Characterization of transgenic sugarcane lines with perturbed pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity.

by

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DECLARATION

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

Signed: …… Date: ……

A.L. Spracklen

ABSTRACT

Pyrophosphate fructose-6-phosphate 1-phosphotransferase (PFP) is an important glycolytic enzyme and catalyses the reversible conversion of fructose-6-phosphate (Fr-6-P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate (Fr-1,6-P_2) and inorganic phosphate (Pi). Sugarcane PFP has been inversely correlated with sucrose content across segregating F1 varieties. The down-regulation of PFP in cultivar NCo310 in a previous study led to an increase in sucrose accumulation and fibre content in immature tissue. Several potential transgenic sugarcane lines from genotypes 88H0019 and N27, transformed with the untranslatable sense sugarcane PFP-β gene, were characterized in this study. Initial screening for transgenesis was determined by slot blot and Southern blot analysis to confirm the presence of the co-transformed selectable marker *npt II* transgene. Northern blot analysis confirmed expression of the 1.2 kb PFP-β transcript in 7 of 9 lines analyzed. Sugar analysis using standard South African Sugarcane Research Institute (SASRI) mill room practices and HPLC was performed on 12 month old pot grown stalks divided into immature and mature tissue sections. The analysis of wild type 88H0019 showed an average sucrose content of 17.84 and 30.76 g sucrose/stalk in immature and mature tissue, respectively. However, no significant difference between the putative transgenic plant values and wild type controls was seen. PFP specific activity was determined in these tissues using enzymatic assay analysis and although levels obtained in immature tissue were between 5-18 nmol/min/mg protein, they were less than values previously reported in sugarcane. The results indicated that no down-regulation of PFP in immature tissue occurred when comparing transgenic and wild type plants.

A more discrete internodal tissue sampling method was used to overcome the difficulty of detecting small changes in PFP enzyme activity in bulked stalk tissue sections. Fine analysis of PFP was conducted on specific developmental tissues and single stalks were divided into immature (internodes 1-3), maturing (internodes 4-5) and mature (internodes 7-8) regions. Sucrose analysis was performed using HPLC and PFP activity was determined enzymatically on each tissue type. The analysis of discrete developmental tissues showed specific PFP activity of 60-80 nmol/min/mg protein in young tissue, an amount which falls in the range previously obtained for sugarcane. However there was no significant difference between PFP or sucrose in the transgenic lines when compared with the wild type controls in any of the three developmental tissues examined. Western blotting and densitometric analysis of the blots confirmed the lack of PFP down-regulation in immature tissue in all lines. A final analysis of PFP

in immature stalk tissue on selected lines was performed using quantitative PCR, which became available near the end of the study. The fold change of each transgenic line indicated that there was a minor increase in PFP confirming the lack of effect of transgenesis.

Although evidence for the expression of the PFP-β transgene was seen in the northern blot, no further evidence for transgenesis could be found to support the desired effect of down-regulation of PFP. Characterization of transgenic stalks in this study was hindered by a limited number of lines available for analysis and large variability between replicate samples. Sampling techniques employed in an attempt to make use of existing standard SASRI mill room practices for sugar analysis highlighted the need for a more precise sampling method, specifically when determining the effects of an enzyme manipulation such as PFP. A refined approach has been developed which will assist researchers in the choice of analytical techniques for screening and characterization of potential transgenic lines in the future.

OPSOMMING

Pirofosfaat fruktose-6-fosfaat 1-fosfotransferase (PFP) is ‘n belangrike glikolitiese ensiem wat die omkeerbare reaksie tydens die omskakeling van fruktose-6-fosfaat (Fr-6-P) en pirofosfaat (PPi) na fruktose-1,6-bisfosfaat (Fr-1,6-P_2) en anorganiese fosfaat (Pi) kataliseer. Suikerriet PFP vlakke vertoon ‘n omgekeerde korrelasie met suikerinhoud oor seggregerende F1 variëteite. ‘n Vorige studie het aangetoon dat die afregulering van PFP uitdrukking in die kultivar NCo310 tot ‘n verhoging in sukrose akkumulasie en veselinhoud in onvolwasse weefsel gelei het. Verskeie transgeniese suikerrietlyne, wat vanaf genotipe 88H0019 en N27 getransformeer is met ‘n nie-transleerbare sense suikerriet PFP-β geen, is in hierdie studie gekarakteriseer. Aanvanklike bepaling van transgenese is deur middel van gleuf en Southern blot analise uitgevoer om bevestiging te kry dat die ko-getransformeerde selekteerbare merkergeen *npt II* teenwoordig was. Northern blot analise het die uitdrukking van die 1.2 kb PFP-β transkriptoom bevestig in 7 van die 9 lyne wat ondersoek is. Analise van suikerinhoud is uitgevoer volgens standaard SASRI meulkamer prosedures sowel as met hoëdruk vloeistof chromatografie op stamme van 12 maande oue potplante, wat verdeel is in onvolwasse en volwasse gedeeltes. Analise van die wildetipe (88H0019) het ‘n gemiddelde suikerinhoud van 17.84 g sukrose per stam in die onvolwasse gedeelte teenoor 30.76 g sukrose per stam in die volwasse gedeelte aangetoon. Geen betekenisvolle verskil in suikerinhoud het tussen transgeniese lyne en die wildetipe kontroles voorgekom nie. Die spesifieke aktiwiteit van PFP is in dieselfde stamweefsel bepaal deur middel van ensiematiese analise. Die spesifieke aktiwiteit van PFP het tussen 5-18 nmol/min/mg proteïen gewissel. Hierdie aktiwiteitswaardes was laer as dié wat voorheen vir suikerriet gerapporteer is. Hierdie resultate dui daarop dat geen afregulering van PFP uitdrukking in onvolwasse stamweefsel plaasgevind het nie wanneer transgeniese en wildetipe plante met mekaar vergelyk word nie.

‘n Meer verfynde monsternemingsmetode is gevolg om te verseker dat klein veranderinge in PFP ensiemaktiwiteit bepaal kon word wat nie noodwendig sigbaar sou wees wanneer lang stamgedeeltes, wat uit talle litte bestaan, gebruik is nie. Analise is uitgevoer op enkelstamme wat verdeel is in onvolwasse (litte 1-3), gedeeltelik-volwasse (litte 4-5), en volwasse (litte 7-8) gedeeltes. Suikerinhoud en PFP aktiwiteit op hierdie verskillende ouderdomsgedeeltes van die stam is onderskeidelik bepaal met behulp van hoëdruk vloeistof chromatografie en ensimatiese analise. Die analise van onvolwasse stamweefsel het ‘n spesifieke PFP aktiwiteit van 60-80 nmol/min/mg proteïen opgelewer wat goed ooreenstem met resultate wat voorheen vir suikerriet

gerapporteer is. Daar was geen betekenisvolle verskil tussen PFP aktiwiteit of sukroseinhoud in die transgeniese lyne wanneer die waardes in die drie verskillende weefselgedeeltes met dié van die wildetipe kontroles vergelyk is nie. Western blot analise van die PFP proteïen, gevolg deur kwantifisering van die antiserumsein met behulp van desitometriese analise, het bevestig dat daar nie voldoende afregulering van PFP in die onvolwasse weefsels van al die suikerrietlyne wat ondersoek is plaasgevind het nie.

Verdere analise van PFP in onvolwasse stamweefsel van geselekteerde lyne is uitgevoer deur gebruik te maak van kwantitatiewe polimerase ketting reaksie. Die klein veelvoudige verandering in elk van die trangeniese lyne het daarop gedui dat daar ‘n klein verhoging in PFP uitdrukking was. Hierdie resultaat het die gebrek aan transgenese bevestig. Alhoewel bewyse van uitdrukking van die PFP-β transgeen aangetoon is tydens northern blot analise kon daar geen verdere bewyse van transgenese gevind word wat die gewensde effek van PFP afregulering ondersteun het nie. Die karakterisering van transgeniese suikerriet in hierdie studie was bemoeilik deur die groot mate van variasie wat voorgekom het tussen eksperimentele herhalings. Die gebruik van standaardanalise om suikerinhoud te bepaal, soos wat uitgevoer word deur die SASRI meulkamer, dui daarop dat ‘n meer noukeurige analitiese metode verlang word, veral wanneer spesifieke effekte van ensiemmanipulering, soos byvoorbeeld deur PFP, bestudeer word. ‘n Meer verfynde metode is gedurende hierdie studie ontwikkel wat in die toekoms navorsers se keuse van analitiese metodes vir die identifisering en karakterisering van potensiële transgeniese suikerrietlyne sal vergemaklik.

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ABBREVIATIONS

2,4-D	2,4-dichloro-phenoxyacetic acid
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
cm	centimetre
CWI	cell wall invertase (EC 3.2.1.26)
µCi	microCurie
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetra acetic acid
EGTA	ethyleneglycoltetra acetic acid
e.g.	for example
Emu	Emu synthetic monocotyledonous promoter
ERC	estimated recoverable crystal
FBPase	fructose-1,6-bisphosphatase (EC 3.1.3.11)
FC	fold change
F pr	probability density function
Fr	fructose
Fr-6-P	fructose 6-phosphate
Fr-1,6-P_2	fructose 1,6-bisphosphate
Fr-2,6- P_2	fructose 2,6-bisphosphate
FW	fresh weight
Gl	glucose
Gl-1-P	glucose-1-phosphate

Gl-6-P	glucose-6-phosphate
Glycerol-3-P DH	glycerol-3-phosphate dehydrogenase
GM	genetically modified
g	gravitational force
g	gram
kg	kilogram
mg	milligram
µg	microgram
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	hexokinase (EC 2.7.1.1)
HM	HEPES magnesium buffer
HPI	hexose phosphate isomerase (EC 5.3.1.9)
HPLC	high performance liquid chromatograph
IDV	integrated density value
IPB	Institute of Plant Biotechnology
IgG	immunoglobulin G
IU	international enzyme unit (one micromole substrate transformed per minute)
K_a	concentration of effector that produces half maximal activation
kb	kilo base pair
kDa	kilo Dalton
Km	substrate concentration producing half maximum velocity
KOH	potassium hydroxide
L	litre
LiCl	lithium chloride
mRNA	messenger RNA
ml	milliliter
MS	Murashige and Skoog medium
MIOP	*myo*-inositol oxygenation pathway

µM	micromolar (10^{-6}M)
mM	millimolar (10^{-3}M)
nM	nanomolar (10^{-9}M)
min	minute
nd	not determined
nm	nanometer
NaCl	sodium chloride
NADH	reduced nicotinamide-adenine dinucleotide
NaOH	sodium hydroxide
NI	neutral invertase EC 3.2.1.26
npt II	neomycin phosphotransferase gene
OPP	oxidative pentose phosphate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Q-PCR	quantitative reverse transcriptase polymerase chain reaction
PEG	polyethyleneglycol
PEP	phosphoenolpyruvate
PFP	pyrophosphate: fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90)
PFK	ATP: fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11)
PGM	phosphoglucomutase (EC 2.7.5.1)
Pi	inorganic phosphate
Pol	total dissolved matter
PPi	inorganic pyrophosphate
PPO	polyphenol oxidase (EC 1.14.18.1)
PTGS	post transcriptional gene silencing
PVPP	polyvinyl polypyrrolidone
RAPD	random amplified polymorphic DNA
RT	reverse transcriptase
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
RNAi	interference RNA
rpm	revolutions per minute
SA	South Africa
SASRI	South African Sugarcane Research Institute
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
SAI	soluble acid invertase (EC 3.2.1.26)
spp.	species
SPS	sucrose phosphate synthase (EC 2.4.1.14)
SSC	sodium saline citrate
SuSy	sucrose synthase (EC 2.4.1.13)
TBST	tris-buffered saline with Tween-20
TE	tris-EDTA buffer
TENS	tris-EDTA-NaCl-SDS buffer
TG	transgenic
TPI	triosephosphate isomerase (EC 5.3.1.11)
TGS	transcriptional gene silencing
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tween 20	polyoxyethylene sorbitan monolaurate
UDP	uridine 5'-diphosphate
UDP-Gl	uridine 5'-diphosphoglucose
UDPGl-DH	uridine 5'-diphosphoglucose dehydrogenase
UV	ultra violet
V	volts
V_{max}	maximum velocity
WT	wild type

GENERAL INTRODUCTION

Sugar supplies about 13 percent of all energy that is derived from human foods (Escalona 1952) and is the 3rd largest agricultural commodity exported in South Africa, which is ranked as the 13th largest sugar producer in the world (Anonymous 2006). The South African sugar industry is responsible for producing cost-competitive high-quality sugar, with an annual average income of R6 billion (http://www.sasa.org.za/sugarbusiness/sugarindustry.asp). The estimated tonnage of sugar produced per season is 2.5 million, of which 50% is marketed within South Africa. The remaining 50% of sugar is exported to countries in the Middle East, Asia, Africa and North America (Anonymous 2006). The contribution the sugar industry made to South Africa's foreign exchange earnings in 2004/05 was R2.38 billion. It therefore makes a significant contribution to the national economy (Anonymous 2006).

The South African sugar industry is a diverse one as it combines the agricultural activities of sugarcane along with the industrial factory production (Anonymous 2006) and much is being done to ensure that it remains competitive as a leading sucrose producer. The South African Sugarcane Research Institute (SASRI) is responsible for most of the agricultural research and development required to improve sugarcane production in SA (Snyman *et al.* 2008). The research done at SASRI is aimed at providing sustainable development, optimising resource utilization and agronomic practices as well as resolving logistical problems involved in growing and harvesting sugarcane (Snyman *et al.* 2008). One of the most important areas of research undertaken at SASRI is plant breeding and some advances in sugarcane variety improvement have been made, by both conventional (Butterfield *et al.* 2007) and biotechnological means (Snyman *et al.* 2001, Leibbrandt and Snyman 2003, Sooknandan *et al.* 2003, Snyman 2004).

Sugarcane is part of the *Saccharum* complex, and Bull and Glasziou (1963) proposed that it has the potential to store more than 25% sucrose on a fresh weight (FW) basis, which is almost double the current commercial yield. There is therefore a large amount of economic appeal in exploiting the modern gene transfer techniques of biotechnology to enhance the field performance of sugarcane and ultimately increase sucrose yields. A significant amount of the progress in increasing sugarcane crop productivity has been as a result of the genetic improvement of sugarcane via conventional breeding (Berding *et al.* 1997, Hogarth *et al.* 1997, Moore *et al.* 1997, Butterfield *et al.* 2001). However, crop improvement by the manipulation of a single enzyme to increase the accumulation of sucrose by genetic modification techniques has

been reported (Groenewald 2006, Bekker 2007, Rossouw *et al*. 2007) although no genetically modified (GM) cane is grown commercially. Over-expression of a native or foreign gene encoding a rate-limiting step has been used as an attempt to increase flux in certain biochemical pathways (Grof and Campbell 2001). In addition gene suppression by antisense or co-suppression mechanisms has been used in order to down-regulate the activity of a targeted enzyme, to ultimately reduce the flux through a specific pathway (Groenewald 2006).

The enhancing of sugar yield by genetic manipulation of sugar metabolism is difficult with both conventional and molecular breeding as the presence of alternative pathways often results in an unpredictable outcome (Lakshmanan *et al.* 2005). In this regard, it has become evident that only a combined approach of conventional and molecular breeding strategies will enable a successful increase in crop productivity (Groenewald and Botha 2001). Increasing the yield of sucrose and/or fibre (biomass) in sugarcane will have implications for the sugar industry and may potentially increase the contribution this industry makes to the national economy.

There are currently three main targets for molecular manipulation when increasing sucrose yield per plant or elucidating sucrose metabolism. These include: (i) the manipulation of the sucrose synthesis pathway, (ii) the increase in expression or activity of proton-sucrose transporters and (iii) the decrease in expression or activity of sucrose hydrolysing enzymes (Grof and Campbell 2001). A particular interest at SASRI includes engineering sugarcane to either over- or under-express glycolytic enzymes, which will ultimately redirect the flux of carbon into economically important end products such as sucrose and biomass (reviewed by Grof and Campbell 2001, Groenewald 2006).

An important glycolytic enzyme in the sucrose metabolic pathway, is the pyrophosphate dependant phosphofructokinase (PFP; EC 2.7.1.90) (Carnal and Black 1979), which catalyses the reversible conversion of fructose-6-phosphate (Fr-6-P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate (Fr-1,6-P_2) and inorganic phosphate (Pi) (Reeves *et al.* 1974). Evidence for the physiological role of PFP provided by transgenic plants exhibiting as much as 99% down-regulation of PFP has been contradictory. Transgenic tobacco plants with altered PFP levels demonstrate that, during photosynthetic sucrose synthesis, PFP does not make an essential contribution to carbon flow and PPi turnover (Paul *et al.* 1995). Hajirezaei *et al.* (1994) and Paul *et al.* (1995) concluded that in transgenic potato and tobacco plants, PFP catalyses a net glycolytic reaction under non-stressed conditions. Down-regulation of PFP in transgenic potato

(Hajirezaei *et al.* 1994) and tobacco (Paul *et al.* 1995, Nielson and Stitt 2001) appears to have no significant impact on the metabolite concentrations and fluxes in the plant.

Sugarcane PFP, however, appears to play an important role in sugarcane sucrose accumulation as it is inversely correlated to sucrose content across commercial varieties (Whittaker and Botha 1999). The reaction that sugarcane PFP catalyses is also close to equilibrium *in vivo*, indicating that sugarcane PFP may indeed have two roles, namely the regulation of carbon flow between sucrose synthesis and accumulation and the supply of carbon for respiration and to other biosynthetic pathways (Groenewald and Botha 2007). The down-regulation of PFP activity by up to 70% in transgenic glasshouse-grown sugarcane plants, results in an increase in sucrose content in immature internodes and significantly higher fibre content in both immature and mature internodes (Groenewald 2006). This increase in sucrose content in immature but not mature tissue, significantly contributed to an increase in sucrose purity, which resembled an early ripening phenotype. These sugarcane transgenic lines also presented no visible change in phenotype or any significant difference in growth and development when compared with the wild type material (Groenewald and Botha 2007), suggesting that PFP has a direct influence on the ability of young biosynthetically active sugarcane stalk tissue to accumulate sucrose.

