive to sulfonylurea class herbicides, specifically chlorsulfuron and rimsulfuron as well as imidazolinones class herbicides, such as imazapyr. Other monocotyledonous plant species have been shown to be susceptible to the DPE class herbicides, for example rice to oxyfluorfen and maize to butafenacil (Lee *et al.*, 2000; Li *et al.*, 2003). However, prior to this study no published data was available in regard to sugarcane's susceptibility towards DPE herbicides. Recent work performed at the IPB showed that NCo310 sugarcane callus is highly susceptible to fomesafen, leading to cell death, but not lactofen, which lead to the choice of *PPO* as target endogenous plant gene for inducing herbicide resistance (Van der Vyver, personal communication).

The tobacco *PPO-1* gene (Y13465) was isolated and targeted mutagenesis was performed (Y437M + S316L) to replicate the two mutations (Y426M + S305L) in the *Arabidopsis PPO-1* gene, described by Li *et al.*, (2003). However, sugarcane callus transformed with the *NtPPO1-DM* gene construct failed to regenerate on medium containing fomesafen. Callus browning and necrosis started to occur around 6 to 8 weeks of selection on the herbicide, indicating fomesafen sensitivity, but no new callus regenerated. The probable reasons for the lack of tissue development after exposure to the herbicide might be that the biolistic transfer of the transgene was unsuccessful. However, this is unlikely since sugarcane calli are routinely genetically transformed at the IPB with high rates of success where transgenes are driven by the constitutive ubiquitin promoter. It is possible that sugarcane is not sensitive to the specific double mutation, even though it conveys herbicide resistance in other monocots such as maize as described by Li *et al.* (2003).

Another possibility is that the encoded protein needs to be translocated to the plastid. The mechanism of PPO inhibition by DPE herbicides involves the leaking of protoporphyrinogen IX substrate into the cytoplasm, where it is oxidised to protoporphyrin IX, which in turn reacts with reactive oxygen species leading to membrane damage and cellular death (Jacobs *et al.*, 1991). Thus, protoporphyrinogen IX needs to be oxidised within either the chloroplast or mitochondria organelles before leaking into the cytoplasm. The double mutated *NtPPO-1*

construct created in this study contained the same putative target peptide N terminal sequence, 192 bp long, as the *NtPPO-1* (Y13465) isolated by Lermontova *et al.*, (1997). However, it might be that a dicotyledonous transit peptide is unable to function within a monocotyledonous species and this might cause the transgenic enzyme to not be translocated to the sugarcane chloroplast. This is unlikely, however, since Li *et al.*, (2003) successfully utilized a mutated *Arabidopsis PPO-1* in transgenic maize, which suggests that a dicotyledonous transit peptide can still function within a monocotyledonous species. It is also possible that the sequence for the complete transit peptide, which has not been characterised so far, was not included in the sequence region in front of the *PPO* start codon, regardless of the Lermontova *et al.* (1997) claim. Furthermore, it should be noted that the Y13465 sequence was isolated using a cDNA library from the tobacco cultivar Samsun. This is in contrast to the study by Lee *et al.*, (2000) where they attempted to PCR amplify the N terminal segment in the Y13465 sequence from *Nicotiana tabacum* cv. KY160 gDNA to isolated a N terminal segment (NCBI accession number: AF225963.1) containing a 33 nucleotide long insertion which might be essential for plastid translocation, but which was not present in the gene fragment used in this study. Two constructs were transformed into rice by Lee *et al.*, (2000), namely the *B. subtilis PPO* fused with the isolated N terminal segment AF225963.1 and *B. subtilis PPO* without a transit sequence. They showed that transgenic rice containing the *B. subtilis PPO* targeted to the plastid with the AF225963.1 transit sequence were more resistant to oxyfluorfen herbicide than transgenic rice containing the *B. subtilis PPO* targeted to the cytoplasm. This provided evidence that translocation of PPO enzymes to the plastid organelle is of significance when trying to confer resistance to DPE herbicides (by Lee *et al.*, 2000).

In the future a solution for the non-functionality of the *PPO* gene in sugarcane might be to directly isolate the *NtPPO-1* chloroplast transit peptide from the KY160 tobacco cultivar and fuse this to the double mutated *NtPPO-1* construct. This new construct can be transformed into sugarcane callus and its effectiveness at conferring herbicide resistance re-assessed. Another solution to consider is to isolate a known chloroplast transit peptide from a monocotyledonous species, such as sorghum, maize or sugarcane and fuse these to the *NtPPO-1* double mutated construct. Alternatively, once a full length *SoPPO-1* is isolated, this sequence could also be overexpressed or mutated in sugarcane callus in order to induce resistance to the fomesafen herbicide. Overexpression of a un-mutagenized *SoPPO-1* in sugarcane would allow any transit peptides present in the sequence to function in their own biological background. Overexpression of un-mutagenized *PPO* sequences has also been shown to confer certain levels of resistance to DPE herbicides (Li and Nicholl, 2005). For

example, tobacco plants overexpressing the *Arabidopsis PPO-1* were shown to be fivefold more resistant to aciflourfen in a study conducted by Lermontova and Grim, (2000).