Data obtained by Dennis and Greyson (1987) support the proposed role of PFP in sugarcane as a potential bypass to an irreversible adenosine triphosphate (ATP)-dependant phosphofructokinase (PFK; EC 2.7.1.11) during times of increased metabolic flux (Groenewald and Botha 2007), however the effects of PFP down-regulation on the metabolites and enzymes associated with carbon partitioning in sugarcane have yet to be determined. It is thought that an increase in sucrose synthesis via the down-regulation of PFP in transgenic sugarcane plants (Groenewald and Botha 2007) may be via the stimulation of sucrose phosphate synthase (SPS; EC 2.4.1.14), due to increased levels of its allosteric activator glucose-6-phosphate (Gl-6-P) (Reimholz *et al.* 1994) and its substrates (Hajirezaei *et al.* 1994). An increase in fibre content may be explained by an increase in the hexose-phosphate pools in sink tissues (Hajirezaei *et al.* 1994, Paul *et al.* 1995), stimulating an increase in the cell wall pre-cursors, such as UDP-glucose (UDP-Gl) (Groenewald and Botha 2007).

Transgenic sugarcane research has therefore provided valuable insight into the nature of sucrose accumulation metabolism. However there are many difficulties in the investigation of sucrose accumulation in the internodal tissues of the sugarcane stalk. This is due to the hard rind on the stalk and fibrous nature of the tissue. As the whole stalk represents one organ it is

necessary to sacrifice the entire plant when sampling (Moore 1995). In addition, long periods of growth are required before sampling. Two different approaches have therefore been followed in attempt to overcome these problems, namely tissue discs of internodal tissue (Bieleski 1962, Sacher *et al.* 1963, Hawker 1965, Bindon and Botha 2002) and cell suspension cultures derived from callus material (Maretzki and Thom 1972, Komor *et al.* 1981, Wendler *et al.* 1990, Rossouw *et al*. 2007). Sampling techniques different to those used conventionally for standard mill analysis where stalks are bulked and divided into two halves may also need to be considered when analysis of transgenic sugarcane is required.

The purpose of this study was to determine the effects of down-regulation of PFP on sucrose concentrations in transgenic sugarcane lines previously created at SASRI. The specific aims of this study were:

1. To investigate expression of the PFP-β transgene in genotypes 88H0019 and N27 and compare PFP specific activity in transgenic and wild type lines.
2. To determine the effects of altered PFP activity on sucrose levels throughout the sugarcane stalk, specifically immature tissue.
3. To evaluate a range of techniques for rapid, high throughput testing of transgenic metabolic manipulations of plants created at SASRI.

LITERATURE REVIEW

1. Sugarcane as a crop

Sugarcane belongs to the grass family (Poaceae) and is common in tropical and sub-tropical countries throughout the world where it is grown commercially. The main product of sugarcane is sucrose, which contributes greatly to the calorie consumption of the average person. Sucrose is used in numerous ways namely, as a sweetening agent for foods, in the manufacture of cakes and candies, preservatives, alcohol, soft drinks and numerous other foods (Braun 1999). Seventy percent of the proportion of the world's sucrose is obtained from sugarcane (http://www.sasa.org.za/sugarbusiness/sugarindustry.asp).

After harvesting and milling, sugarcane extracts e.g. molasses, which is produced from the raw sugarcane juice and bagasse, are used for many different purposes. Molasses may be sold as syrup, used to flavour rum and other foods, used in animal feed or even as an additive for ethyl alcohol (Harris and Staples 1998). The major by-product of milling sugarcane however is the fibrous residue of cane stalks left over after the crushing and extraction of juice, known as bagasse (Pandey *et al.* 2000).

Sugarcane bagasse is a complex material and is comprised of 50% cellulose, 25% hemicellulose and 25% lignin (Pandey *et al.* 2000). It is used for electricity generation by the sugar factory as boiler fuel or in the generation of steam and power required to operate the sugar mill. Several other products have been made from sugarcane bagasse, either as is or through fermentation. This includes the production of pulp and paper, particleboard and furfural (a selective solvent) (Patarau 1986) or fermentation products, such as the production of protein enriched cattle feed and enzymes (Pandey *et al.* 2000). Because of its low ash content, sugarcane bagasse offers many advantages over other crop residues such as rice straw and wheat straw (Pandey *et al.* 2000). One of the potentially most important uses of sugarcane and one which is currently fervently studied is its use as a substrate in ethanol production e.g. nearly half of Brazil's sugarcane is used for ethanol production (Bolling and Suarez 2001).

2. Sucrose metabolism

Sucrose metabolism involves a large network of reactions which may be positively or negatively influenced by a variety of different factors. Sucrose accumulation may loosely be described as "the difference between the amount of sucrose produced in the leaf by photosynthesis and the amount of this sucrose that is removed by metabolism to produce carbon compounds and energy for the growth and development of the plant" (Moore 2005). Sucrose metabolism however, is not this simple, as sucrose appears to play a far more important role in the plant.

2.1. Regulation of plant sucrose synthesis and degradation

Sucrose is the main storage sugar in plant cells and has therefore attracted a lot of attention since its discovery (Kruger 1990). There is evidence that the accumulation of sucrose in plant cells may be regulated by a rapid cycle in which sucrose is synthesised and then degraded again, allowing for the net rate of sucrose accumulation to respond very sensitively to small changes in the concentrations of substrates and products involved in these reactions (Figure 1) (Dancer *et al.* 1990). For example, the accumulation of sucrose would stop and consequently the levels of the degradative enzymes increase as the rate of degradation increases (Dancer *et al.* 1990).

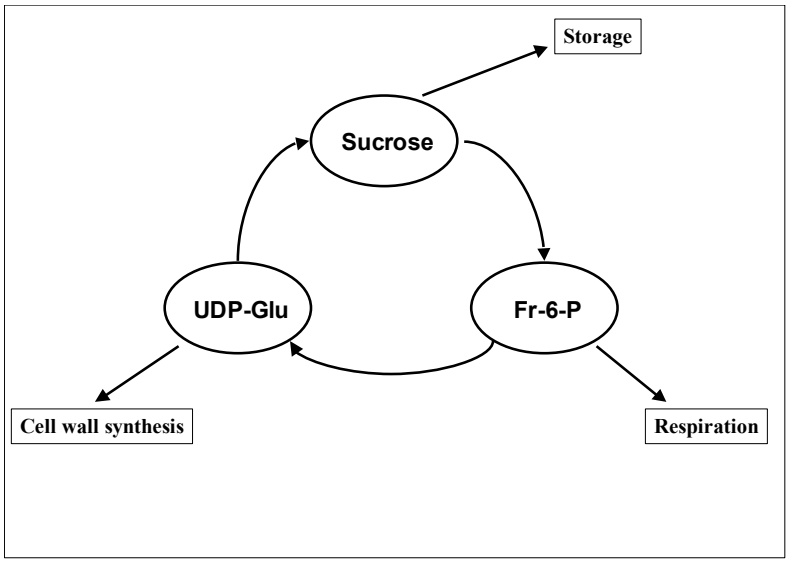

Figure 1. Schematic representation of the carbon cycle in C4 plants.
There are three main demands on carbon when entering the cell, namely for sucrose synthesis, to be used in respiration and in the synthesis of cell wall pre-cursors.

There are two cytoplasmic pathways responsible for the synthesis and degradation of sucrose. These pathways consist of a glycolytic and gluconeogenic flow, which are affected by the interconversion of Fr-6-P and Fr-1,6-P_2. One of the pathways is known as the maintenance pathway and is catalysed by PFK glycolytically and FBPase gluconeogenically, both of which are non-equilibrium reactions (Black *et al.* 1987). The second pathway is known as the adaptive pathway and is catalysed by PFP in an equilibrium reaction (Black *et al.* 1987), which seems to conserve overall energy for plants (Carnal and Black 1979). A regulator cycle is present to control the two aforementioned pathways in which changing levels of fructose-2,6-bisphosphate (Fr-2,6-P_2) serve as the regulator of both pathways (Black *et al.* 1987).

The concentration of the enzymes involved in glycolysis or gluconeogenesis varies according to the specific cell cycle, type and developmental stage of the tissue, and also as a result of the plant's adaptation to an environmental or nutrient status (Dennis and Miernyk 1982, Dennis and Emes 1990, Dennis *et al.* 1991, Botha *et al.* 1992, Miernyk and Dennis 1992, Sangwan *et al.* 1992, McHugh *et al.* 1995). Therefore, due to the range of plant sucrose concentrations and the rapid daily fluxes that can occur, it has been proposed that plant cells must have molecular mechanisms for reacting to differing sucrose concentrations (Dancer *et al.* 1990). The enzymes involved in the regulation and degradation of sugar are also involved in sugar sensing and signalling which occurs throughout the entire life cycle of the plant (reviewed by Plaxton 1996). These enzymes are either under coarse- or fine-regulatory control (Copeland and Turner 1987, Plaxton 1990). There are a wide variety of genes involved in the coarse-regulation of sugar occurring at the level of transcription (Rolland *et al.* 2002). The sugars produced may either induce or repress various enzymes (Rolland *et al.* 2002). Two enzymes in particular are exceptionally responsive to their sucrose supply, namely SPS and PFP (Black *et al.* 1987, Sung *et al.* 1990, Xu *et al.* 1989) (Figure 2).

Fine regulatory control has appeared to evolve in order to regulate glycolysis (Plaxton 1996). The majority of fine systems control seen in plants is exerted on the enzymes catalyzing reactions involved in the conversion of hexoses to hexose-phosphates i.e. Fr-6-P to Fr-1,6-P_2 (Figure 2) and phosphoenol pyruvate (PEP) to pyruvate (Copeland and Turner 1987, Kubota and Ashihara 1990, Miernyk 1990). Fine control is usually determined by factors such as substrate concentration, pH variation, metabolite effectors, subunit association/dissociation and covalent modification which may be reversible by phosphorylation (Plaxton 1996). PFP is under fine metabolic control by Fr-2,6-P_2 which contributes to the coordination of sucrose synthesis in

plants (Stitt 1990, Claassen *et al.* 1991). After a certain concentration of sucrose has been reached some of the common precursor Fr-6-P will be converted to Fr-2,6-P_2 (Figure 2) (Claassen *et al.* 1991). Numerous studies have shown that an increase in the level of Fr-2,6-P_2 increases starch formation, and a decrease leads to an increase in sucrose formation (Kruger and Scott 1994, Scott *et al.* 2000, Draborg *et al.* 2001).

Plant cells have four distinguishable activities responsible for cleaving sucrose. This includes sucrose synthase (SuSy; EC 2.4.1.13) which is the predominant enzyme responsible for sucrose breakdown activity (Sung *et al.* 1989) and three separate invertase activities: neutral invertase, (NI; EC 3.2.1.26); soluble acid invertase (SAI; EC 3.2.1.26), and cell wall invertase (CWI; EC 3.2.1.26). Sucrose synthase and alkaline invertase have the ability to readily increase their activity over a wide physiological range of sucrose concentrations (Sung *et al.* 1989). Sucrose is degraded by SuSy to UDP-Gl and fructose or by invertase to both invert sugars (glucose and fructose) (Figure 2). Most cell wall pre-cursors are derived from UDP-Gl which is therefore considered as the primary precursor for the synthesis of sucrose and structural polysaccharides (Kruger 1990). UDP-glucose is also a respiratory substrate (Figure 2) (Turner and Botha 2002).

Due to the action of phosphoglucomutase (PGM; EC 2.7.5.1.) and hexose phosphate isomerase (HPI; EC 5.3.1.9), the hexose phosphates (glucose-1-phosphate (Gl-1-P), Gl-6-P and Fr-6-P), are generally thought to be close to equilibrium (ap Rees 1980, Kruger 1990) and therefore form a pool of intermediates. They are derived from either the breakdown of sugars and polysaccharides or from triose-phosphates and may be used for the synthesis of carbohydrates or for catabolism (Figure 2) (Kruger 1990). An important factor in the metabolic control of the net rate of sucrose degradation may in fact be the cytosolic levels of the hexose-phosphate pool (Renz and Stitt 1993). In most plant cells, the main drain on the hexose phosphate pool is glycolysis as it is the prominent pathway of carbohydrate oxidation (Kruger 1990). An increase in the cytosolic hexose-phosphate pool will lead to a decrease in the net rate of sucrose degradation (Geigenberger *et al.* 1994, Hajirezaei *et al.* 1994) and as a result will lead to a slight increase in starch accumulation (Hajirezaei *et al.* 1994). The accumulation of sucrose and/or starch signifies the difference between the rate of synthesis and degradation of these products in plant cells (Dancer *et al.* 1990).

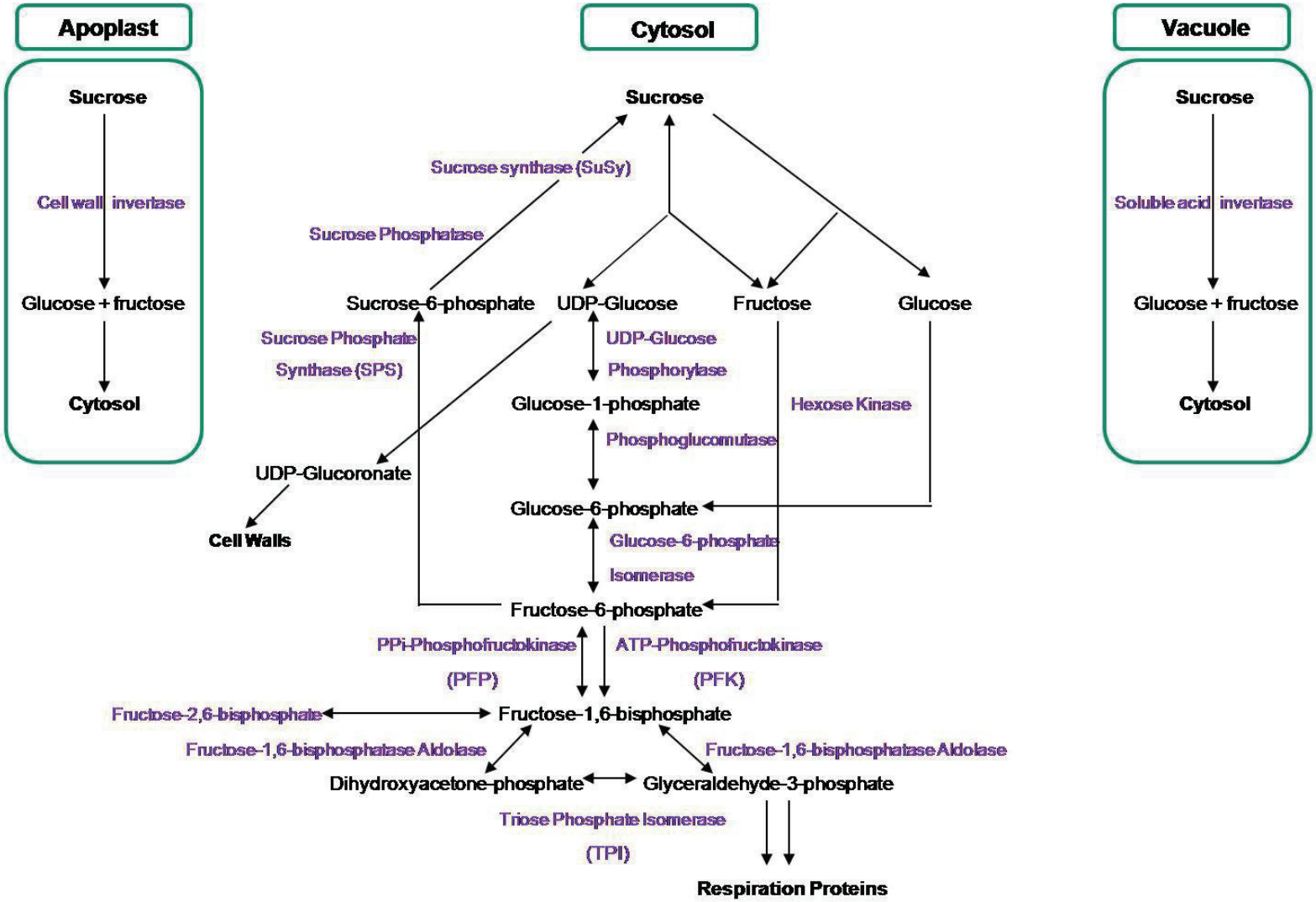

Figure 2. A diagrammatic representation of the essential reactions which occur in sucrose metabolism in sink tissues of sugarcane (modified from Groenewald 2006).

2.2. Sugarcane sucrose metabolism

Sugarcane sucrose metabolism is highly complex and the biochemical basis for sucrose accumulation is not fully understood. Upon maturation of the sugarcane stalk either seasonally or developmentally, an increase in sucrose content coincides with a redirection of carbon partitioning from insoluble matter and respiration towards sucrose (Whittaker and Botha 1997). As a result, a relatively small allocation of carbon to respiration and cell wall synthesis in mature parenchyma cells is seen (Whittaker and Botha 1997). Bindon and Botha (2002), using a tissue disc system, reported that the allocation of carbon into fibre was two-fold less than the allocation of carbon into sucrose in immature sugarcane tissue. This carbon allocation decreased with tissue maturity, as did the allocation into starch (Bindon and Botha 2002). The rate of carbon cycling throughout the sugarcane stalk may therefore depend on the age of the cane (Bindon and Botha 2002).

Previous studies on carbon cycling between sucrose and the hexose sugars in sugarcane have revealed that a rapid cycle of sucrose synthesis and degradation exists (Sacher *et al.* 1963, Wendler *et al.* 1990, Komor *et al.* 1996). This rapid cycling allows for small changes in the enzymes and metabolites of the pathway to induce significant changes in the rate of synthesis or degradation of sucrose (Dancer *et al.* 1990). The allocation of carbon to fibre in sugarcane is a significant drain on sucrose accumulation levels, consuming 16% of the incoming carbon in immature tissue, representing a significant sink (Bindon and Botha 2002). Sucrose accumulation in mature tissues could be as a result of a decrease in sucrose degradation leading to a higher net storage of sucrose (Bindon and Botha 2002). The storage cells of mature sugarcane tissue contain a vacuole which occupies approximately 90% of the total cellular space and is found to contain high concentrations of solutes, predominantly sucrose (Bull and Glasziou 1963, Welbaum and Meinzer 1990). Water content generally increases down the sugarcane stalk with a corresponding increase in sucrose content (Bull and Glasziou 1963). High sugar genotypes need at least 70% moisture content in mature internodes (Bull and Glasziou 1963) and the vacuole therefore represents an important storage compartment and in mature tissues may contain over 21% of the stored sucrose (Welbaum and Meinzer 1990).

3. PFP: Characteristics and potential roles in sucrose metabolism

3.1. Characteristics and differential subunit expression

Plant PFP was first identified in pineapple leaves (Carnal and Black 1979) and has since been identified in a variety of different tissues from a number of plant species including watermelon (Botha and Botha 1991b), potato (Kruger and Dennis 1987), bean and rice seeds (Botha and Small 1987, Blakely *et al.* 1992), tomato (Wong *et al.* 1990), carrot roots (Wong *et al.* 1988), tobacco (Paul *et al.* 1995) and sugarcane (Whittaker and Botha 1997). It is widely distributed among photosynthetic organisms (Black *et al.* 1982) and is the only phosphotransferase present in plants (Black *et al.* 1995). PFP is found exclusively in the cytosol in plant tissue and exists predominantly as a heterotetramer comprising two regulatory α subunits and two catalytic β subunits (Yan and Tao 1984, Kruger and Dennis 1987, Cheng and Tao 1990, Wong *et al.* 1990, Botha and Botha 1991a, Nielson 1994). PFP has a native molecular mass of approximately 265 000 Daltons (Kruger and Dennis 1987, Botha *et al.* 1988), a single transcript of 2.3 kb for the α-subunit gene and a single 2.1 kb transcript for the β-subunit gene (Carlisle *et al.* 1990). Sugarcane PFP consists of two polypeptides of 63.2 and 58.0 kDa (Groenewald and Botha 2007) which have been found at different concentrations in several sugarcane tissues (Suzuzki *et al.* 2003).

The PFP protein appears to differ structurally between plant species, perhaps explaining the different kinetic properties described for PFP (Kombrink *et al.* 1984, Yan and Tao 1984, Wu *et al.* 1984, Botha *et al.* 1986, Macdonald and Preiss 1986**,** Botha *et al.* 1987). The physical properties of PFP not only vary among plant species but also between tissue types which suggest that differential expression of the genes of the two subunits may exist (Blakely *et al.* 1992). There is clear evidence for differential expression of the PFP α- and β-subunit genes as well as tissue specific expression of these genes during seedling development in castor beans, which is consistent with a glycolytic role of PFP in this tissue (Blakely *et al.* 1992). Results suggest the presence of one gene for each of the subunits of castor PFP (Blakely *et al.* 1992). However conflicting results have been found with potato PFP, where it has been suggested that subunits are not likely to share extensive amino acid homology, since antibodies raised to each subunit do not cross-react (Kruger and Dennis 1987).

PFP also appears to be an adaptive enzyme whose activity and subunit structure change in response to environmental stresses and developmental changes (Plaxton 1996). Changes in the

activity of PFP coincide with a change in the isoform detected (Botha and Botha 1991b). One of the isoforms of PFP contains only the α-subunit whilst the second larger isoform consists of both the α- and the β-subunits (Yan and Tao 1984). The isoform in which PFP is present appears to be dependent on the concentration of the subunits (Kruger and Dennis 1987). Subunit availability might also be an important factor in determining the isoform in which PFP is present (Botha and Botha 1991b). In wheat seedlings both isoforms of the enzyme are activated by Fr-2,6-P_2, however the smaller form is activated to a lesser degree than the larger form (Yan and Tao 1984). The stability of the smaller α-subunit can actually be increased by Fr-1,6-P_2 (Wang and Shi 1999). This would enable PFP to act in the gluconeogenic direction or PPi turnover, despite the lack of activation by its activator Fr-2,6-P_2 (Wang and Shi 1999). The α-subunit may therefore be the controlling agent of PFP activity (Yan and Tao 1984).

Studies have also shown that PFP can be reversibly converted from a high to a low molecular form, depending on the presence of PPi and Fr-2,6-P_2 (Wu *et al.* 1984, Kruger and Dennis 1987). PFP either dissociates into a dimer in the presence of PPI, which is an inherent property of barley PFP (Nielson 1994) or aggregates into a tetramer in the presence of Fr-2,6-P_2 in potato (Kruger and Dennis 1987). In developing castor bean seed and *Citrullus lanatus*, the PFP subunits are not co-ordinately expressed in all tissues (Botha and Botha 1991b, Blakely *et al.* 1992). The levels of PFP activity in sugarcane appear to be controlled by the expression of the 63 kDa β-subunit. Most of the measurable PFP activity in fact, is associated with the β-subunit (Suzuzki *et al.* 2003).

3.2. Metabolic interactions and potential roles in sucrose metabolism

Activation of PFP occurs at nanomolar concentrations of the regulatory metabolite Fr-2,6-P_2 (Sabularse and Anderson 1981a, 1981b, Cséke *et al.* 1982, van Shaftingen *et al.* 1982, Kombrink *et al.* 1984). The levels of Fr-2,6-P_2 found in sugarcane are sufficient to fully activate PFP (Whittaker and Botha 1997) and a relatively broad pH optimum of between 6.7 and 8.0 was discovered for sugarcane PFP in both the forward and reverse reactions, in the presence of Fr-2,6-P_2 (Groenewald and Botha 2007). Fr-2,6-P_2 also influenced the aggregation state of sugarcane PFP in that it had a significant effect on the molecular weight of the enzyme (Whittaker and Botha 1997) and may cause an association of PFP into the bigger, most active form of the enzyme (Wu *et al.* 1983, 1984). This indicates a gluconeogenic/glycolytic regulatory mechanism, as PFP associates and glycolysis is favoured when the levels of Fr-2,6-P_2 in the plant cell increases (Wu *et al.* 1983, 1984). The process of glycolysis is reversed when Fr-2,6-P_2

levels decrease and gluconeogenesis is favoured (Wu *et al.* 1983, 1984). The presence of PFP would therefore make the regulation of glycolysis and gluconeogenesis increasingly more subtle as Fr-2,6-P_2 stimulates PFP activity by a 10-fold increase in the maximum velocity (V_{max}) of PFP (Botha *et al.* 1986). The physiological significance of the activation of PFP by nanomolar concentrations of Fr-2,6-P_2 is still not fully understood although it has been determined as having an important role in the regulation and accumulation of sucrose (Stitt 1998). Kinetic results obtained from barley however, indicate that PFP may be allosterically activated by Fr-1,6-P_2 which substitutes for Fr-2,6-P_2 as an activator (Figure 2) (Nielson 1995) at the same time as it is a substrate for PFP (Nielson and Wischmann 1995), although Nielson (1995) however found that the total degree of activation was greater with Fr-2,6-P_2.

The exact physiological function of PFP however is not yet known and as a result a great deal of interest has been expressed in elucidating the function of this enzyme. Although evidence obtained for the role of PFP in plants so far has been somewhat contradictory, several potential roles have been suggested. These include the regulation of glycolytic carbon flow (Carnal and Black 1979, Hajirezaei *et al.* 1994, Whittaker and Botha 1999) as evidence for the role of PFP in increased glycolytic flux includes the following two observations from numerous plant sources: (i) PFP is present in tissues in which a net gluconeogenic flux is unlikely i.e. Fr-1,6-P_2 to Fr-6-P (ap Rees *et al.* 1985, Wong *et al.* 1988, Mertens 1991, Tobias *et al.* 1992) and (ii) FBPase and PFP appear to co-exist in the same compartment and are inversely regulated by Fr-2,6-P_2 (Mertens 1991).

PFP has been implicated in PPi metabolism (ap Rees *et al.* 1985, Black *et al.* 1987). Although research provides support for the function of PFP in the production of PPi for the net breakdown of sucrose via the sucrose synthase pathway (ap Rees *et al.* 1985, Dancer and ap Rees 1989, Xu *et al.* 1989), the key role of PFP may be in the maintenance of the cytosolic PPi concentration (Stitt 1989). As PFP is thought to be involved in stress metabolism, it implies that this enzyme is an important sensor of environmental changes and may be involved in mobilizing energy reserves during unfavourable environmental conditions (Murley *et al.* 1998, Teramoto *et al.* 2000, Kovács *et al.* 2006). PFP may therefore confer a significant bioenergetic advantage in organisms which contain both PFP and PPi (Murley *et al.* 1998). PFP has also been associated with sink strength (Edwards and ap Rees 1986, Botha and Botha 1991b, Black *et al.* 1995) and may play a role during wound respiration (van Schaftingen and Hers 1983).

PFP may serve as an alternate enzyme to PFK in glycolysis. Studies on maize have deduced that PFK is the main enzyme responsible for glycolysis whilst PFP activity increases above PFK activity when starch accumulation increases (Tobias *et al.* 1992). The presence of PFK in both the chloroplast and the cytoplasm (Kelly and Latzko 1977) and the presence of PFP in the cytoplasm only, suggest the presence of two glycolytic pathways in green cells (Carnal and Black 1983). PFK-catalysed glycolysis may therefore serve mainly to support energy production, whilst PFP-catalysed glycolysis may be to contribute primarily to the generation of biosynthetic intermediates for cellular growth and development (Tobias *et al.* 1992). Changes in PPi and PFP levels in sugarcane tissue are therefore more likely to be associated with glycolysis (Lingle and Smith 1991) as PFP plays an important role in glycolytic flux when high flux is required (Groenewald and Botha 2008).

Wong *et al.* (1988, 1990) suggested that the kinetic characteristics of PFP in sucrose-storing plants might be adapted to favouring the gluconeogenic reaction. This would maintain the substrate levels needed for sucrose synthesis. In several plant species studied the highest levels of PFP activity were seen at a peak in gluconeogenesis (Wong *et al.* 1988, Botha and Botha 1993) as an increase in Fr-2,6-P_2 occurs with an increase in PFP activity as well as an increase in gluconeogenic flux (Botha and Botha 1993). This was confirmed by Bindon and Botha (2002) who argued that PFP is the main enzyme catalyzing gluconeogenic flux from triose-phosphates. Further studies suggested that PFP may form part of a highly responsive system in which it could react in a flexible manner to changes in these metabolic concentrations (Groenewald and Botha 2008).

3.3. Sugarcane PFP

Sugarcane PFP is closely associated with sucrose accumulation as it is inversely correlated with sucrose content across commercial varieties and F1 segregating populations (Whittaker and Botha 1999). A reason for this inverse relationship between PFP and sucrose content may be due to an increase in the utilisation of sucrose for biosynthesis in some of these varieties (Whittaker and Botha 1999). Sugarcane PFP activity has been positively related to carbon partitioning into respiration and its activity decreases with tissue maturity (Xu *et al.* 1989, Whittaker and Botha 1999). An increase in PFP activity has been associated with a decrease in the ability of the stalk to accumulate sucrose and there is a positive relationship between PFP activity and increased carbon flux into respiration (Whittaker and Botha 1999). This indicates a

strong inverse correlation between the ability of the stalk to store sucrose and the levels of PFP activity in the plants (Whittaker and Botha 1999).

Developmental differences exist in the metabolic activities of the storage tissues from one internode to another (Rae *et al.* 2005). Between internodes 4 and 7 (Figure 3), there appears to be a distinct increase in the rate of sucrose accumulation (Whittaker and Botha 1997) as PFP activity was found to decrease with stalk maturity (Whittaker and Botha 1999). Whilst there is an inverse correlation between sucrose and PFP activity in different varieties of sugarcane, total respiration has been positively correlated to PFP activity (Whittaker and Botha 1999). A decrease in the levels of Fr-2,6-P_2 coincides with an increase in tissue maturity, which may therefore down-regulate PFP in these tissues (Bindon and Botha 2002). It is expected then, that PFP regulates the balance of sucrose demand and supply in respiration and biosynthesis in sugarcane (Bindon and Botha 2002). The reaction which sugarcane PFP catalyses is close to equilibrium *in vivo* at all stages in the sugarcane stalk as the theoretical equilibrium value of 3.3 (Stitt 1989) was calculated for the reaction (Whittaker and Botha 1997). This indicates that sugarcane PFP may be responsible for the regulation of carbon flow between sucrose synthesis or accumulation and the supply of carbon for respiration and to other biosynthetic pathways (Groenewald and Botha 2007).

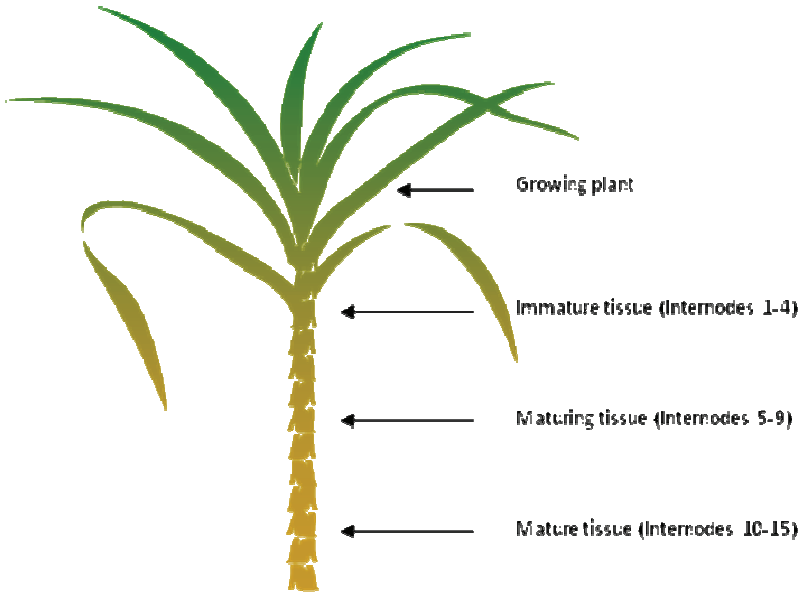

Figure 3. Diagrammatic representation of tissue maturity throughout the sugarcane stalk.
PFP is found at highest concentrations in immature and maturing tissue (Whittaker and Botha 1997).

4. Transgenesis as a tool to elucidate plant metabolism

Transgenic plants provide a powerful means to analyze the role of enzymes. The role of an enzyme in the regulation of a particular process in plant metabolism may be studied by engineering plants that demonstrate an increase or decrease in the respective enzyme activity and by evaluating the impact of this manipulation on the pathway flux in the plant (Herbers and Sonnewald 1996, reviewed by Iyer *et al.* 2000). Transgenic research has also been used to alter metabolic fluxes in the plant, to increase the production or yield of a particular product, or introduce novel functions into the plant to obtain products other than sucrose (Herbers and Sonnewald 1996). Transgenic research has been performed in the major graminaceous monocotyledonous plants such as rice (Cao *et al.* 1992, Chamberlain *et al.* 1994), barley (Lazerri *et al.* 1991), oats (Somers *et al.* 1992), maize (Klein *et al.* 1990, Zhang *et al.* 1996) and sugarcane (Bower and Birch 1992, Bower *et al.* 1996, Snyman *et al.* 1996, Groenewald and Botha 2001, 2008, Ferreira 2008).

4.1. Methods in transgenesis

Techniques used to introduce foreign/novel genetic elements in plants include methods such as electroporation (Zhang *et al.* 1988), PEG-mediated transformation (Li *et al.* 1990) and particle bombardment of regenerable tissues (Cao *et al.* 1992, Bower *et al.* 1996). The isolation of novel genes used in transgenesis involves the identification of suitable gene promoter elements to direct cell or tissue specific expression and/or identification of suitable targeting sequences to direct the gene product to the appropriate sub cellular location (Grof 2001). The majority of transgenic plants with reduced enzyme activity are created with sense or antisense suppression (Kreft *et al.* 2003) where RNA interference mechanisms (RNAi) are being used as a powerful initiator for the gene silencing of expression in many organisms (Xiong *et al.* 2004). The introduction of RNAi into a cell is an efficient way of shutting down gene expression (Xiong *et al.* 2004) as it is closely related to the post transcriptional gene silencing (PTGS) seen in plants (Fire 1999, Sharp and Zamore 2000, Sijen and Kooter 2000). An advantage of using RNAi when compared with conventional gene knockouts is that a relatively small gene sequence of usually 20-100 bp is adequate to silence a gene (Xiong *et al.* 2004).