### 3.4.2 Identification of mutations that confer DPE herbicide resistance in *NtPPO-1*

As seen in other studies, certain mutations can disable the inhibitory effects of herbicides, yet still allow for the enzyme to perform their intended function. An example of this can be seen with mutations identified in the *ALS* gene that confer herbicide resistance in plants (Chaleff and Mauvais 1984; Falco and Dumas 1985; Yadav *et al.* 1986; Van der Vyver *et al.*, 2013). The *Arabidopsis PPO-1* (Y426M + S305L) double mutant, isolated by Li *et al.*, (2003), was produced by random mutagenesis and screening in a heme deficient *E.coli* mutant. Thus, the second objective of this study was to identify any new mutations in the tobacco PPO-1 gene that would confer herbicide resistance to eukaryotes.

The XL1-Red strain is described as being deficient in three of the primary DNA repair pathways resulting in a 5000 fold higher mutation rate than observed in wild type *E. coli* cells, thus making it suitable for random mutagenesis of gene fragments (Li *et al.*, 2003). The *NtPPO-1* gene was subjected to random mutagenesis in XL1-Red. However, in order to identify clones with desired mutations, an efficient screening / selection system must be in place to identify useful phenotypes. For this purpose the *E. coli* VSR-800 strain was purchased from the National Institute of Genetics (NIG), Japan.

The *E. coli* VSR-800 strain contains a mutation in the *hemG* gene making it defective in the heme biosynthesis pathway. This defect causes the strain to grow poorly on normal LB medium (Narita *et al.*, 1996). This poor growth phenotype allows screening for *PPO* genes, where complementation by a functional gene restores growth of cells. To name a few examples, complementation of *E.coli hemG* mutants grown on LB medium was used successfully by Narita *et al.*, (1996) and Lermontova *et al.*, (1997) to isolate plant *PPO* sequences. However, screening of *hemG* mutants on LB medium would not differentiate between a normal *PPO* gene and one that is resistant to *PPO* inhibiting herbicides. Li *et al.*, (2003) showed that heme deficient SASX38 cells transformed with a functional plant *PPO* gene, were able to grow on normal LB medium, but not on LB medium containing butafenacil herbicide. Thus, PPO enzymes containing mutations that confer DPE herbicide resistance could be screened on LB medium containing a DPE herbicide. Prior to screening of randomly mutated inserts the VSR-800 strain was tested to see that it displayed the advertised

poor growth phenotype. The *NtPPO1-DM* gene construct was also planned to be tested to see whether it could complement the growth defect in VSR-800. However, growth analysis showed that the VSR-800 strain did not display the heme deficient phenotype. This is most likely due to contamination of the cell stocks upon arrival or upon handling of mutant strain cells. Thus work was halted until a new heme deficient strain is obtained for screening purposes.

### 3.4.3 Isolation of a full length sugarcane *SoPPO-1*

Although the genomes of other Poaceae members such as rice, sorghum and maize are available, the sugarcane genome has not been fully sequenced (Grivet and Arruda, 2001; Goff *et al.*, 2002; Paterson *et al.*, 2009; Schnable *et al.*, 2009; Souza *et al.*, 2011). Difficulties encountered with sequencing the sugarcane genome include its substantial size and its complex structure, which is highly polyploidy (Souza *et al.*, 2011). Resources have been made available in the form of expressed sequence tags (ESTs) databases, such as the SUCEST database, which contains a substantial amount of sugarcane sequence data (Carson and Botha, 2000; Casu *et al.*, 2001; Grivet and Arruda, 2001). Isolation of genes in organisms with sequenced genomes is substantially easier due to online algorithms such as BLAST, which enable quick identification of putative sequences *in-silico*, whereupon one can design primers to isolate target sequences via PCR. However, other strategies have to be considered for gene sequence isolations from an organism with a non-sequenced genome.

The only sugarcane *PPO* sequence that was initially available on the NCBI database was a partial *SoPPO* sequence (BD291972), isolated by Johnson *et al.*, (2003). Aligning this sequence with *PPO* sequences from sorghum and maize, *SbPPO-1* and *ZmPPO-1*, respectively, showed that a potential 1303 bp of the sugarcane *PPO-1* gene still had to be determined, which should include a putative NADP binding domain (Marchler-Bauer *et al.*, 2013). Using the BD291972 sequence as a search query in nucleotide BLAST searches of the SUCEST EST database did not yield any additional sequence information. Strategies that were considered for the isolation of a full length sequence included the screening of a sugarcane cDNA library, performing PCR amplification with primers based on either the *SbPPO-1* or *ZmPPO-1* or amplification of the 5' end of the sequence using 5' RACE.

5' Rapid amplification of cDNA ends (5' RACE) was used in order to attempt isolating a full length *SoPPO-1* gene. 3' RACE involves synthesizing cDNA and amplifying a gene with a

polydT primer and a gene-specific forward primer. On the other hand, 5' RACE involves the attachment of a custom priming site on the 3' end of RNA sequences. Following reverse transcription, custom priming sites situated on the 5' ends of synthesized 5' RACE cDNA should be created. PCR can then be performed using a gene-specific reverse primer and the forward primers designed to bind at the custom binding sites. 5' RACE and 3' RACE had been used before to isolate the spinach *PPO-1* and *PPO-2* sequences (Watanabe *et al.*, 2001).