Genetic manipulation in sugarcane however is complicated as its genome is the most complex of all crop plants (Grivet and Arruda 2002). This is largely due to the fact that commercial sugarcane cultivars are derived from initial crosses between *Saccharum officinarum* and *S.*

spontaneum, followed by a number of backcrosses to *S. officinarum*. As a result sugarcane varieties are interspecific polyaneuploid hybrids with an excess of 100 chromosomes (Butterfield *et al.* 2001, Grof *et al.* 2006). The first successful transformation of sugarcane through particle bombardment was reported in 1992 (Bower and Birch 1992). Subsequently, optimized protocols have been published (Bower *et al.* 1996, Snyman *et al.* 1996, Elliott *et al.* 1999). Although successful incorporation of foreign genes can be achieved with *Agrobacterium* (Dong *et al.* 1996, Arencibia *et al.* 1998, Elliott *et al.* 1998), the most widely used method of transformation in sugarcane is particle bombardment of embryogenic callus (Bower *et al.* 1996, Snyman 2004). The maize Ubiquitin promoter is the most effective transgene promoter used in sugarcane (Christenson *et al.* 1992).

Certain transgene manipulations for altering plant metabolic pathways may not always be successful as it has been found that many transgenes do not express as expected (Napoli *et al.* 1990, Kinney 1998). This may also be due to metabolic compensation by the plant, rendering the manipulation useless (Napoli *et al.* 1990, Kinney 1998). It has been observed that over-expressing single enzymes prematurely in metabolic pathways may be of limited effectiveness in increasing the overall flux (Kinney 1998). This may be as a result of silencing of the transgene, which occurs by either transcriptional gene silencing (TGS) or PTGS, or both (Iyer *et al.* 2000*)*. There is also the stochastic nature of silencing: genetically identical plant siblings may exhibit differences in gene silencing or reactivation, or the way in which they inherit the silencing and/or expression characteristics (Iyer *et al.* 2000). It is thought that after insertion of transgenic DNA further arrangements or eliminations may occur. Duplication or deletion of the transgene during meiosis is possible. However, little is known about the processes by which transgene DNA is incorporated into the plant genome (Iyer *et al.* 2000). Stability of expression is essential for successful regulation or transformation for future increases in the performance of economically important crops. One of the major difficulties in the genetic modification of plants is to overcome in the short-term the effective control of gene expression and phenotypic changes, which may be a result of tissue culture or transformation process and might negatively influence the intended manipulation (Grof and Campbell 2001, Vickers *et al.* 2005a).

4.2. Analysis of techniques for the identification of transformed plants

Within a population of plants obtained from the same experiment, variation in phenotype is often seen (Hoekema *et al*. 1989, Conner *et al*. 1994, Bregitzer *et al*. 1998, Singh *et al*. 1998, Kaniewski and Thomas 1999, Shu *et al*. 2002). Transformed plants must therefore be carefully screened to identify those presenting the desired traits only (Kumar *et al*. 1998, Dear *et al*. 2003). It is also important to screen sufficient numbers of potential transgenic lines so that only insertion events resulting in desired phenotypes are chosen for potential commercialization (Wilson *et al.* 2006). Insertion events obtained by standard particle bombardment procedures are usually characterized by either DNA or RNA analysis or in some instances, both. The techniques chosen for the analysis of the transformed plants will depend on the intended genetic manipulation and must include the appropriate controls.

Southern blot analysis (Sambrook and Russell 2001) on restricted DNA is especially useful when a novel gene has been inserted as it is able to reveal multiple copies and insertion patterns of the foreign transgene. It is unable however, to identify mutations created at a transgene insertion event (Jakowitsch *et al*. 1999, Mehlo *et al*. 2000, Svitashev and Somers 2001, Svitashev *et al*. 2002). Large scale PCR of genomic DNA is a rapid and powerful technique for the *in vitro* amplification and analysis of novel DNA (Mullis *et al*. 1986, Gibbs 1990) and subsequent DNA sequence analysis of amplified fragments may also be used to screen potential transgenic plants. Few studies described in literature however, use PCR and DNA sequence analysis to characterize transformed plants (Shimizu *et al*. 2001, Windels *et al*. 2001, Svitashev *et al*. 2002, Ulker *et al*. 2002, Makarevitch *et al*. 2003). Random amplified polymorphic DNA (RAPD) or restriction fragment length polymorphism (RFLP) analyses may indicate numerous genomic differences between control plants and transgenic plants and also indicate undesirable mutations (reviewed by Sala *et al*. 2000).

The conventional northern blot allows for a direct comparison of messenger RNA between samples on a single membrane (Sambrook and Russell 2001). It is a widely accepted, well regarded method and is used as a confirmation of transgene expression. This method of RNA analysis to identify transgene expression is less sensitive than the quantitative real-time PCR (RT-PCR) analysis of expression and any degradation by RNases however slight, will negatively affect the quality and quantitation of the expression data.

Fluorescent quantitative RT-PCR analysis of transgenic and control samples is a highly valuable tool for the rapid screening of tissues as it identifies differences in the level of gene expression of the gene of interest in small amounts of mRNA (Freeman *et al.* 1999). It therefore has potential for the high-throughput analysis of gene expression in research and routine diagnostics when screening transgenic plants. Although this technique allows for of a large number of samples to be screened (including numerous genes) in one experiment, allowing for more flexibility which is unavailable in conventional methods such as northern and Southern analyses, errors may occur when amplifying the target gene which could result in large variability between samples and ultimately decrease the reliability of the quantification (reviewed by Freeman *et al.* 1999). In addition, mathematical and statistical analysis of the large amount of data created by RT (Q)-PCR may also lead to inefficient evaluation of the reaction (Muller *et al.* 2002).

4.3. Importance of transgenic sugarcane research and the effects of enzyme manipulations on sucrose accumulation

A major focus of sugarcane genetic manipulation research has been in the control of sucrose accumulation and yield (reviewed by Grof and Campbell 2001). A potentially important way to attain this is by genetic improvements in photosynthetic efficiency or carbon partitioning among metabolic pools (Inman-Bamber *et al.* 2005). This approach has been supported by transgenic work where specific enzymes have either been up- or down-regulated and the effects on sucrose metabolism investigated (Hajirezaei *et al.* 1994, Paul *et al.* 1995, Rossouw 2006, Ferreira 2008, Groenewald and Botha 2008).

Transgenic sugarcane research does not only aim to increase sugar content throughout the stalk. Overexpression or underexpression of polyphenol oxidase (PPO; EC 1.14.18.1) results in a darker or lighter colour of sugarcane juice and raw sugar (Vickers *et al.* 2005a). Work done to increase sugar content in the stalk includes work done on sorbitol (Chong *et al.* 2007) and SPS (Vickers *et al.* 2005b, Grof *et al.* 2006) in which increased SPS activity was correlated with higher final sucrose content (Grof *et al.* 2006). Research performed in order to increase sucrose in plants has also included doubling the sugar content in the stalk tissue modified to produce a sucrose isomer (Wu and Birch 2007). These plants had a remarkable increase in total stored sugar levels and a decrease in mature stalk water content. This resulted in an increase of up to double the amount of sucrose in harvested juice and a net increase of 15-115% in the total sugar concentration in the harvested juice (Wu and Birch 2007). The table below (Table 1)

highlights some areas of sugarcane manipulation undertaken by SASRI and the Institute of Plant Biotechnology (IPB), Stellenbosch University, over the last 2-3 years.

Table 1. Examples of genetic engineering research in sugarcane performed at the IPB.
Carbon flux is directed into important end products or to elucidate the sucrose accumulation pathway.

Enzyme	Manipulation
Neutral Invertase (Rossouw 2006)	Down-regulation
PFP (Groenewald 2006)	Down-regulation
UDP-Glucose Dehydrogenase (Bekker 2007)	Down-regulation
β-amylase (Ferreira 2008)	Up-regulation
ADP-glucose pyrophosphorylase (Ferreira 2008)	Down-regulation

A substantial amount of transgenic work performed in sugarcane has been to investigate the role of PFP in sucrose metabolism. Constitutive expression of an untranslatable form of the sugarcane PFP-β gene (GenBank AA525655) using anti-sense or co-suppression technologies (reviewed by Iyer *et al.* 2000), resulted in the down-regulation of PFP in varying levels i.e. a reduction of up to 40% and 80% in leaf roll and immature tissue respectively (Groenewald and Botha 2001). In the transgenic plants the extent of reduction of PFP was dependant on the developmental stage or maturity of the tissue (Groenewald and Botha 2008). Minimal levels of activity were detected in mature tissue and there was a concurrent decrease in PFP-β protein content in these tissues (Groenewald and Botha 2008). A significant increase in sucrose accumulation in the transgenic sugarcane stalks was also detected (Groenewald 2006). This is similar to results seen in transgenic potato (Hajirezaei *et al.* 1994) and tobacco (Paul *et al.* 1995) where co-suppression and antisense technologies were used to induce gene silencing.

4.4. Transgenic manipulation of PFP activity in plants

Evidence for the physiological role of PFP provided by transgenic plants exhibiting as much as a 99% down-regulation of PFP has been contradictory. Analysis of PFP in transgenic plants exhibiting down-regulated levels of the enzyme indicates that it is acting in a net glycolytic reaction, thereby consuming pyrophosphate (Hajirezaei *et al.* 1994). PFP would therefore be unable to support sucrose degradation (Fernie *et al.* 2002). Data from both transgenic potato and tobacco studies together provide evidence that it is unlikely that PFP supplies PPi for sucrose degradation (reviewed by Fernie *et al.* 2002). Work done on transgenic potato plants led scientists to believe that PFP catalyses a net glycolytic flux in general (Plaxton 1996), although it

does not control the rate of this flux (Hajirezaei *et al.* 1994, 2003), nor is it essential for Pi control (Theodorou *et al.* 1992). Under conditions of altered Pi levels, there was no significant change in the physiological response of enzyme activities suggesting that PFP may play a less important role in Pi stress than previously thought (Theodorou *et al.* 1992). Potato tubers with a 70-90% decrease in PFP activity contained 20-50% less starch and a parallel reduction in starch and sucrose was seen in these plants (Hajirezaei *et al.* 1994). PFP may therefore provide excess capacity and flexibility for the plant to be able to adapt to non-optimal conditions or changes in the environment (Hajirezaei *et al.* 1994).

Transgenic tobacco plants with a decrease in PFP expression of up to 80%, using antisense or co-suppression technology, demonstrated that during photosynthetic sucrose synthesis PFP does not make an essential contribution to carbon flow and PPi turnover during non-stressed conditions (Paul *et al.* 1995). This suggests the reaction catalysed by PFP is in the glycolytic direction (Paul *et al.* 1995). There was a negligible difference between the hexose and triose phosphates between wild type and transgenic PFP plants, suggesting that PFP does not really play an important role during growth (Paul *et al.* 1995). PFP could therefore provide a bypass for glycolysis under stressful conditions (Paul *et al.* 1995).

The fact that both transgenic tobacco and potato studies with decreased PFP levels (Hajirezaei *et al.* 1994, Paul *et al.*1995, Nielson and Stitt 2001) failed to produce a plant with a dramatic change in phenotype suggests that PFP either does not play an essential role in metabolism, or the plant has other reactions which compensate for the decrease in PFP (Hajirezaei *et al.* 1994, Paul *et al.*1995, Nielson and Stitt 2001). In the work done on both transgenic potato (Hajirezaei *et al.* 1994) and tobacco (Paul *et al.* 1995) a reduction in PFP activity led to an increase in the hexose-phosphate pools in sink tissues which could result in a decrease in the rate of sucrose degradation (Hajirezaei et al 1994, Paul *et al.* 1995). PFP has been up-regulated in developing transgenic tobacco seed tissues where alterations in the onset and extent of storage lipid deposition were evident (Wood *et al.* 2002).

The study by Groenewald and Botha (2008) on transgenic sugarcane where down-regulation of PFP activity by up to 70% in transgenic sugarcane resulted in an increase in sucrose content in immature internodes and a significantly higher fibre content (Groenewald and Botha 2007). This implicated PFP in a role in glycolytic carbon flow, as the down-regulation of PFP suggests that the enzyme has an effect on the ability of young biosynthetically active tissue to accumulate

sucrose (Groenewald and Botha 2007). An increase in the hexose-phosphate pool could also lead to an increase in the cell wall precursors, via UDP-Gl, hence resulting in the increase in fibre content seen in these plants (Groenewald and Botha 2008). In mature internodes, there is a decrease in the demand for respiratory flux (Bindon and Botha 2002). The remaining PFP and PFK activity is sufficient to allow the system to revert back to its state of equilibrium, hence the sugar levels seen in these transgenic plants is similar to the natural plant (Groenewald and Botha 2008). This supports the suggested role of PFP by Dennis and Greyson (1987), that PFP is a bypass to PFK at times of high metabolic flux in biosynthetically active tissues. PFP may therefore have an effect on carbon partitioning in sugarcane (Groenewald and Botha 2008).

As it was established that an inverse relationship between PFP and sucrose content in internodal tissue exists (Whittaker and Botha 1999), down-regulation of PFP in sugarcane should ultimately lead to an increase in stalk sucrose concentrations (Groenewald and Botha 2001, 2008). Importantly, these plants showed no visible phenotypic or significant differences in growth and development (Groenewald and Botha 2008) suggesting that PFP has a direct influence on the ability of young biosynthetically active sugarcane stalk tissue to accumulate sucrose. The down-regulation of PFP activity influences the metabolism of sucrose in transgenic plants as a significant increase in sucrose concentration was present in immature tissue, increasing juice purity in the sugarcane tissues and representing an early ripening phenotype (Groenewald and Botha 2008) which was seen in 50% of the transgenic lines used in the study. The inverse relationship between PFP activity and sucrose concentration observed supported the role of PFP in sucrose accumulation (Groenewald and Botha 2008).

MATERIALS AND METHODS

1. Chemicals and reagents

All auxiliary enzymes, cofactors and substrates used for enzyme assays and metabolite determinations were of molecular biology grade and were obtained from Sigma-Aldrich Fine Chemicals (St Louis, Missouri, USA), Roche Diagnostics (Manheim, Germany) or Fermentas (Inqaba Biotechnical Industries, South Africa). All other solvents and chemicals were of analytical grade. Kits used in this study were from QIAGEN (Southern Cross Biotechnology, South Africa), Promega (Madison, Wisconsin, USA), AEC Amersham Biosciences (Johannesburg, South Africa), BioRad Laboratories (Hercules, California, USA), Applied Biosystems (Foster, California, USA), or KAPA Biosytems (Boston, Massachusetts, USA). Radioactively labeled [α-^{32}P] was obtained from Izotop, Institute of Isotopes Co., Ltd. (Budapest, Hungary). Primers were generated by Inqaba Biotechnical Industries (South Africa). Antiserum from potato PFP-β was obtained from Dr H Groenewald (Institute of Plant Biotechnology, Stellenbosch University) and has previously been described (Kruger and Dennis 1987).

2. Plant material and sample preparation

2.1. Production of transgenic material used in this study

Embryogenic callus of sugarcane (*Saccharum* spp. hybrid) varieties 88H0019, N27 and NCo310 (SASRI, Mt Edgecombe, Kwazulu-Natal, South Africa) were co-transformed by microprojectile bombardment using the following two plasmid constructs: pEmuKN, which confers resistance to the selection agent geneticin due to the presence of the *npt II* gene and is under control of the Emu monocot specific promoter; and pUSPc510, which contains the untranslatable sense sugarcane PFP-β gene under the maize Ubiquitin promoter (Figure 4) (Groenewald and Botha 2001). Selection and regeneration methods are as described by Snyman (2004).

2.2. Plant tissues and sample preparation

Transgenic and non-transgenic mature (12 month old) non-flowering, pot-grown (SASRI, Mt Edgecombe, Kwazulu-Natal, South Africa) sugarcane (*Saccharum* spp. hybrid) varieties 88H0019, N27 and NCo310 were selected and harvested. A tissue culture control available for

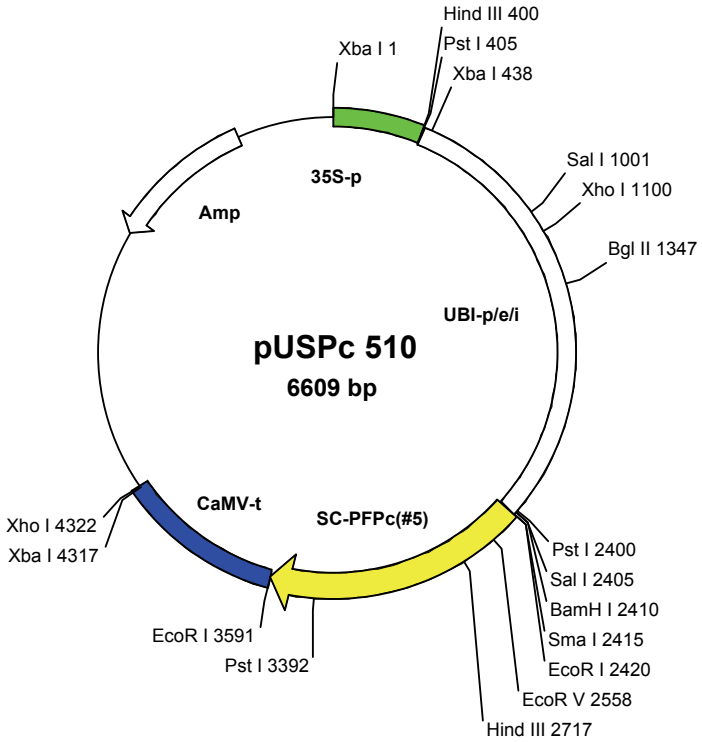

Figure 4. Plasmid map of construct pUSPc510 containing the untranslatable sense PFP-β transgene. GenBank AA525655; 875bp [53%] at the 3'-end of the coding sequence (Groenewald and Botha 2001).

N27 only, where the stalk had gone through the tissue culture process but had not been transformed, was also included. Stalk samples were harvested in triplicate from 3 pots, which were arranged in a randomized design. All mature stalks per pot were harvested and bundled for mill room analysis. The bundles from each of three pots of the same sugarcane line represented biological replicates.

In the laboratory, immature, maturing and mature internode groups were identified and excised from three single young stalks for fine analysis. The leaf with the uppermost visible dewlap and the internode it was attached to was defined as internodes 1-3, according to the system of Kuijper (van Dillewijn 1952) and represented immature tissue. Maturing and mature tissue was defined as internodes 4-5 and 6-7, respectively. The tissue was ground to a fine powder in liquid nitrogen and stored at -80°C till further use.

3. Molecular characterization of transgenic pot-grown sugarcane lines

3.1. RNA extraction

RNA was extracted from frozen cells according to a modified method of Bugos *et al*. (1995). Two grams of frozen tissue preparation was added to 10ml homogenization buffer, containing 10ml TENS Buffer (0.1M Tris-HCL (pH 7.5), 1mM EDTA, 0.1M NaCl, 0.1% SDS) and 70µl β-mercaptoethanol. Samples were vortexed and 700µl 3M sodium acetate (pH 5.2) was added, followed by an incubation period of 15 minutes on ice. Extracts were centrifuged at 12 000*g* for 15 minutes and the aqueous phase removed. An equal volume of isopropanol was added and the samples were incubated at -70°C for 30 minutes. Precipitated RNA was recovered by centrifugation at 10 000*g* for 10 minutes (4°C) and the pellets washed with 70% ethanol. The samples were centrifuged again at 10 000*g* for 5 minutes and the RNA pellet resuspended in 750µl diethyl pyrocarbonate (DEPC) treated water. Insoluble material was recovered by centrifugation at 10 000*g* for 5 minutes and the RNA precipitated with the addition of 167µl of 12M LiCl and 83µl DEPC-treated water. Samples were incubated overnight at 4°C. The precipitated RNA was recovered by centrifugation at 12 000*g* for 15 minutes. The pellets were rinsed in 70% ethanol, centrifuged at 10 000*g* for 5 minutes and dried briefly. Remaining insoluble material was removed by a final centrifugation at 10 000*g* for 5 minutes and resuspension of the pellet occurred in 20-50µl DEPC-treated water.

3.2. RNA quantification

Total RNA concentration was determined spectrophotometrically at an absorbance of 260nm using a Synergy HT_x microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA). The purity of RNA was determined using a ratio of the absorbance readings obtained at 280:260nm.

3.3. Northern blot analysis

Total RNA (20µg per sample) was loaded onto a 1.2% formaldehyde gel. After resolution, the gel was rinsed in DEPC-treated water for 15 minutes with agitation and then pre-equilibrated in 20x SSC-buffer for 15 minutes. This was repeated. The RNA was transferred to a positively charged nylon membrane (Amersham Hybond^{TM} –N^+, Amersham Biosciences) overnight, using a downward capillary blot, with 20x SSC-buffer at room temperature. After transfer, the RNA was UV cross-linked to the membrane for 1 minute at 70 000 µJ/cm^2 on a Hoefer uvc-500 UV cross linker (Hoefer Scientific Instruments, San Francisco). A partial sequence of the sugarcane PFP-

β gene (GenBank AA525655; 875bp [53%] at the 3'-end of the coding sequence) was isolated using a set of degenerate primers, based on the consensus sequence of the castor bean and potato PFP-β gene sequences available in the GenBank database system (Z32850 and M55191) (Groenewald 2006). The sugarcane PFP-β transgene was isolated from the pUSPc510 plasmid using restriction digest by *Eco*R1 (Fermentas) with overnight incubation at 37ºC. The restriction digestion was then resolved on a 1% agarose gel at 75V for 1 hour. The respective DNA fragment was gel-purified using the QIAquick gel extraction kit (QIAGEN). The PFP-β gene was then radioactively labelled using a Megaprime DNA labelling system (Amersham Biosciences) and 25µCi [α-^{32}P] dCTP (Izotop). Membranes were hybridized in 5x SSC-buffer, 5x Denhardt's solution, 0.5% (w/v) SDS and 200µg/ml denatured herring sperm DNA and incubated at 65°C for 4 hours. The PFP-β probe was boiled for 5 minutes before adding to the buffer and was incubated with the membrane at 65°C overnight. The membrane was washed twice in wash buffer 1 (1x SCC, 0.1% SDS) for 10 minutes at 65°C and twice in wash buffer 2 (0.1x SSC, 0.1% SDS) for 10 minutes at 65°C. The membrane was exposed to a supersensitive Cyclone storage Phosphor screen (Packard) for 24 hours. The results were visualized using the Cyclone™ Storage Phosphor System (Packard Instrument Co. Inc., Meriden, USA).

3.4. Q-PCR

Q-PCR reactions were carried out on total RNA (Section 3.1). RNA (4µg) was treated with a DNAse I, RNAse-free kit (Fermentas) to remove any contaminating genomic DNA. The RNA was incubated with 2µl 10x reaction buffer with $MgCl_2$, 12µl DEPC water and 5µl DNAse I and RNAse-free (1U/µl) at 37°C for 30 minutes. The reaction was stopped with the addition of 1µl 25mM EDTA and incubated for 10 minutes at 65°C. The pre-treated RNA was used to synthesise cDNA suitable for PCR amplification using a First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Primers for the target gene (sugarcane PFP) forward: PFP-1F 5' GCATGGTGGAAACTGAACTG 3' and reverse: PFP-1RB 5' CCTGTCTTCCCACTTTGGAG 3', as well as for the reference gene, 18s sugarcane ribosomal subunit forward: BP18s 5' AAATTTCATCGTGATGGGGA 3' and reverse: BP18s 5' GTACAAAGGGCAGGGACGTA 3' were generated (Inqaba Biotechnical Industries (Pty) Ltd) and optimized to an annealing temperature of 54°C. Conditions for all PCR reactions were optimized in a MyCycler™ thermal gradient cycler (BioRad) with regard to KAPATaq DNA polymerase (KAPA Biosystems), forward and reverse primers, $MgCl_2$ concentrations (KAPA Biosystems), dNTP concentrations (Roche diagnostics) and various annealing temperatures (49.6-65.6°C).

The cDNA was then analyzed using a SYBR® Green Jumpstart™ Taq readyMix™ and a Q-PCR Real-time system (Bio-Rad) machine in a 25µl reaction. 12.5µl SYBR Green JumpStart Taq readyMix, 0.8µl 6mM forward primer and 0.8µl 6mM reverse primer, 9.5µl water and a 1/100 dilution of the template cDNA. The reaction parameters were as follows; 98°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds with a final extension of 72°C for 1 minute. The melt curve of the amplified product was determined with a 1°C increment from 65°C to 95°C. A melt temperature of 81°C indicated that the PFP product had been obtained.

Q-PCR amplifications were examined by agarose gel electrophoresis. After ethidium bromide staining, bands were visible only at the expected molecular weights for the PFP cDNA and internal reference products were present (Figure 5). This fragment was excised and gel extracted using the QIAquick Gel Extraction Kit protocol (QIAgen) according to the manufacturer's extractions. In order to determine if the product had been amplified, the DNA was sequenced using the ABI Prism 310 Genetic Analyzer (Applied Biosystems) at the SASRI sequencing unit. The DNA Cycle sequencing reactions were prepared using the BigDye™ Terminator v3.1 Cycle Sequencing Ready reaction Kit (Applied Biosystems) according to the manufacturer's instructions. Removal of excess dye terminators was performed using ethanol precipitation and the pellet resuspended in 20µl Hi-Di formamide. Once the PFP fragment had been verified using a BLAST analysis, the fold change (FC) between the transgenic and control sample was determined and quantified using the ΔΔCT method (Livak and Schmittgen 2001). If ΔΔCt is negative, there is a fold change increase and the equation used was: 2^-[average ΔΔCt]. If ΔΔCt is positive, there is a fold change decrease and the equation used was: -(2^[average ΔΔCt]).

3.5. DNA extraction

DNA was extracted from frozen cells using a method modified from Dellaporta *et al*. (1983). Frozen tissue (1g) was added to 9ml extraction buffer (100mM Tris-HCl (pH 8.0), 500mM NaCl, 50mM EDTA (pH 8)) and 90µl β-Mercaptoethanol, after which 0.9ml 20% SDS was added and the samples were incubated at 70°C for 1-1.5 hours. To this, a volume of 1.8ml 5M potassium acetate was added followed by an incubation on ice for 20 minutes and centrifugation at 8 000 rpm for 15 minutes. The supernatant was then added to 2.5ml isopropanol. Precipitated DNA was resuspended in 600µl TE buffer (1M Tris-HCl (pH 7.4), 0.5M EDTA) and incubated at 37°C

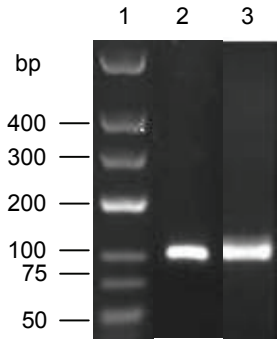

Figure 5. Gel photo of ethidium bromide stained reaction products obtained using Q-PCR.
Lane (1) 2 µl O'GeneRuler^{TM} DNA Ladder, Low Range ready-to-use molecular marker (Fermentas), (2) 81 bp PFP reaction product, (3) 109 bp 18s ribosomal subunit reaction product.

overnight. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the DNA was centrifuged at 5 000 rpm for 10 minutes. The aqueous phase was removed and stored at –20°C.

3.6. DNA quantification

Total DNA concentration was determined spectrophotometrically at an absorbance of 260nm using a Synergy HT_x microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA). The purity of DNA was determined using a ratio of the absorbance readings obtained at 260:280nm.

3.7. Southern blot analysis

Genomic DNA (10µg) was digested with *Bam*H1 (Fermentas) at 37°C overnight. Digested DNA was precipitated with 5M NaCl and absolute ethanol at -80°C for one hour. DNA was recovered with centrifugation at 12 000 rpm for 30 minutes and the pellet resuspended in 20µl TE buffer overnight at 37°C. The restricted DNA was resolved on a 0.8% agarose gel at 35V overnight. The gel was rinsed briefly in distilled water and incubated in depurination solution (125mM HCl) for 10 minutes with gentle agitation, after which it was rinsed in distilled water again and incubated in denaturation buffer (0.5M NaOH, 1.5M NaCl) for 30 minutes with gentle agitation. DNA was transferred to a positively charged nylon membrane (Amersham Hybond^{TM} –N^+, Amersham Biosciences) overnight using a downward capillary blot and a transfer buffer the same as the denaturation buffer (at room temperature). The membrane was UV cross-linked and hybridization was carried out as described for RNA (Section 3.3.). The *npt II* gene was PCR amplified from the pEmuKN plasmid, using the following primer pair: 5' AGA CGC TAT TCG

GCT ATG AC 3' and 5' CCA TGA TAT TCG GCA AGC AG 3' according to Snyman (2001). The *npt II* gene was radioactively labeled and incubated overnight with the membrane as described in Section 3.3. Visualization was determined in the same manner as Section 3.3.

3.8. Dot blot analysis

Genomic DNA (2.5µg) was diluted in 100µl distilled water (20µl of this was added to 5µl loading dye and separated on a 1% agarose gel). To the remaining 80µl of diluted DNA, 120µl 0.4M NaOH was added and the samples heated to 95ºC for 2 minutes. The samples were cooled on ice and vortexed before slot blot analysis. DNA was transferred to a positively charged nylon membrane (Amersham Hybond^{TM} –N^+, Amersham Biosciences) using the Bio-Dot$^{®}$ SF Microfiltration Apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions. The membrane was rinsed in 5 x SSC for 5 minutes, allowed to dry and then UV cross-linked as before (Section 3.3.). Hybridization was carried out as described for RNA (Section 3.3.). The slot blot was probed with the *npt II* gene according to Section 3.7.

3.9. Protein extraction and quantification

Crude protein was extracted from 30mg of frozen cells per sample, on ice, in 2.5 volumes of fresh protein extraction buffer (50mM HEPES/KOH (pH 7.5), 10mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 10% glycerol, 0.1% Triton X-100, 5mM DTT, 1x Complete^{TM} protease inhibitor cocktail (Roche) and 3% (m/v) PVPP) whilst mixing vigorously. Cell debris was collected by centrifugation for 15 minutes at 10 000*g* (4°C). Clear supernatant was used as the crude protein extract in enzymatic assays. Protein concentration was determined according to the method of Bradford (1976) using BSA as a standard in the Bio-Rad microassay (Bio-Rad Laboratories).

3.10. SDS-PAGE and western blot analysis

Protein extract (20µg) and 1µg of purified protein were resolved on an 8-16% Tris-HCl Criterion^{TM} pre-cast SDS-polyacrylamide gel (Bio-Rad Laboratories) according to the manufacturer's instructions. The separated polypeptides were transblotted onto Immun-Blot^{TM} PDVF membrane (Bio-Rad) using the Criterion^{TM} Blotter system (Bio-Rad Laboratories). The membrane was blocked overnight in Tris buffered saline with Tween-20 (TBST, 20mM Tris (pH 7.6), 137mM NaCl, 0.1% Tween-20) containing 3% Blotto (Whitehead Scientific) at 4°C and incubated in a 1:200 dilution of the specific antiserum (potato PFP-β, (Kruger and Dennis 1987)). The blot was washed in TBST and incubated for 45 minutes in a 1:1000 dilution of goat anti-rabbit IgG-alkaline phosphatase conjugate (Roche). Detection was induced with NBT/BCIP

Ready-to-Use Tablets (Roche) by adding 1 tablet to 10ml dH_2O, until bands were visualized. The addition of TE buffer was used to stop the reaction.

4. Enzymatic analysis of transgenic sugarcane lines

*4.1. Enzymatic determination of PFP activity**

PFP activity was assayed in the forward (glycolytic) direction as described by Kruger and Dennis (1987) by following the oxidation of NADH at 340 nm, using a Synergy HT_x microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA). Reactions were carried out in a final volume of 250µl at 30°C. The standard reaction contained 50mM HEPES/KOH (pH 7.5), 2mM $MgCl_2$, 0.05% Triton X-100, 0.2mM NADH, 5mM Fr-6-P, 10µM Fr-2,6-P_2, 1 IU aldolase, 1 IU glycerol-3-phosphate dehydrogenase, 10 IU triose-phosphate isomerase (TPI) and 5-20µl protein extract (depending on the tissue type). PFP activity was initiated by the addition of 1mM PPi (Kruger *et al.* 1983).

*4.2. Sugar extraction and quantification by HPLC**

Soluble sugars were extracted in 10 volumes of 30% (v/v) HM buffer (100mM HEPES, 0.5M $MgCl_2$, pH 7.8) and 70% (v/v) ethanol and incubated overnight at 70°C. The sugar extracts were centrifuged at 12 000rpm for 20 minutes and the supernatant immediately used for HPLC. Glucose, fructose and sucrose concentrations were determined on a Waters Associates (Millford, MA) HPLC system equipped with a 1500 series pump and a SAT/IN™ module. Samples were diluted 100 times and 5µl were injected into the system using a Waters 717 plus Autosampler (Massachusetts, USA). The sugars were separated on a Carbopak™ PA1 (Dionex) analytical column. Peaks were detected using a Waters® 2465 Electrochemical Detector (Massachusetts, USA), with a Waters® 1525 Binary HPLC Pump (Massachusetts, USA).

*4.1 and 4.2 were performed on both pooled stalk and specific intermodal tissue sections.

5. Mill Room analysis

The pot-grown sugarcane lines were harvested after 12 months and 1.0-2.0 kg bundles from each pot were harvested and selected. Excess leaf material and the tops of the stalks were removed at the natural breaking point of sugarcane, which is routine in commercial harvesting. Immature and mature regions of the stalk were separated by dividing the stalk into two

approximate halves as the number of internodes per stalk varied greatly. Sample bundles were sent to the SASRI mill room where total dissolved matter (Pol % cane), sucrose content (ERC % cane (estimated recoverable crystal) and gram sucrose per stalk) and fiber (fiber % cane) analysis were performed using standard industry-scale procedures (Thompson 1991). Data collected also included the total yield of soluble sugars (Brix % FW and Brix % DW cane) and purity %.

6. Data collection and statistical analysis

Statistical analysis was performed on all data. Means were compared with a one-way analysis of variance (ANOVA) using a Holm-Sidak pairwise and Dunnets t statistic test. Log_{10} or square transformations were performed to normalize data where necessary. Means were considered statistically different at the 5% level of probability. Data is expressed as the mean of the standard deviation (± SD). Statistical analysis was performed using computer programme using GENSTAT Version 2.0 (data analysis software system), was used throughout for all statistical analysis. Statistical significance was defined as $P \leq 0.05$.

RESULTS

1. Genomic characterization of transgenic sugarcane lines

For the purposes of this study, the transgenic and wild type sugarcane lines analyzed were labeled transgenic (TG) and wild type (WT). A tissue culture control (TC) where the stalk had gone through the tissue culture process but had not been transformed was included. Transgenic lines of three different sugarcane cultivars, namely 88H0019, N27 and NCo310, generated in the SASRI Biotechnology laboratory were analyzed (the latter created by Groenewald and Botha 2001) (Table 2). The transformed and wild type lines were grown in pots under ambient conditions. This allowed for a larger number of replicates to be grown under conditions reflecting those found in the field to be studied in contrast to the limitations of glasshouse trials. In addition, high variability can be seen in plants grown under containment (Groenewald 2006). These transgenic and wild type sugarcane lines were analyzed using a suite of techniques to genotypically and phenotypically characterize the plants.

Table 2. Cultivars used in this study are listed. Lines are labeled TG for transgenic and WT for wild type. Positive transgenic controls, NCo310TG1 and NCo310TG2 were produced previously (Groenewald and Botha 2001).