For 5' RACE a gene-specific reverse primer was designed based on the partial *SoPPO-1* sequence (BD291972). A 5' RACE fragment was initially isolated, which revealed 1006 bp of previously unknown sequence. Alignments with *SbPPO-1* and *ZmPPO-1* showed a potential 297 bp of sequence to still be missing (Paterson *et al.*, 2009; Soderlund *et al.*, 2009). However, regardless of numerous additional 5' RACE attempts no more sequence was uncovered. The additional sequence added with 5' RACE included a putative in frame ATG start codon at nucleotide position 73 when compared with *SbPPO-1* and *ZmPPO-1* sequences. This start codon would result in the transcription of a potential protein with only four amino acids of a normally 68 amino acid long NADP binding domain as seen for other monocotyledonous species, such as in *SbPPO-1* and *ZmPPO-1*. Thus the protein from the 5' RACE fragment would encode a putative truncated *SoPPO-1* protein, which excludes a functional NADP binding domain. Whether the encoded protein would be functional without a complete NADP binding domain is unknown. Most prokaryotic PPO enzymes do not require a NADP binding domain and instead use the cell respiratory chain as an electron acceptor when protoporphyrinogen IX is oxidized (Dailey *et al.*, 1994). However, eukaryotic PPO enzymes use oxygen as a final electron acceptor during the oxidation of protoporphyrinogen IX and therefore would most likely require a working NADP binding domain.

The newly isolated *SoPPO* partial fragment showed a very high sequence similarity (97%) with the known sorghum *SbPPO-1* sequence. A large degree of similarity exists between the Andropogoneae tribe plant species, such as sugarcane, sorghum and maize and for this reason these plants have been described as good models for genomic studies performed in sugarcane (Grivet and Arruda, 2001; Paterson *et al.*, 2009). Yet the sugarcane genome is estimated to be substantially larger (10 Gbp) than the maize (2.3 Gbp) and sorghum (730 Mbp) genomes (Grivet and Arruda, 2001; Paterson *et al.*, 2009; Schnable *et al.*, 2009). In a second attempt to obtain the full length *SoPPO-1* gene, a strategy was followed where primers based on the sorghum sequence were used in PCR. This strategy of using primers based on a sorghum sequence led to the isolation of an approximately 1700 bp sequence which, although the



expected size, did not encode a PPO. Despite numerous attempts using either sugarcane leaf cDNA or 5' RACE cDNA as a template, different PCR buffers and the addition of DMSO to aid DNA strand separation, no new sequence was added to the already known *SoPPO* sequence fragment. This study only utilized cDNA, synthesized from RNA extracted from the young leaves of healthy growing sugarcane, which was the most likely tissue type where *PPO* expression involved in the chlorophyll synthesis pathway should be found. Different templates, such as cDNA from internode or mature leave tissue, might yield higher concentrations of *SoPPO-1*, which might aid future sequence isolation attempts. Furthermore, testing different primers sites, as seen in the successful isolation attempts of the *SoTPS* genes, might aid in isolating an *SoPPO-1* gene sequence fragment. In the future, once a full length *SoPPO-1* sequence is isolated, work can commence to adapt the sequence to be used as an *in vitro* cisgenic selection gene that confers herbicide resistance in sugarcane callus and crop plantings.

### 3.5 Conclusions

A double mutated tobacco PPO-1 gene was expressed in sugarcane callus and selected on fomesafen-containing medium. Mutations induced in this study replicated those used by Li *et al.*, (2003) where a mutagenized *Arabidopsis PPO-1* gene was used as a selectable marker in maize. Transgenic sugarcane callus containing the mutagenized tobacco *PPO-1* gene were incapable of regeneration on medium containing the fomesafen herbicide. In the future, targeting of the protein to the chloroplast might aid in regeneration of transgenic sugarcane callus on medium containing herbicide. Specifically, fusing a chloroplast transit peptide from a closely related monocotyledonous species has been considered for use in creating a new transformation construct. Random mutations were furthermore introduced into the tobacco *PPO-1* gene to try and identify novel mutations that could enable the tobacco *PPO-1* gene to induce herbicide resistance in sugarcane. Work was halted at the screening phase, since the *E. coli* mutant to be used did not possess the advertised mutant phenotype.

5' RACE was used in attempts to isolate a full length *SoPPO-1* sequence, but was only successful in adding 1006 bp of previously unknown sequence to the known sugarcane partial sequence. PCR can be repeated in the future with new primers based on either the sorghum or maize *PPO-1* sequences, since high similarity was displayed between the sugarcane partial *PPO-1*, sorghum *PPO-1* and maize *PPO-1*.