Cultivar 88H0019	Cultivar N27	Cultivar NCo310
88HTG1	N27TG1	NCo310TG1 (Positive control)
88HTG2	N27TG2	NCo310TG2 (Positive control)
88HTG3	N27TG3	NCo310WT
88HTG4	N27TC (Tissue Culture control)	
88HTG5	N27WT	
88HTG6		
88H0019WT		

1.1. Analysis of DNA to confirm the presence of the co-transformed npt II *gene*

Screening of a large number of transgenic lines is time consuming and a rapid method is therefore required. To identify transgenic plants in this study, two methods were chosen: a dot blot analysis performed on unrestricted DNA and a Southern blot, which is the more conventional method of DNA analysis. The transgene probed was the co-transformed selectable marker *npt II* gene, as PFP is an endogenous gene, which makes the identification of the PFP-β transgene in genomic DNA difficult. As the co-transformation frequency in sugarcane is usually 90% (Bower *et al.* 1996), this screening step can identify transformed plants.

The dot blot analysis identified the presence of the *npt II* transgene in all putative transgenic lines (Figure 6A). No signal was present in the wild type controls 88H0019WT (dot 8), N27WT (dot 9) and NCo310WT (dot 10), nor in the tissue culture control N27TC (dot 5). High signal levels were seen in the positive controls NCo310TG1 and NCo310TG2 (dots 6 and 7) and in the transgenic lines 88HTG6 (dot 17), N27TG2 (dot 19) and N27TG3 (dot 20) (Figure 6A). The Southern blot analysis (Figure 6B) supported the results seen in the dot blot and the presence of the *npt II* gene was confirmed in all the transformed lines (lanes 2 to 10). Although detailed information on the *npt II* gene copy number and integration pattern in this study is not pertinent, the same lines that demonstrated strong binding with the probe in the dot blot analysis showed numerous bands in the Southern blot, suggesting multiple insertions of the transgene (Figure 6B). No fragments in the tissue culture, N27TC and wild type controls, 88H0019WT, N27WT and NCo310WT hybridized with the probe.

1.2. RNA analysis to confirm the presence and expression of the PFP-β transgene

Northern blot analysis served to confirm the presence and expression of the PFP-β transgene. PFP is an endogenous sugarcane gene, so the introduction of an untranslatable transgene is hypothesized to cause gene silencing (Groenewald and Botha 2001). The northern blot reflects both endogenous and transgenic transcripts, with a size difference between the endogenous (2.3 kb) and transgenic (1.2 kb) messenger PFP RNA being used in the interpretation of the results (Figure 7A). The gel photo of ethidium bromide stained RNA verifies (i) equal loading of the total RNA and (ii) presence of both the 28s and 18s ribosomal RNA bands (Figure 7B).

Figure 7A indicates expression levels of the 1.2 kb PFP-β transcript in all but two of the transgenic lines, namely 88HTG4 (lane 4) and N27TG1 (lane 8) where only endogenous PFP expression similar to the wild type controls was seen. Expression of the PFP-β transcript should result in lowered levels of the endogenous PFP expression. This expected effect of the transgene was seen only in lines 88HTG3 (lane 3) and the positive controls NCo310TG1 and NCo310TG2 (lanes 12 and 13). The remaining transgenic lines where expression of the 1.2 kb transgene was evident also showed high endogenous PFP levels when compared with the wild type. The N27TC line (lane 11) presented the same level of expression in endogenous PFP as seen in the wild type controls, N27WT and NCo310WT (lanes 15 and 16) (Figure 7A).

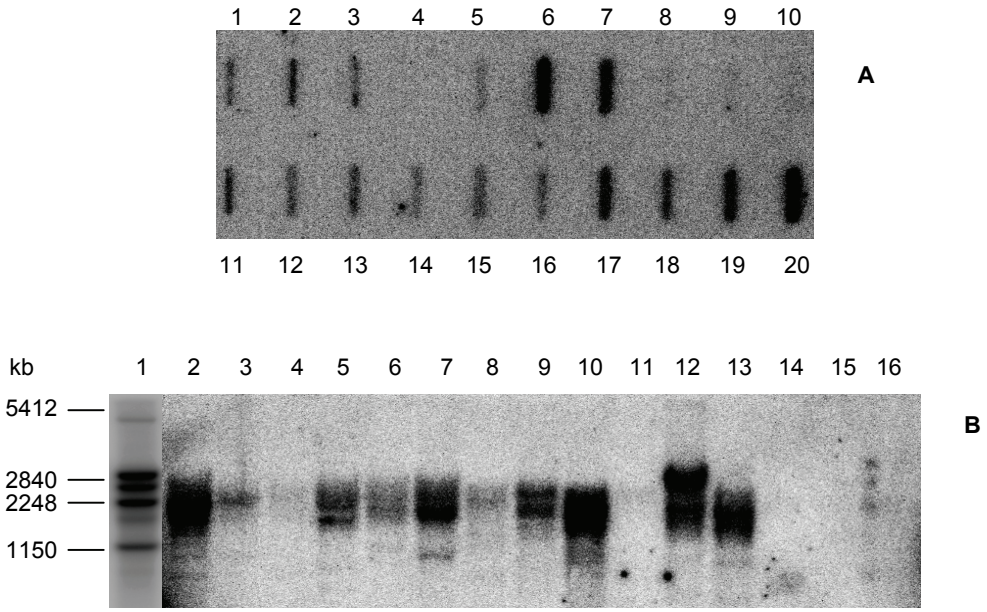

Figure 6. Genomic DNA characterization of transgenic and wild type lines of 88H, N27 and NC0310. The blots were probed with a 685bp PCR-generated *npt II* probe.

A. Dot blot analysis of presumptive transgenic sugarcane lines. Unrestricted DNA (2.5µg) from leaf roll tissue of selected lines was transferred by vacuum to a positively charged nylon membrane. Lanes (1), (2), (3), (11) plasmid pEmuKN (positive control), (4) distilled water, (5) N27TC, (6) NCo310TG1, (7) NCo310TG2, (8) 88H0019WT, (9) N27WT, (10) NCo310WT, (12) 88HTG1, (13) 88HTG2, (14) 88HTG3, (15) 88HTG4, (16) 88HTG5, (17) 88HTG6, (18) N27TG1, (19) N27TG2, (20) N27TG3.

B. Southern blot analysis of restricted DNA (10µg) from leaf roll tissue, was digested with *Bam*H1, electrophoresed and transferred to a positively charged nylon membrane. Lanes (1) λ P*st* molecular weight marker (2) 88HTG1, (3) 88HTG2, (4) 88HTG3, (5) 88HTG4, (6) 88HTG5, (7) 88HTG6, (8) N27TG1, (9) N27TG2, (10) N27TG3, (11) N27TC, (12) NCo310TG1, (13) NCo310TG2, (14) 88H0019WT, (15) N27WT, (16) NCo310WT.

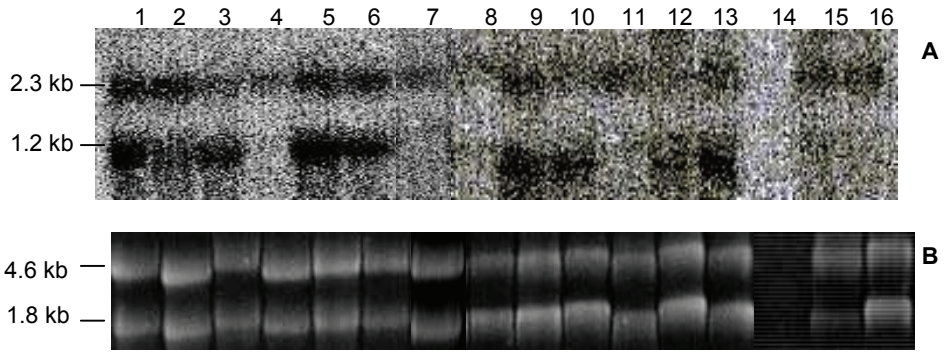

Figure 7. Northern blot analyses showing endogenous and transgenic PFP transcripts in the transformed and wild type 88H0019, N27 and NCo310 lines.

A. 20µg of total RNA from leaf roll tissue of selected lines was electrophoresed and transferred to a positively charged nylon membrane. The blot was probed with an *EcoR1* digested 1061 bp sugarcane PFP-β fragment from the pUSPc510 plasmid. The untranslatable transcript is 1.2 kb in size, whilst the endogenous transcript is 2.3 kb in size. Lanes (1) 88HTG1, (2) 88HTG2, (3) 88HTG3, (4) 88HTG4, (5) 88HTG5, (6) 88HTG6, (7) 88H0019WT, (8) N27TG1, (9) N27TG2, (10) N27TG3, (11) N27TC, (12) NCo310TG1, (13) NCo310TG2, (14) Blank (15) N27WT, (16) NCo310WT.

B. The gel photo confirms equal loading of total RNA between lanes and the bands represent the 28s (4.6 kb) and 18s (1.8 kb) ribosomal RNA bands, which have been stained with ethidium bromide.

2. Analysis of sucrose concentrations and PFP activity in pooled, pot-grown stalk samples of transgenic sugarcane lines

2.1. Use of conventional milling methods to characterize transgenic sugarcane lines

The performance of a new sugarcane cultivar produced by conventional breeding is usually evaluated by quantifying parameters such as biomass, sucrose content, purity % (which represents the ratio of sucrose to invert sugars), Brix (which indicates all dissolved solids) and fiber % cane on milled stalk material. The research mill at SASRI performs these measurements on crushed tissue regularly. This system would therefore be ideal for the evaluation of large samples of transgenic sugarcane. Mature (12 month old) stalks were sent to the research mill and divided into top (immature) and bottom (mature) halves. PFP activity is more prominent in immature tissue on a protein basis and therefore the potential effect of down-regulation of PFP on this tissue needs to be identified separately from mature tissue. A decrease in PFP activity could result in higher levels of sucrose in immature stalk sections where one would usually expect a low sucrose content (and high level of invert sugars) and an increase in the purity in immature tissue in transgenic lines would be expected.

An analysis of variance (ANOVA) was conducted on the results generated by the reasearch mill. The standard errors obtained for each mean (Table 3) indicated high variability between replicates making the comparison and interpretation of these results difficult. The analysis determined that there was no significant increase in sucrose, fiber or purity in any of the three cultivars analyzed in the immature tissue, as indicated by an F probability of greater than 0.05 (Table 3). Cultivar 88HTG3 presented a significant decrease in sucrose concentration, whilst cultivar 88HTG4 and 88HTG6 presented a significant decrease in fiber % in immature tissue compared with the wild type control. Analysis of mature tissue in a similar manner revealed that no significant increase in purity, sucrose content or fiber existed in any of the transgenic lines when compared with the wild type controls in this tissue (Appendix 1).

2.2. HPLC analysis for sucrose

A rapid and highly sensitive assay used in the determination of sugars is high performance liquid chromatography (HPLC). HPLC analysis allows for detection of minute changes in sucrose levels which mill room analysis may not detect, therefore increasing the accuracy of comparison between the transgenic and wild type samples. The variability observed from mill room measurements of pooled tissues was decreased by using this more sensitive detection method (Figure 8).

Soluble sugars from each of the pooled immature and mature tissue samples harvested for mill room analysis were extracted and quantified using HPLC in order to investigate the potential role of down-regulation of PFP activity in sucrose accumulation. In comparison with the wild types there was no significant increase in sucrose (g/g fresh weight) in either the immature or mature tissue in any of the transgenic lines studied when the results were subjected to ANOVA (Figure 8). Cultivars 88HTG4 and N27TG2 had significantly less sucrose in mature tissue when compared with their wild type controls 88H0019WT and N27WT, respectively. In all but three of the lines, namely 88HTG2, 88HTG4 and N27TG2, a higher concentration of sucrose was seen in mature tissue when compared with immature tissue. This trend of sucrose accumulation occurs in conventional sugarcane stalks and is seen in the wild type controls. Although the increase of sucrose in immature tissue in the 88HTG2, 88HTG4 and N27TG2 lines is not significantly different from the wild type stalks, they are indicative of the desired effect of PFP down-regulation in sucrose accumulation in immature tissue.

Table 3. Conventional mill analyses of pooled, crushed immature stalk tissue.

Results represent an average of 3-5 pooled stalk samples from each pot (n=3 ± SE). F probability (F pr) was calculated by ANOVA.

Line Designation	Weight (kg per bundle)	Sucrose * (g sucrose/g.FW)	Purity (%)	Fiber % cane
88HTG1	0.55 ± 0.21	15.67 ± 6.91	76.42 ± 3.85	11.43 ± 1 .01
88HTG2	0.86 ± 0.41	33.84 ± 17.69	82.45 ± 7.03	13.57 ± 0.55
88HTG3	0.35 ± 0.13	6.47 ± 1.13 [a]	76.20 ± 5.31	12.23 ± 0.72
88HTG4	0.58 ± 0.15	16.35 ± 5.68	77.00 ± 7.19	8.63 ± 0.95 [a]
88HTG5	0.31 ± 0.07	7.45 ± 0.83	76.64 ± 3.84	12.20 ± 0.49
88HTG6	0.40 ± 0.09	11.66 ± 5.17	75.06 ± 8.69	10.63 ± 0.50 [a]
88H0019WT	0.88 ± 0.16	17.84 ± 6.27	78.13 ± 6.58	11.20 ± 0.39
F pr	nd	0.88	0.17	0.06
N27TG1	0.63 ± 0.15	14.66 ± 0.78	86.14 ± 11.64	13.80 ± 0.22
N27TG2	0.92 ± 0.03	25.08 ± 4.16	89.54 ± 2.78	13.08 ± 0.57
N27TG3	0.77± 0.13	21.77 ± 4.16	88.34 ± 0.46	12.17 ± 0.91
N27TC	0.58 ± 0.16	16.58 ± 4.97	91.07 ± 7.11	11.63 ± 0.73
N27WT	0.98 ± 0.04	23.59 ± 1.04	86.38 ± 1.70	13.50 ± 0.36
F pr	nd	0.76	0.46	0.27
NCo310TG1	0.52 ± 0.07	11.93 ± 2.45	81.32 ± 1.09	10.21 ± 0.81
NCo310TG2	0.48 ± 0.11	11.22 ± 1.74	83.76 ± 2.47	12.49 ± 1.14
NCo310WT	0.67 ± 0.03	18.04 ± 2.35	77.72 ± 8.09	9.56 ± 0.48
F pr	nd	0.82	0.694	0.25

nd = not determined

Data underlined as a point of reference

[a] = significantly less than wild type

* = data was $\log_{10}$ transformed for statistical analysis, but non-transformed data is shown in the table

The invert sugars (glucose and fructose) for each tissue were also analyzed by HPLC and compared statistically using ANOVA (results not shown). Although there were no significant differences in the invert sugar levels obtained for any of the transgenic lines when compared to the wild type in both tissue types, the higher levels of glucose and fructose in immature tissue compared to mature tissue were in accordance with the expected trend of the invert sugar proportion seen in the wild type controls.

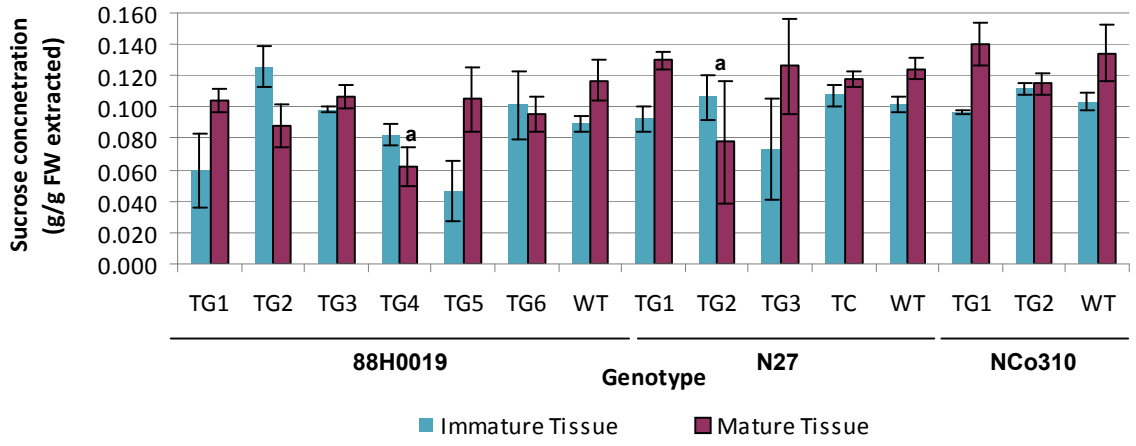

Figure 8. Sucrose concentrations obtained from HPLC analysis of 88H0019, N27 and NCo310 transformed and wild type pooled tissue samples of immature and mature tissue.

Values are expressed as a function of fresh weight and are representitive of the mean of 3 replicate samples. Standard error is indicated by lines and [a] = significantly less than wild type.

2.3. PFP specific activity enzyme determination

Although sucrose determinations revealed that there was no significant increase in sucrose concentration in any of the transgenic lines analyzed, it was still necessary to determine whether or not PFP activity had been down-regulated in these tissue samples. A highly sensitive enzymatic assay designed to detect the change in NADH coupled to PFP activity, is the standard method used when determining PFP levels. In order to assay for PFP activity, soluble protein content was extracted and quantified from each of the replicate immature and mature pooled mill room samples. The crude protein extracts were assayed for PFP activity and the results are reflected in Figure 9. An ANOVA was conducted using three replicate data values from each cultivar which confirmed that expression of the untanslatable sense PFP-β transgene did not result in a decrease in the levels of PFP activity in either the immature or mature tissue of the 88H0019, N27 and NCo310 transformed lines (Figure 9). The level of PFP activity was higher in immature tissue than mature tissue as was reported by Whittaker 1997, which conforms to the trend obtained in most of the transgenic and wild type lines.

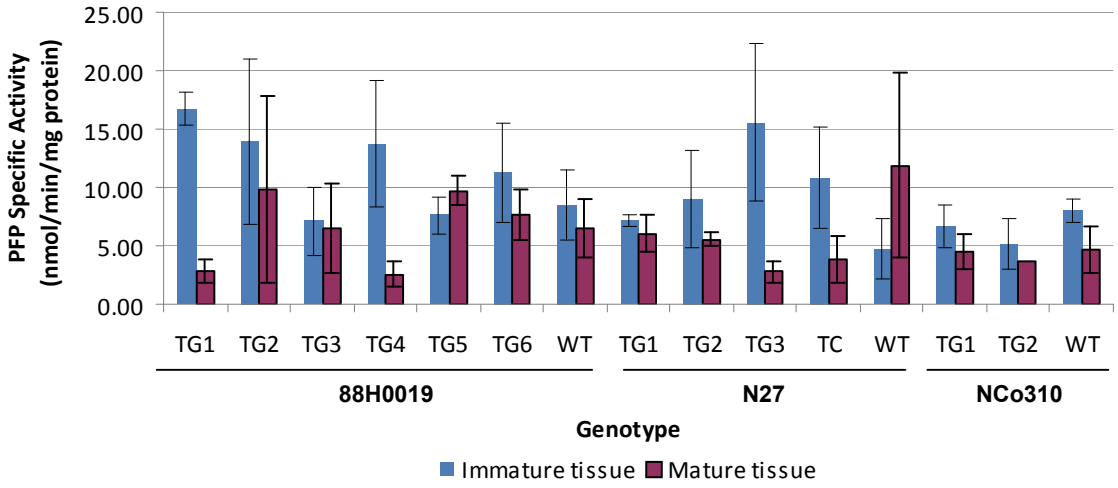

Figure 9. PFP specific activity in immature and mature pooled tissue sample of transgenic and wild type lines of 88H0019WT, N27WT and NCo310WT.

Crude protein extracts from each tissue type were assayed enzymatically to determine PFP activity. The values are representitive of the mean of 3 pooled sample tissues. Standard error is indicated by lines.

The standard error obtained for each of the 88H0019, N27 and NCo310 pooled tissue samples was high, making comparison and interpretation of this data difficult. The levels of extractable PFP activity in each of the transgenic lines was considerably lower than previously described in literature (Whittaker 1997). The most likely reason for this, is the way in which the tissues from which PFP was extracted were sampled, as an equal division of the stalks into two tissue halves did not represent the specific developmental stages present in sugarcane. The slight decrease in PFP in immature tissue of the positive controls NCo310TG1 and NCo310TG2 relative to the wild type, although not significant, was representative of the desired effects of expression of the PFP-β transgene.

3. The effect of more discrete and developmentally representative tissue on sucrose content and PFP activity determinations in selected transgenic lines.

The use of conventional sampling for analysis of stalks using mill room measurements was unsatisfactory as material had large variability between replicates. In addition, no clear evidence for the down-regulation of PFP activity could be obtained using larger sections of sugarcane stalks. It was decided that a more defined sampling approach to analyze enzyme down-

regulation effects in the transgenic stalks would be required. As PFP has been found to be at its highest activity level in young tissue (Whittaker 1997), internodal sections representative of different developmental stages in maturing stalks were harvested for analysis. Single stalks of each transgenic and wild type cultivar were selected for analysis and divided into three developmental sections as follows: internode sections 1-3 were pooled and defined as immature tissue, internodes 4-5, maturing tissue and internodes 6-7, mature tissue. As 6 month old stalks were used, the number of internodes available was less than in the mature, 12 month old stalks previously harvested.

Specific lines chosen for this fine analysis of internodal tissue were based on high expression levels of the PFP-β transgene as determined by northern blot analysis (Figure 7A, page 50): 88HTG2, 88HTG3, 88HTG5, 88HTG6, the positive controls NCo310TG1 and NCo310TG2 and corresponding wild type stalks were used.

3.1. Sucrose determination in internodal tissues using HPLC analysis

Immature, maturing and mature internodal tissue samples from single stalks were analyzed using HPLC. The results obtained correspond to those seen in the pooled mill room tissue analysis (Section 2.1, page 50). Although the variability between replicates was reduced by the more precise sampling method, statistical analysis (ANOVA) revealed that no significant increase in the sucrose content of immature tissue in the 88H0019 transgenic lines when compared to the wild type had occurred (Figure 10). The expected increase in sucrose accumulation from immature to mature tissue was seen in all samples except 88HTG6, where the highest amount of sucrose was obtained in the maturing tissue sample. A slight increase in sucrose in immature and maturing tissue was observed in the positive control line NCo310TG2 compared with the wild type. A decrease in sucrose in mature tissue in both NCo310TG1 and NCo310TG2 was seen when compared with the wild type NCo310WT. However, as only two replicate stalks for each of these controls were available for sampling, it was not possible to perform statistical analysis on these results.

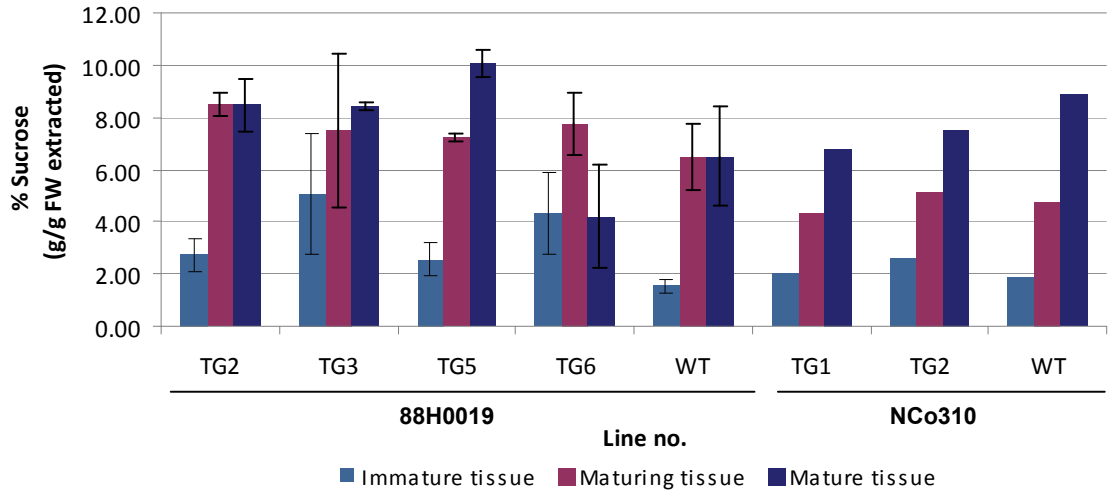

Figure 10. Sucrose concentrations, expressed as % of fresh weight measured in grams, obtained from HPLC analysis of selected 88H0019 and NCo310 transformed and wild type immature, maturing and mature internodal tissue samples.

Values expressed for the 88H0019TG and WT samples are representitive of the mean of 3 tissue samples. Standard error is indicated by lines. The positive control lines NCo310TG and WT represent an average of 2 tissue samples.

3.2. Internodal PFP specific activity enzyme determination

The more defined method of internodal tissue sampling resulted in higher extractable levels of PFP than in the pooled stalk sections (Section 2.3, page 53) and the expected activities obtained were similar to the activity values seen in literature (Whittaker 1997). The data revealed however, that there was no significant decrease in the levels of PFP expression in immature, maturing or mature tissue in the 88H0019 transgenic lines relative to the control (Figure 11). PFP results corroborate the sucrose data in the above section.

Positive control lines NCo310TG1 and NCo310TG2 showed a decrease in the level of PFP expression when compared to the wild type control NCo310 in all internodes (Figure 11). Due to the availability of only two replicate stalk samples for these positive lines no statistical analysis could be performed on these results (the individual values of PFP activity for each replicate are indicated in Appendix 2). The data suggested a down-regulation of PFP activity in the immature, maturing and mature tissue of NCo310TG1 and NCo310TG2 when compared with the wild type control.

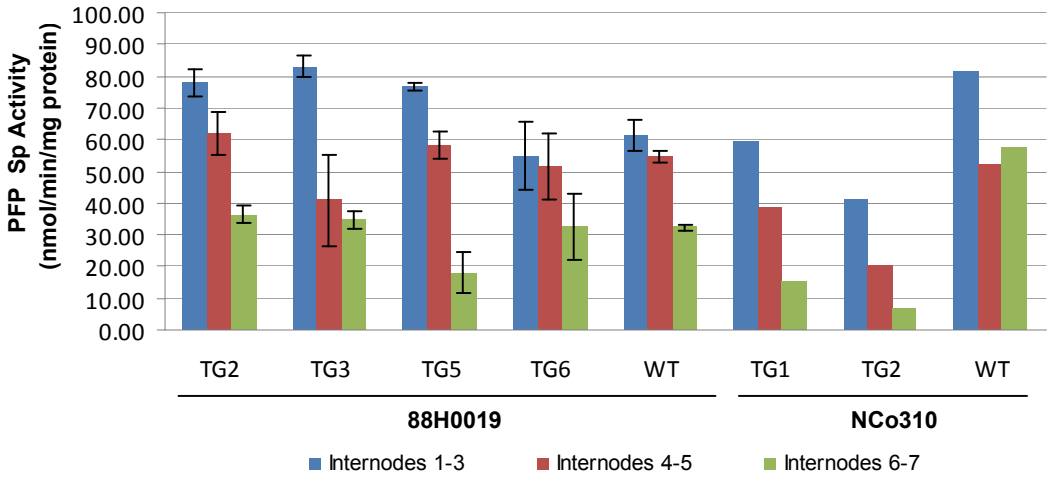

Figure 11. PFP specific activity was determined enzymatically in immature, maturing and mature internodal tissue samples of selected transgenic and wild type 88H0019 and NCo310 pooled internodal tissue samples.
Crude protein extracts from each tissue type were used to determine PFP activity. In the 88H0019 lines, the values are representitive of the mean of 3 internodal tissue samples (± SE). In the NCo310 lines, n=2.

3.3. Western blot analysis to determine levels of extractable PFP in young developmental tissue sections

The effect of the expression of the PFP-β transgene at this stage remained unclear. Therefore to verify the levels of extractable PFP protein, a western blot was performed. Total protein was extracted from each of three immature tissue replicates and equal amounts were pooled. These protein samples were resolved on an 8-16% SDS polyacrylamide gel (Figure 12A) and blotted onto a nylon membrane. The blot was incubated with an antibody specific for the PFP-β protein sub-unit (Figure 12B).

The detection of PFP on the western blot membrane (Figure 12B) indicated that there was no visual reduction in the β-subunit in the 88H0019 lines compared with the wild type control. However, when a densitometric analysis of the blot was done to quantify band intensity further, cultivar NCo310TG2 had a lower amount of the PFP-β subunit compared with the wild type (Figure 12C).

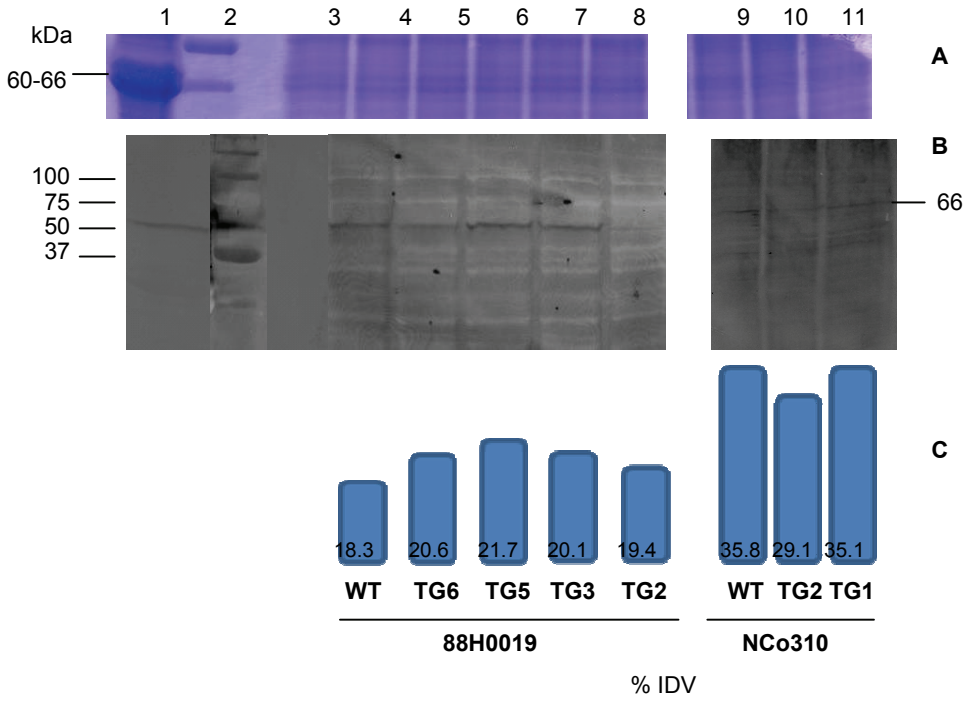

Figure 12. Western blot analysis of pooled extractabe PFP activity in immature internodal tissue of selected 88H0019 and NCo310 transgenic lines.

A. Crude protein from immature tissue was extracted. 20µg crude protein was loaded per lane, electrophoresed on an 8-16% SDS-PAGE gel. Lane (1) purified PFP protein standard (5-10µg), (2) Precision Plus Protein™ Standards Dual Colour molecular marker (Biorad), (3) blank (4) 88H0019WT, (5) 88HTG6, (6) 88HTG5, (7) 88HTG3, (8) 88HTG2 (9) NCo310WT, (10) NCo310TG2, (11) NCo310TG1.

B. Resolved protein bands were transferred to a nylon membrane and probed with a polyclonal rabbit anti-sugarcane PFP-β antibody.

C. Densitometric results of western blot analysis of immature tissue of 88H0019 and NCo310 transformed and wild type samples performed on the nylon membrane after blotting and development. Values plotted represent % integrated density values (% IDV).

3.4. Quantitative Real-Time PCR analysis of internodal tissues

Despite the expression of the PFP-β transgene seen in the northern blot (Figure 7A) and the possible down-regulation of total PFP content in NCo310TG2 indicated in the western blot (Figure 12B), no solid evidence for successful phenotypic change was found. Therefore to identify possible impacts of the insertion of the PFP-β transgene on total PFP expression levels, quantitative PCR (Q-PCR) was performed on the immature 88H0019 and NCo310 transgenic and wild type internodal tissue samples.

Analysis of immature tissue samples of 88H0019 and NCo310 transgenic and wild type lines by Q-PCR showed that there was a minor up-regulation of total PFP mRNA expression levels in the selected transgenic lines when compared to the total PFP mRNA expression levels in the wild type (Table 4). ΔΔCT values obtained for each sample were negative indicating a fold change increase, where the fold change denotes the ratio of average normalized expression level in the reference gene compared to the wild type. The fold change (FC) and ΔΔCT values obtained for the transgenic lines are presented in Table 4. A fold change increase of 0.01 seen in 88HTG5 would not have a significant up-regulation impact on total PFP expression levels whereas a fold change of 0.48 seen in 88HTG2 would imply a minor increase in PFP activity.

Table 4. Quantification of PFP-β expression by quantitative PCR (Q-PCR) using 18sRNA as the reference gene. The fold change (FC) between PFP as compared to the wild type is shown here. Negative ΔΔCT values indicate an increase in fold change i.e. up-regulation; where positive ΔΔCT values indicate a decrease in fold change i.e. down-regulation.

Line Designation	ΔΔCT	**Q-PCR FC**	**Line Designation**	ΔΔCT	**Q-PCR FC**
88HTG2	-1.071	0.48	**NCo310TG1**	-1.647	0.32
88HTG3	-4.047	0.06	**NCo310TG2**	-1.461	0.36
88HTG5	-6.483	0.01	**NCo310WT**	0	0
88HTG6	-2.909	0.13			
88H0019WT	0	0			

The slight up-regulation of total PFP expression in the transgenics compared with the wild type would explain the lack of down-regulation of PFP activity seen in the protein and enzymatic analysis of these lines when using either of the two stalk sampling methods.

DISCUSSION AND CONCLUSIONS

1. PFP as a target enzyme to perturb sucrose metabolism: myth or future possibility?

Preliminary determinations confirming the presence of the co-transformed *npt II* gene (Figure 6) and expression of the PFP-β transgene in the northern blot (Figure 7A) indicated that all transgenic lines, except two, contained the transgene. Despite these results, no further evidence for the down-regulation of PFP could be found.

The analysis of these transgenic lines using the conventional rapid and well developed sampling practices at SASRI for routine milling analysis was unsatisfactory for PFP activity determination. Pooled stalk sampling with immature top and mature bottom separated provided no clear evidence as to whether or not PFP had been down-regulated (as shown by specific activity seen in Figure 9 and 11) nor was it sufficiently possible to determine the effects of transgenesis on sucrose (Table 3, Figure 8 and 10) and fibre accumulation (Table 3). PFP activity in mixed developmental tissues where down-regulation was intended was diluted over the two stalk halves. Interpretation of results was further confounded as extractable PFP levels were much lower in the pooled immature and mature stalk tissue sections (5-18 nmol/min/mg protein and 2-12 nmol/min/mg protein, respectively) harvested in this study (Figure 9). The values previously reported for sugarcane, where discrete sampling of specific developmental stalk regions was undertaken, were found to be in the range of 30-60 nmol/min/mg in internodes 6 and 7 representing immature material (Whittaker 1997, Groenewald & Botha 2008).