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### 3.7 Annexures

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GAATTC CCGTCTACAAGTCAGGCAGTC ATG ACAACAACCTCCATCGCCAATCATCCTAATATTTTCACTCACCAAGTCGTCGTCATCGCCATTGGCATTCC
TTAAACCGTACGAGTTTCATCCCTTCTCTTCAATCTCCAAGCGCAATAGTGTCAATTGCAATGGCTGGGAAACACGATGCTCCGTTGCCAAAGATTAC
ACAGTTCCCTTCTCAGCGGTCGACGGCGGACCCGCCGGAGCTGGACTGTGTTATAGTTGGAGCAGGAATTAGTGGCCCTGCAATTGCCAGGTGAT
GTCGCGTAATTACCCCAATTTGATGGTAACCGAGGCGAGAGATCGTGCCGGTGGCAACATAACGACTGTGAAAAGAGACGGCTATTTGGGAAGAA
GGTCCCAACAGTTTCCAGCCGTCGATCCTATGTTGACTATGGCAGTAGATTGGATTGAAGGATGATTGGTGTGGGAGATCCTAATGCGCCCCGT
TTCGTTTTGTGGAAGGTAATAAAGGCCGTCCTCAAAAACCTCATCTTCCCTTTTTGATTTGATGAGCATTCCTGGCAAGTTGAGAGCTGGTT
TTGGTGCATTGGCCCTCCGCCCTTACCTCCAGGTCATGAGGAATCAGTTGAGCAGTTCGTGCGTCGTAATCTTGGTGGCGAAGTCTTTGAACGCTTGA
TAGAACCTTTTGTCTGGTGTATGCTGGTGATCCCTCAAAACTGAGTATGAAAGCAGCATTGGGAAAAGTTGGAAAGTTGGAAGAAACTGGTGGT
AGCATTATTGGAGGAACCTTTAAAGCAATAAAGGAGAGATCCAGTACACCTAAAGCGCCCGCATCCGCTTACCTAAAACAAAAGGACAGACAG
TTGGATCATTAGGAAGGTTCTCAGAGTGTGCCGGATGCAATCAGTGAAGATTGGGAAGCAAAATAAAACATCATGGAAGCTT CTCAGCATTACT
AAGTCAGAAAAGGAGGATATCACTTGACATACGAGACACCAGAAGGAGTAGTTTCTTCAAAGTCGAAGCATTGTCATGACTGTCCATCCTATGT
AGCAAGCAACATATTACGCTCTCTTTCGGTTGCCGACGAGATGCACCTTCAAATTTCTACTATCCCCAGTTGGAGCAGTCAAAATTCATATCCCA
AGAAGCTATTCGTGATGAGCGTCTGGTTGATGGTGAACATAAAGGGATTGGGCAGTTGCATCCACGTACACAGGAGTGGAAACACTAGGAACGATA
TATAGTTCACTCTTCCCTAACCGTCCCAAAAAGTCCGGTCTACTCTTGAAC ATG ATTGGAGGAGCAAAAATCCTGAAATTTGTCTAAGACG
GAGAGCAACTTTGGGAAGTAGTTGATCGTGACCTCAGAAAATGCTTATAAAACCCAAAGCTCAAGATCCTCTTGTGGTGTGCGAGTATGGCC
ACAAGCTATCCACAGTTTTTGGTGGTCACTGGATACGCTAAGTACTGAAAAGCTGCTATGAATGATAATGGGCTTGAAGGGCTGTTTCTGGGGG
TAATTATGTGTCAGGTGATAGCATTGGGGAGGTGTTGAAGGTGCTTATGAAGTGCATCCGAGGTAAACAGGATTTCTGTCTCGGTATACATACAAAT
GAAACCTGTGTTGGGGG TAG GAATTC

```

**Figure A3.1:** pUBI 510::*NtPPOI-DM* construct sequence. The *NtPPOI-DM* insert was ligated into the pUBI 510 + vector using an *EcoRI* restriction site present within the multiple cloning site of the pUBI 510 + plasmid. *EcoRI* restriction sites are highlighted in light blue and the start and stop codons are highlighted in green and red respectively. The nucleotides changed by targeted mutagenesis are highlighted in yellow.

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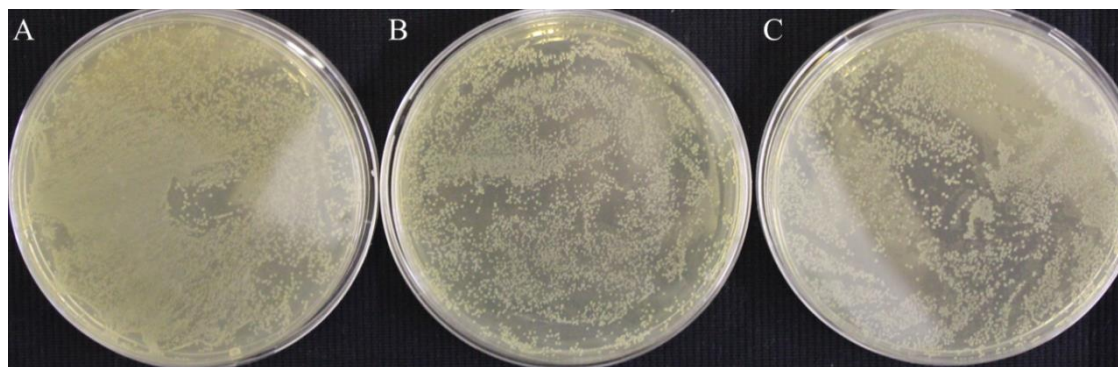
TCTAGA GGATCTACTAGTCATATCGATAGAAATCCGGTCTACAAGTCAGGCAGTC ATG ACAACAACCTCCATCGCCAATCATCCTAATATTTTCACTCA
CCAGTCGTCGTCATCGCCATTGGCATTCTTAAACCGTACGAGTTTCATCCCTTCTCTTCAATCTCCAAGCGCAATAGTGTCAATTGCAATGGCTGGAG
AACACGATGCTCCGTTGCCAAAGATTACACAGTTCCTTCTCAGCGGTCGACGGCGGACCCGCCGGAGCTGGACTGTGTTATAGTTGGAGCAGGAA
TTAGTGGCCCTCTGCATTGGCAGGTGATGTCGCTAATACCCCAATTTGATGGTAACCGAGGCGAGAGATCGTGCCGGTGGCAACATAACGACTGTG
GAAAAGAGACGGCTATTTGTGGGAAGAAGTCCCAACAGTTTCCAGCCGTCGATCCTATGTTGACTATGGCAGTAGATTGGATTGAAGGATGATT
GGTGTGGGAGATCCTAATGCGCCCCGTTTCGTTTTGTGGAAGGTAATAAAGGCCGTCCTCAAAAACCTCACTGATCTTCCCTTTTTGATTTGATG
AGCATTCTGGCAAGTTGAGAGCTGGTTTTGGTGCATTGGCCCTCCGCCCTTACCTCCAGGTCATGAGGAATCAGTTGAGCAGTTCGTGCGTCGTAAT
CTTGGTGGCGAAGTCTTTGAACGCTTGATAGAACCATTTTGTCTGGTGTATGCTGGTGATCCCTCAAAACTGAGTATGAAAAGCAGCATTGGGAAA
GTTTGGAAAGTTGGAAGAACTGGTGGTAGCATTATTGGAGAACCTTTAAAGCAATAAAGGAGAGATCCAGTACACCTAAAGCGCCG-CGCATCCGC
GTTTACCTAAACAAAAGGACAGACAGTTGGATCATTAGGAAGGGTCTCAGAGTGTGCCGGATGCAATCAGTGAAGATTGGGAAGCAAAATTA
AACTATCATGGAA-GCTTTTACGATTACTAAGTCAGAAAAGGAGGATATCACTTGACATACGAGACACCAGAAGGAGTAGTTTCTTCAAAGTGC
AAGCATTGTGATGACTGTGCCATCCTATGTAGCAAGCAACATATTACGCTCTCTTTCGGTTGCCGACGAGATGCACCTTCAAATTTCTACTATCCCC
AGTTGGAGCAGTCACAATTCATATCCTCAAGAAGCTATTCGTGATGAGCGTCTGGTTGATGGTGAACATAAAGGGATTGGGCAGTTGCATCCAGTA
CACAGGGAGTGGAAACACTAGGAACGATATATAGTTTCATCACTTCCCTAACCGTCCCAAAAAGGTCGGGTGCTACTCTTGAATACATTGGAGGA
GCAAAAATCCTGAAATTTGTCTAAGACGGAGACCACTTGTGGAAGTAGTTGATCGTGACCTCAGAAAAATGCTTATAAAACCCAAAGCTCAAG
ATCCTCTTGTGGGTGTGCGAGTATGGCCAAAGCTATCCACAGTTTTTGGTGGTCACTGGATACGCTAAGTACTGAAAAGCTGCTATGAATG
ATAATGGGCTTGAAGGGCTGTTTCTTGGGGTAATAATGTGTCAGGTGATGACATTGGGGAGGTGTTGAAGGTGCTTATGAAGTTGCATCCGAGGTA
CCAGGATTTCTGTCTCGGTATACATACAAATGAAACCTGTGTTGGGGG TAG GAATTCGATCGGATCCCCGGTACC

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**Figure A3.2:** pBK-CMV::*NtPPO-1*. The tobacco *NtPPO-1* gene (Y13465.1) was ligated directionally into the pBK-CMV vector using *KpnI* and *XbaI* restriction sites. *KpnI* and *XbaI* restriction sites are highlighted in light blue and the start and stop codons are highlighted in green and red respectively.

TCTAGAAGGATCTACTAGTCATATCGATAGAATTCCGGTCTACAAGTCAGGCAGTCATGACAACAACCTCCCATCGCCAAATCATCTAATATTTTCACTCA  
 CCAGTCGTCGTCATCGCCATTGGCATTCTTAAACCGTACGAGTTTCATCCCTTCTCTTCAATCTCCAAGCGCAATAGTGTCAATTGCAATGGCTGGAG  
 AACACGATGCTCCGTTGCCAAAAGATTACACAGTTCTCTCTCAGCGGTCGACGGCGGACCCGCCGCGGAGCTGGACTGTGTTATAGTTGGAGCAGGAA  
 TTAGTGGCCCTCTGCATTGGCGCAGGTGATGTCGCTAATTACCCCAATTTGATGGTAACCGAGGCGAGAGATCGTGGCGTGGCAACATAACGACTGTG  
 GAAAGAGACGGCTATTTGTGGGAAGAAGGTCCCAACAGTTTCCAGCCGTCGATCCTATGTTGACTATGGCAGTAGATTGTGGATTGAAGGATGATT  
 GGTGTGGGAGATCCTAATGCGCCCGTTTCGTTTTGTGGAAGGTAATAAAGGCCCGTCCCTCAAACCTCACTGATCTCCCTTTTTGATTTGATG  
 AGCATTCTGGCAAGTTGAGAGCTGGTTTTGGTGCATTGGCCTCCGCCCTCACCTCCAGGTCATGAGGAATCAGTTGAGCAGTTCTGCGTCGTAAT  
 CTTGGTGGCGAAGTCTTTGAACGCTTGATAGAACCATTTTGTCTGGTGTATGCTGGTATCCCTCAAACCTGAGTATGAAAGCAGCATTGGGAAA  
 GTTTGGAAGTTGGAAGAACTGGTGGTAGCATTATTGGAGGAACCTTTAAAGCAATAAAGGAGAGATCCAGTACACCTAAAGCGCCCGCATCCGC  
 GTTTACCTAAACAAAAGGACAGACAGTTGGATCATTAGGAAGGTTCTCAGAGTGTCCGGATGCAATCAGTGAAGATTGGGAAGCAAATTA  
 ACTATCATGGAAGCTTCTCAGCATTACTAAGTCAGAAAAAGGAGGATATCATTGACATACGAGACACCAGAAAGGAGTGTCTTCAAAGTCGAA  
 GCATTGTCATGACTGTGCCATCTATGTAGCAAGCAACATATTACGTCCTCTTTCGGTTGCCGACGAGATGCACTTTCAAATTTCTACTATCCCCAGT  
 TGGAGCAGTCAAAATTCATATCCTCAAGAAGCTATTCGTGATGAGCGTCTGGTGTGATGGTGAACAAAGGGATTGGGCAGTTGCATCCAGTACAC  
 AGGGAGTGGAAACACTAGGAACGATATATAGTTCATCACTCTTCCCTAACCGTGCCCAAAAGTCCGGTGTACTCTTGAACATGATTGGAGGAGCA  
 AAAAACTCTGAAATTTTGTCTAAGACCGGAGAGCCAACTTGTGGAAGTAGTGTACGTGACCTCAGAAAAATGCTTATAAAAAACCAAAGCTCAAGATCC  
 TCTTGTGTGGGTGTGCGAGTATGCCACAAGCTATCCACAGTTTTGGTTGGTCATCTGGATACGCTAAGTACTGCAAAAAGCTGTATGAATGATAA  
 TGGGCTTGAAGGGCTTTCTTGGGGTAATATGTGTGAGGTGTAGCATTGGGGAGGTGTGTTGAAGGTGCTTATGAAGTTGCATCCGAGGTAACAG  
 GATTTCTGTCTCGGTATACATACAATGAACTGTGTTGGGGTAGGAATTCGATCGGATCCCCGGTACC

**Figure A3.3:** pBK-CMV::NtPPO1-DM. The NtPPO1-DM insert was ligated directionally into the pBK-CMV vector using *KpnI* and *XbaI* restriction sites. *KpnI* and *XbaI* restriction sites are highlighted in light blue and the start and stop codons are highlighted in green and red respectively. Nucleotides changed by targeted mutagenesis are highlighted in yellow.



**Figure A3.4:** VSR-800 *E. coli* cells grown on LBA medium containing increasing amounts of fomesafen herbicide. (A) LBA medium. (B) LBA medium containing 10 mg/L fomesafen. (C) LBA medium containing 40 mg/L fomesafen.

GAGCGCCCGAGGAAGGGTACCCTGGGAGGAGGGTCCCAACAGCTCCAGCCATCCGACCCCGTCTCACCATGGCTGTGGACAGCGGGCTGAAGG  
 ATGACTTGGTTTTGGGGACCCCAACGCGCCGCGGTTTCGTGCTGTGGGAGGGGAAGCTGAGGCCGTGCCATCCAAGCCCGCCGACCTCCCGTCTTC  
 GATCTCATGAGCATCCCTGGCAAGCTTAGGGCCGGTCTCGGCGCGCTTGGCATCCGCCCGCTCTCCAGGCCGCGAGGAGTCAAGTGGAGGAGTTCGT  