The subsequent change in sampling approach using more defined internodal stalk tissues, namely immature (internodes 1-3), maturing (internodes 4-5) and mature (internodes 6-7) tissue allowed for the detection of a 3-5 fold increase in PFP activity in the immature tissues (Figure 11) and these matched the range of activity obtained by Whittaker in immature tissue (1997). However no evidence of a down-regulation in PFP in transgenic lines was observed when compared with the wild type controls. Although sucrose determinations by HPLC analysis (Figure 8) followed the conventional sucrose accumulation trend with higher concentrations of sucrose in mature stalk tissues, there were no differences between transgenic lines and their wild type counterparts. When a Western blot and Q-PCR analysis (Figure 12, Table 4) was undertaken, a minor up-regulation of PFP expression was observed in the transgenic lines when compared with the wild type using the latter technique. Unexpectedly, the formerly characterised

transgenic lines, NCo310TG1 and NCo310TG2 included in this study as positive controls did not respond as reported previously, no down-regulation of PFP activity or increase in sucrose content was observed (Groenewald and Botha 2008). Possible reasons for these results and those observed in presumptive transgenic genotypes 88H0019 and N27 may include the following:

- Expression of the PFP-β transgene seen in the northern blot (Figure 7A) without consequent down-regulation of the enzyme in any of the developmental tissues analyzed may be as a result of the position effect and copy number of the transgene, which could vary the level of expression within different transformed lines (Bajaj *et al.* 1999). This may not be applicable to the results seen for the positive transgenic control lines NCo310TG1 and NCo310TG2, compared with the wild type, as previous analysis of these lines (Groenewald 2006) showed a down-regulation of PFP activity. It has been shown that transgenic rice plants containing multiple copies of the transgene exhibited recurrent onset of silencing and instability of expression (Kumpatla and Hall 1998, Morino *et al*. 1999). It is desirable to select transgenic lines with single copies of the PFP-β transgene as a high-level of transcript expression has been achieved by selecting transformed plants that contain only one transgene copy, thereby minimizing the occurrence of gene silencing (Bajaj *et al.* 1999). However, as PFP is an endogenous enzyme, integration patterns of the PFP-β transgene are difficult to determine. The use of the copy numbers seen for the *npt II* transgene determined in the Southern blot (Figure 6B) may have indicated potential PFP-β transgene copy number due to the high co-transformation frequency observed in sugarcane (Bower *et al.* 1996).

- The lack of effects of PFP-β transgene expression observed in this study may have been due to gene silencing mechanisms not operating as predicted. Post- transcriptional gene silencing (PTGS), also referred to as co-suppression, is a specific silencing phenomenon occurring in plants and involves sequence- specific RNA degradation (Fire 1999). The production of RNAi is also responsible for gene silencing in plants (Hamilton and Baulcombe 1999, Matzke *et al.* 2001) and often occurs when transgene transcripts are abundant (reviewed by Taylor 1997). These two mechanisms of gene silencing result in the loss of expression of a transgene and therefore appear to be related (Dernburg *et al.* 2000, Fagard *et al.* 2000, Hammond *et al.* 2000, Ketting and Plasterk 2000, Sijen and Kooter 2000, Sharp 2001). As they are responses to various types of foreign nucleic

acids including transgenes (Dalmay *et al.* 2000), inoperation or inefficiency of either of these phenomena may be responsible for the lack of silencing predicted after transcription of the PFP-β mRNA.

- Field trial analysis of transgenic stalks in previous sugarcane studies was performed on an average of 191 and 50 lines per transformation respectively (Gilbert *et al.* 2005, Vickers *et al.* 2005a). The availability of only 6x 88H0019 and 3x N27 transgenic lines for analysis in this study may have been too few to evaluate a complex down-regulation process. In addition, the large variability obtained in the analyses of these pot grown plants, indicated by the high standard errors obtained for the PFP activity (Figure 9 and 11) and sucrose data (Table 3, Figure 8, Figure 10), indicates that perhaps too few stalk replicates were available to accurately determine effects of insertion of the PFP-β transgene into the plant genome on PFP activity levels and sucrose accumulation patterns.

- Manipulation of PFP activity was previously observed in glasshouse grown sugarcane stalks (including the positive control lines NCo310TG1 and NCo310TG2) where a decrease of up to 90% PFP activity in 50% of 9 transgenic lines analyzed led to an increase in sucrose and fibre in immature tissues (Groenewald and Botha 2007). Groenewald (2006) went on to demonstrate however, that when field grown stalks were harvested after approximately 15 months, no significant differences in sucrose levels were observed. After a second year of growth (first ratoon) and harvesting after 16 months, all transgenic lines, except 1, showed a significant decrease in sucrose accumulation and yield in both immature and mature tissue (Groenewald 2006). A similar phenomenon may have occurred in plants analyzed in this study where stalks were pot grown, as although northern blot analysis indicated expression of the PFP-β transcript (Figure 7A), no further evidence for transgenesis was found. Stalk growth in pots under ambient conditions was thought to provide suitable growth conditions for transgenic and wild type material. Sugarcane however becomes pot-bound rapidly which may be due to the nature of its growth pattern as there is the formation of multiple tillers, each with their own root system so there is competition for space, light and soil nutrients. This method of stalk growth is perhaps not an ideal system but regulatory restrictions hinder large scale field trials of transgenic material. This study therefore emphasises the need in future to

allow only 1-3 tillers to develop per pot (managed by physical removal of young tillers) in an attempt to limit both plant stress and variability between stalks.

- Although PFP-β mRNA transcript expression was evident (Figure 7A), no decrease in PFP activity was observed in any of the tissues studied. This may be due to be a plant-altered metabolic flux in the sucrolysis pathway as a means of compensation for PFP down-regulation, indicating that PFP may not be a crucial enzyme in sucrose metabolism. This 'tolerance mechanism' has been observed in transgenic potato where PFP activity had been decreased by up to 70-90%, but did not result in a change in the phenotype as was hypothesised i.e. there was no resulting increase in tuber starch content (Hajirezaei *et al.* 1994). The growing potato tubers contained less starch than the wild type (Hajirezaei *et al.* 1994). This decrease in starch accumulation was explained as an indirect effect resulting from an increased rate of sucrose cycling in these plants (Hajirezaei *et al.* 1994). The fact that both transgenic tobacco and potato studies with decreased PFP levels (Hajirezaei *et al.* 1994, Paul *et al.* 1995, Nielson and Stitt 2001) failed to produce a plant with a dramatic change in phenotype, suggests that PFP either does not play an essential role in metabolism or the plant has other metabolic reactions which compensated for the decrease in PFP (Hajirezaei *et al.* 1994, Paul *et al.*1995, Nielson and Stitt 2001). Similarly, transgenic sugarcane plants in which UDP-glucose dehydrogenase (UDP-Gl DH) activity was down-regulated, compensated for the lowered UDP-Gl DH activity levels by up-regulation of a secondary pathway, the *myo*-inositol oxygenation pathway (MIOP) which acts as an alternative pathway for the synthesis of cell wall matrix precursors (Bekker 2007), providing further evidence that plants perhaps have the ability to compensate for abnormal metabolic levels, which may have occurred in this study.

The use of an untranslatable sense PFP-β transgene to down-regulate PFP activity and ultimately increase sucrose accumulation in field grown stalk tissue in this study was unsuccessful. Although this study has highlighted some of the difficulties associated with characterization and determination of effects of transgenes affecting metabolic pathways in plants, this approach may provide a valuable research tool in further understanding the sucrose synthesis pathway and the related intricacies between metabolites and enzymes.

2. Overview of methods used to identify transgenesis and the potential framework for future analysis

A suite of rapid screening techniques is required for the identification and characterization of transgenic plants at SASRI where large numbers of transgenic material are created and need to be analyzed on a continuous basis. The range of analyses performed in order to characterise transgenic lines ultimately depends on the type of genetic manipulation induced in the plants. The analysis of plants that have been transformed with an endogenous plant gene is complex and a variety of techniques must be used to evaluate the effects of the transgene on the endogenous expression levels, whereas characterization of plants is less complicated when a simple input trait such as herbicide resistance has been used (Figure 13).

Genetic transformations resulting in up- or down-regulation of endogenous enzymes involved, for example in sucrose accumulation (Grof 2001, Baxter *et al.* 2003, Ferreira 2008), would require initial analysis of discrete developmental stalk tissue sections rather than pooled stalk sections. The more conventional northern blot analysis (Sambrook and Russell 2001) may then be used to identify plants containing the mRNA transcript. However, based on the range of methods evaluated in this study, Q-PCR would provide the most rapid and desirable method for the screening of transgenic plants. This technique allows for identification of the transgene in presumptive transgenic lines and/or quantifies expression of the manipulated gene within a few hours. The optimization of Q-PCR may be lengthy as housekeeping genes are required as a reference point for comparison with the gene of interest.

Once the gene of interest has been identified in the transformed plants, further molecular analyses may be necessary depending on the intended consequence of the genetic manipulation (Figure 13). Modifications involving up- or down-regulation of genes involved in sucrose accumulation (Grof 2001, Baxter *et al.* 2003, Ferreira 2008) would require protein (western blot), enzymatic and metabolic analyses.

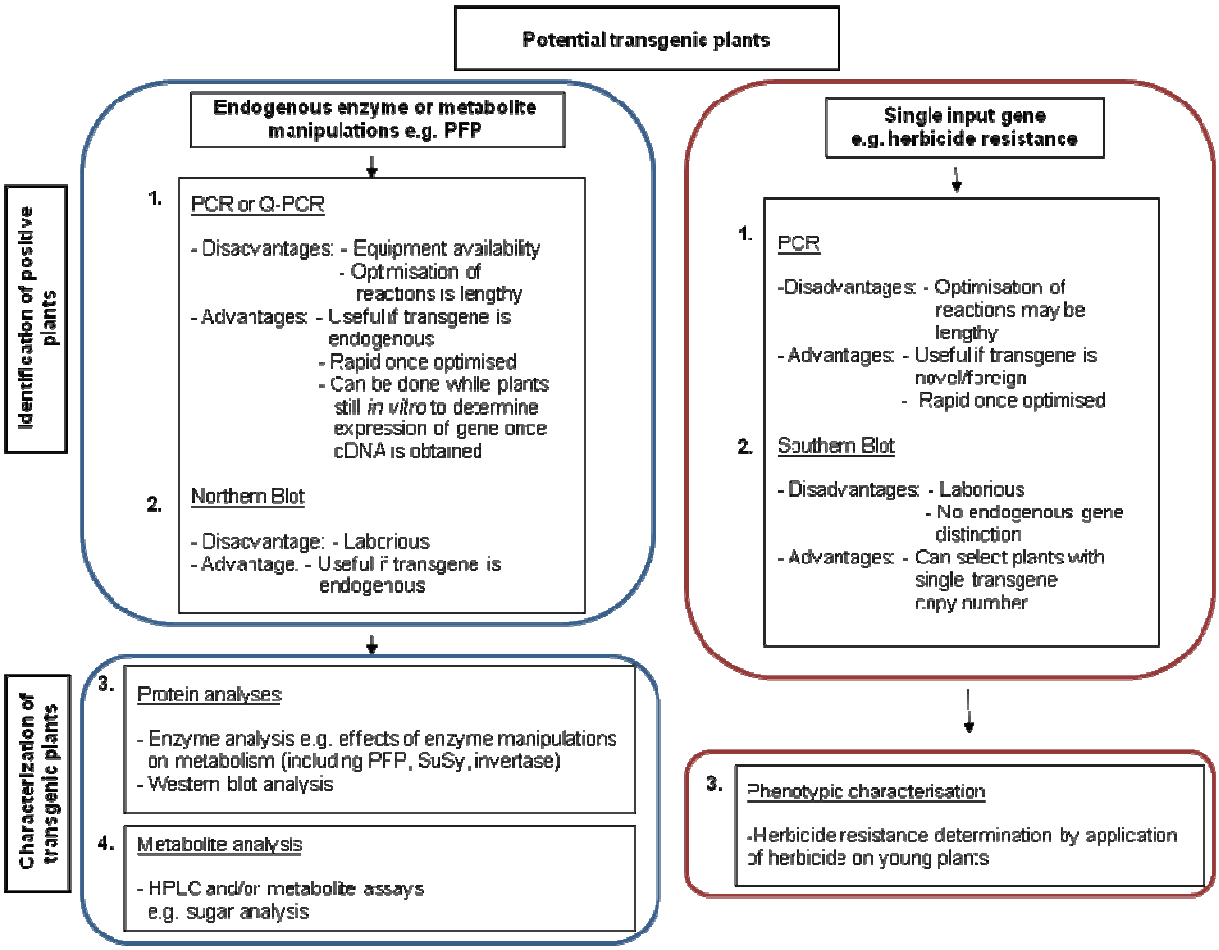

Figure 13. Flow diagram summarizing different methodology available to identify and characterize transgenic sugarcane plants.

The advantages and disadvantages for each technique are given, based on the observations made in this study.

The results obtained in this study, and possibly because of the stresses pot grown sugarcane may be exposed to, indicate that a simpler and more rapid technique using cell suspension cultures may be considered in the future. Sugarcane suspension cultures have previously been used as a model system to investigate cellular sucrose uptake and the regulation of sucrose storage (Maretzki and Thom 1972, Komor *et al.* 1981). More recently, suspension cultures have been used as a system for analyzing single enzymes such as invertase in transgenic sugarcane lines (Rossouw *et al*. 2007). Although there are limitations to this system e.g. atypical sucrose metabolism (Veith and Komor 1993) and 40% lower vacuolisation than in the stalk tissue (Komor 1994), suspension cultures do present several advantages over standard approaches. For example, large numbers of transgenic lines may be grown under constant conditions in smaller

areas, thereby eliminating the need for large amounts of space in glasshouses. Most importantly, suspension cultures may allow for analysis of transgenic lines after only a few weeks, as opposed to analysis of pot grown lines only once the stalk has reached maturity. This novel approach could provide a valuable growth system for initial screening of transgenic plants in the future. *In vitro* methods for plant growth may provide a larger number of replicate samples to be analyzed for desirable traits or manipulation products before glasshouse or field grown stalks are required for testing. This would limit the number of stalks to only those possessing the gene or trait of interest to go through to such trials. Whereas glasshouse grown stalks would provide confirmation of transgenesis on whole plants and field grown material would be required to determine gene stability and possible economic importance.

3. Concluding remarks

Down-regulation of PFP with the use of an untranslatable sense PFP-β transgene in field grown genotypes 88H0019 and N27 varieties was unsuccessful. No significant decrease in PFP activity was observed, nor increase in sucrose content in immature tissue in any of the transgenic lines analyzed when compared with the wild type controls. The analyses of these lines has however, led to improvements in evaluating sampling techniques and methodology for characterizing large numbers of transgenic plants developed at SASRI. SASRI and the IPB continue research into elucidation of aspects of sucrose metabolism by up- or down-regulating enzymes involved in sucrose metabolism.

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Appendices

Appendix 1

Conventional mill analysis of pooled, crushed mature stalk tissue. Results represent an average of 3-5 pooled stalk samples from each pot (n=3 ± SE). F probability (F pr) was calculated by ANOVA.

Line Designation	Weight (kg per bundle)	Sucrose* (g sucrose/g.FW)	Purity (%)	Fiber % cane
88HTG1	0.71 ± 0.31	28.76 ± 14.56	81.11 ± 4.92	14.2 ± 0.8
88HTG2	0.85 ± 0.31	31.61 ± 13.44	82.14 ± 3.36	13 ± 0.51
88HTG3	0.4 ± 0.10	11.92 ± 2.37 [a]	71.35 ± 3.83	12.3 ± 0.23
88HTG4	0.73 ± 0.19	25.34 ± 6.47	81.01 ± 2.50	12.9 ± 0.48
88HTG5	0.38 ± 0.12	13.34 ± 4.01	80.41 ± 1.80	12.5 ± 0.84
88HTG6	0.63 ± 0.15	23.33 ± 8.29	79.14 ± 5.62	9.9 ± 1.13
88H0019WT	1.1 ± 0.28	30.76 ± 10.97	91.06 ± 4.09	14.7 ± 0.77
F pr	nd	0.88	0.17	0.06
N27TG1	0.93 ± 0.22	44.12 ± 12.03	89.51 ± 4.72	13.60 ± 1.00
N27TG2	1.21 ± 0.13	44.23 ± 16.32	86.69 ± 5.28	14.5 ± 1.20
N27TG3	1.13 ± 0.20	29.42 ± 5.45	86.51 ± 1.82	13.96 ± 1.73
N27TC	0.8 ± 0.21	20.67 ± 7.79	85.56 ± 6.37	14.62 ± 0.52
N27WT	1.31 ± 0.04	37.45 ± 2.94	88.31 ± 2.36	14.99 ± 0.69
F pr	nd	0.76	0.46	0.27
NCo310TG1	0.52 ± 0.14	14.56 ± 4.33	88.09 ± 1.49	13.18 ± 0.59
NCo310TG2	0.55 ± 0.10	12.84 ± 2.56	78.82 ± 2.21	15.04 ± 0.58
NCo310WT	0.72 ± 0.08	20.02 ± 4.09	88.9 ± 5.20	14.88 ± 1.46
F pr	nd	0.82	0.694	0.25

nd = not determined

Data underlined as a point of reference

[a] = significantly less than wild type

* = data was $\log_{10}$ transformed for statistical analysis, but non-transformed data is shown in the table

Appendix 2

Individual specific PFP activity results of immature, maturing and mature tissue from two replicate samples analyzed of the positive controls NCo310TG1, NCo310TG2 and NCo310WT. Specific activity values are expressed in nmol/min/mg protein.

Line Designation	Internodes 1-3 (Immature)	Internodes 4-5 (Maturing)	Internodes 6-7 (Mature)
NCo310TG1	72.9	26.2	11.4
	46.3	51.4	19.6
NCo310TG2	43.8	20.29	3.7
	38.8	19.9	9.8
NCo310WT	80.3	47	47.6
	83.1	57.6	67.5