 GCGCCGC AACCTCGGTGCTGAGGTCTTTGAGCGCTCATTGAGCCTTCTGCTCAGGTGTCTATGCTGGTATCCCTCAAAGTCAAGTGAAGGCTG  
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 AGGGATCCCGTCTTCCGAAGCCAAAAGGGCAGACAGTTGCGTCTTTCAGGAAGGGTCTTGCATGCTTCAAATGTATCACATCGAGCTTGGGTAG  
 TAAAGTCAAATCATGGAACCTCAGCAGCATTACAAAATCAGATGGCAAGGGATATGTTTTGGAGTATGAAACGCCAGAAAGAGGTTGTTCCGGTG  
 CAGGCTAAAAGTGTATCATGACCATTCCATCATATGTTGTAGCAACATTTTGGCTCCACTTCAAGCGATGCTGCAGATGCTCTATCAAATTTCTAT  
 TATCCACAGTTGCTGTGTAAGTGTTCGTATCCAAAAGGAAGCAATAGAAAAGAATGCTTAATTGATGGGGAGCTCCAGGGTTTTGGCCAATTACA  
 TCCACGTAGTCAAGGAGTTGAGACATTAGGAACAATATACAGCTCATCACTCTTCAAATCGTCTCCTGCTGGTAGGGTGTACTTCAAATFACAT  
 AGGAGGTGCTACAAACACAGGAATTGTTCCAAAGACTGAAAGTGAAGTGGTGAAGCAGTTGACCGTGACCTCCGGAAAATGCTTATAAATCCTACA  
 GCAGTGGACCTTTAGTCTTGGTGTCCGAGTTTGGCCACAAGCCATACCTCAATTCCTGGTAGGACATCTTGATCTTTGGAGGCCGCAAAATCTGCC  
 CTGGACCGAGGTGGCTACGATGGGCTGTTCTAGGAGGGAACATATGTTGCAGGAGTTGCCCTAGGCAGATGCGTTGAGGGCGCATATGAGAGTGCCG  
 CACAAATATATGACTCTTGAACCAAGTATGCCTACAAGTAGGAAAGTGGAGCGTGTGTTAATTGTTATGTTGCATAGATGAGGTGAGACCA  
 GGAGTAGTAAAAGGCATTACGAGTATTTTCATTCTTATTTGTAATTCAGCTCTGTTTTTTTTTCTGTCAGTAATTAGTTAGATTTTGTCTGTAG

**Figure A3.5:** Partial *SoPPO-1* sequence revealed through 5' RACE PCR. Highlighted in light blue is the partial NAD(P)-binding Rossmann-like domain. Highlighted in yellow are the *SoPPO1* GSP 1 and *SoPPO1* GSP 2 primer sites, which were used in 5' RACE PCR. Highlighted in red is the stop codon of the partial *SoPPO-1* sequence.

## **Chapter 4**

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### **Conclusions**



## 4.1 Genetic modification of sugarcane

Methods for genetically transforming sugarcane were developed in the early 1990's (Franks and Birch, 1991; Bower and Birch, 1992; Bower *et al.*, 1996). Further advancements in improving agricultural traits in sugarcane stem from this work, such as improving insect resistance, increased drought tolerance and increased sugar accumulation (Wu and Birch, 2007; Groenewald and Botha, 2008; Dal-Bianco *et al.*, 2012; Arruda, 2012). Also, new selection systems have been developed for use in creating genetically enhanced sugarcane (Negrotto *et al.*, 2000; Joyce *et al.*, 2010; Van der Vyver *et al.*, 2013). However, the list of available selectable marker systems for sugarcane is still considered to be small. Increasing the range of selection systems available for its transformation would allow for the expression of increased numbers of genes in sugarcane, conferring more than one trait in a single line (Dal-Bianco *et al.*, 2012; Arruda, 2012; Van der Vyver *et al.*, 2013). An additional aim is to utilize sequences that are endogenous in sugarcane when developing new selection systems and genetically modifying sugarcane. The use of cisgenic strategies in future studies might appeal more to public consumers and ease regulation restrictions (Jacobsen and Schouten, 2009). Recent events have already shown that regulations on cisgenic modified crops might be more relaxed than those applying to transgenic crops. Already, the European Food Safety Authority (EFSA) delivered a scientific opinion to the European Commission stating that similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with transgenic plants (Hunter, 2014). More relaxed regulations exist in the USA, where the Environmental Protection Agency has proposed to exempt cisgenic plants from GMO regulations, although only in the context of protecting against pests (Waltz, 2011; Hunter, 2014).

## 4.2 Trehalose-6-phosphate synthesis and its effects on sucrose accumulation

The work described in chapter 2 was aimed at trying to understand the relation between T6P levels and sucrose accumulation in sugarcane. The *otsA* and *otsB* genes from *E. coli* were overexpressed in sugarcane, in order to make initial observations on the effect T6P synthesis has on sucrose accumulation in sugarcane. Nine transgenic *otsA* and three *otsB* sugarcane lines were created. All nine *otsA* lines showed transgene expression, ranging from very low relative expression in *otsA* line 11 to very high relative expression levels in *otsA* line 7. In

contrast, only two of the tree *otsB* transgenic lines showed transgene expression, with very little variation in the relative expression levels between the two lines.

Six out of the nine *otsA* overexpressing lines showed a decrease in sucrose levels, four of which were significantly lower than the non-transgenic control line, while one line showed a significant increase in sucrose levels. Furthermore, eight of the nine *otsA* overexpressing lines showed increases in glucose levels of which four were significant increases. Two of these, namely *otsA* 7 and *otsA* 12 showed significantly increased glucose, but contrasted in sucrose accumulation, where *otsA* 12 was shown to have significant increased and *otsA* 7 decreased sucrose levels. Sq-RT-PCR results showed relatively higher transgene expression occurred in *otsA* 7 than *otsA* 12, suggesting that the level of expression might determine alterations in sucrose content. Future analysis of mature cane will, however, have to include more biological and technical replicates to obtain a statistically sound dataset from where conclusive results can be obtained and consequences of transgene overexpression can be pin pointed regarding relevant enzymatic levels, carbohydrate levels and transgene expression levels.

In two *otsB* overexpressing lines, the first line had a slight increase in sucrose and significantly increased glucose and fructose. However, the second *otsB* overexpression line had significantly decreased sucrose levels with non-significant increases in glucose and fructose. To make sense of these inconsistent results, measurements will have to be repeated to decrease any statistical errors and determining transgenic expression with more quantitative methods might yield a better correlation between transgene expression and sucrose levels in sugarcane. All these measurements were performed on young leaf tissue and different sugar accumulation will occur in mature internode tissue (Bosch, 2005). Future studies will involve measurements of soluble sugars, TPS and TPP enzymatic levels as well as measurements of trehalose and T6P levels in mature internode tissue. Also, more quantitative measurements of expression could provide better correlation of the effects of transgenic overexpression on the accumulation of these sugars.

The second part of chapter 2 describes the isolation of three functional *SoTPS* isoforms, since a functional *SoTPS* can be used to study TPS activity in sugarcane. An initial fragment was isolated using 5' RACE. Three putative *SoTPS* isoforms were then isolated in a PCR reaction, using primers based on the 5' RACE results. These three isoforms, named *SoTPSa*, *SoTPSb* and *SoTPSc*, were transformed in a yeast complementation assay into *tps1Δ* yeast to determine whether these isoforms were functional. All three were shown to be functional.

*SoTPSa* shows high similarity to *TPS* sequences from monocotyledonous plant species, except for an in-frame 75 bp insert absent from other known monocot *TPS* sequences. Similarly, *SoTPSc* shows high similarity to *TPS* sequences from monocotyledonous plant species, but it contains a 126 amino acid long N terminal truncation. This truncated segment removes one of the thirteen amino acids found within the active site of the *TPS* domain (Marchler-Bauer *et al.*, 2013). The *SoTPSa* and *SoTPSc* isoforms indicate the occurrence either of alternative splicing, or production by the different genome copies within sugarcane. Future enzymatic assays will determine the effect that these alterations have on the kinetics of *TPS* activity.

Another consideration is that these isoforms can be used to make constructs to either overexpress or silence *TPS* synthesis in sugarcane, so as to study their effects on sucrose accumulation. *otsB* overexpression was hypothesized to decrease T6P levels, but it could also have caused a flux in T6P and trehalose synthesis. Silencing of endogenous *TPS* genes in sugarcane would cease or lower T6P synthesis, without flux occurring in trehalose synthesis. Use of these genes in sugarcane would also be a step forward toward using a cisgenic strategy to potentially increase sucrose concentrations in sugarcane.

### **4.3 Protoporphyrinogen oxidase as a selectable marker system**

Work detailed in this chapter aimed to create a new *in vitro* alternative selection system for future genetic transformations of sugarcane. Firstly a transgenic strategy was attempted by using a mutagenized tobacco *PPO-1* gene as a selectable marker. This strategy was based on work done by Li *et al.*, (2003) where a mutagenized *PPO-1* gene was used as a selectable marker in maize. Transgenic sugarcane callus containing the mutagenized tobacco *PPO-1* gene were incapable of regeneration on medium containing the fomesafen herbicide.

In future attempts, targeting of the protein to the chloroplast might aid in regeneration of transgenic sugarcane callus on medium containing herbicide. Specifically, fusing a chloroplast transit peptide from a closely related monocotyledonous species has been considered for use in creating a new transformation construct. Random mutations were furthermore introduced into the tobacco *PPO-1* gene to try and identify novel mutations that could enable the tobacco *PPO-1* gene to induce herbicide resistance in sugarcane. Work was halted at the screening phase, since the *E. coli* VSR800 mutant did not possess the advertised

mutant phenotype. Numerous attempts to locate alternative mutant *E. coli* stains that can complement the *hemG* mutations were unsuccessful.

A cisgenic strategy was also considered with the aim of isolating a sugarcane *PPO-1* gene to be developed in the future as an endogenous plant selection gene. Once a sugarcane *PPO-1* gene is isolated this could be manipulated into a selectable marker for *in vitro* selection regimes in sugarcane. 5' RACE was used in attempts to isolate a full length *SoPPO-1* sequence, but was only successful in adding more nucleotides to the known partial sequence. PCR can be repeated in the future with new primers based on either the sorghum or maize *PPO-1* sequences, since high similarity was displayed between the extended sugarcane partial *PPO-1* fragment and the sorghum *PPO-1* and maize *PPO-1* genes.

## 4.4 References

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