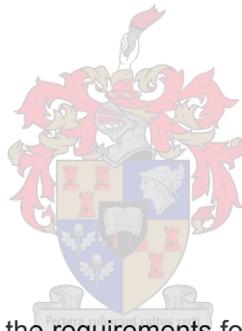


THE MANIPULATION OF FRUCTOSE 2,6-BISPHOSPHATE LEVELS IN SUGARCANE

Nicholas Fletcher Hiten



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

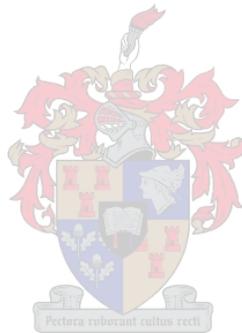
Promoter: Prof. FC Botha

APRIL 2006

DECLARATION

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

Signature: _____ Date: _____



ABSTRACT

Fructose 2,6-bisphosphate (Fru 2,6-P₂) is an important regulatory molecule in plant carbohydrate metabolism. There were three main objectives in this study. Firstly, to determine whether the recombinant rat 6-phosphofructo 2-kinase (6PF2K, EC 2.7.1.105) and fructose 2,6-bisphosphatase (FBPase2, EC 3.1.3.11) enzymes, which catalyse the synthesis and degradation of Fru 2,6-P₂ respectively, showed any catalytic activity as fusion proteins. Secondly, to alter the levels of Fru 2,6-P₂ in sugarcane, an important agricultural crop due to its ability to store large quantities of sucrose, by expressing the recombinant genes. Thirdly, to investigate whether sugar metabolism in photosynthetic- (leaves) and non-photosynthetic tissue (internodes) were subsequently influenced.

Activity tests performed on the bacterially expressed glutathione-S-transferase (GST) fusion 6PF2K and FBPase2 enzymes showed that they were catalytically active. In addition antibodies were raised against the bacterially expressed proteins.

Methods for extracting and measuring Fru 2,6-P₂ from sugarcane tissues had to be optimised because it is known that the extraction efficiencies of Fru 2,6-P₂ could vary significantly between different plant species and also within tissues from the same species. A chloroform/methanol extraction method was established that provided Fru 2,6-P₂ recoveries of 93% and 85% from sugarcane leaves and internodes respectively. Diurnal changes in the levels of Fru 2,6-P₂, sucrose and starch were measured and the results suggested a role for Fru 2,6-P₂ in photosynthetic sucrose metabolism and in the partitioning of carbon between sucrose and starch in sugarcane leaves.

Transgenic sugarcane plants expressing either a recombinant rat FBPase2 (ODe lines) or 6PF2K (OCe lines) were generated. The ODe lines contained decreased leaf Fru 2,6-P₂ levels but increased internodal Fru 2,6-P₂ levels compared to the control plants. Higher leaf sucrose and reducing sugars (glucose and fructose) were measured in the transgenic plants than the control plants. The transgenic lines contained decreased internodal sucrose and increased reducing sugars compared to the control plants. Opposite trends were observed for Fru 2,6-P₂ and sucrose when leaves, internodes 3+4 or internodes 7+8 of the different plant lines were compared. In contrast, no consistent trends between Fru 2,6-P₂ and sucrose were evident in the ODe transgenic lines.

OPSOMMING

Fruktose 2,6-bisfosfaat (Fru 2,6-P₂) is 'n belangrike regulerende molekule in koolhidraat-metabolisme in plante. Hierdie studie het drie hoof doelwitte gehad. Eerstens, om te bepaal of die rekombinante 6-fosfofrukto 2-kinase (6FF2K, EC 2.7.1.105) en fruktose 2,6-bisfosfatase (FBFase2, EC 3.1.3.11) ensieme, wat die sintese en afbraak van Fru 2,6-P₂ onderskeidelik kataliseer, aktief was as fusie proteïene. Tweedens, om die vlakke van Fru 2,6-P₂ in suikerriet, 'n belangrike landbou gewas omdat dit groot hoeveelhede sukrose berg, te manipuleer deur die uitdrukking van die rekombinante gene. Derdens, om die daaropvolgende effek op suikervlakke in fotosintetiese-(blare) en nie-fotosintetiese weefsel (internodes) van suikerriet te ondersoek.

Aktiwiteitstoetse op bakteries uitgedrukte glutatioon-S-transferase (GST) fusie 6FF2K en FBFase2 ensieme het gewys dat die ensieme katalities aktief was. Teenliggame is ook opgewek teen hierdie proteïene.

'n Chloroform/metanol-ekstraksie-metode is ontwikkel wat Fru 2,6-P₂-herwinning van 93% en 85% gelewer het vir suikerrietblare en -internodes onderskeidelik. Daaglikse verskille in die vlakke van Fru 2,6-P₂, sukrose en stysel is gemeet en resultate het daarop gedui dat Fru 2,6-P₂ moontlik 'n rol speel in fotosintetiese sukrose-metabolisme en in die verdeling van koolstof tussen sukrose en stysel in suikerrietblare.

Transgeniese suikerrietplante wat of 'n rekombinante FBFase2 (ODe lyne) of 6FF2K (OCe lyne) uitdruk is gegenereer. Die ODe plante het laer blaar Fru 2,6-P₂-vlakke maar hoër internode Fru 2,6-P₂-vlakke in vergelyking met kontrole plante bevat. Hoër blaar sukrose en reduserende suikers (glukose en fruktose) is gemeet in die transgeniese plante in vergelyking met kontrole plante. Die transgeniese plante het minder sukrose en meer reduserende suikers in hul internodes bevat. Teenoorgestelde tendense is waargeneem vir Fru 2,6-P₂ en sukrose wanneer blare, internodes 3+4 of internodes 7+8 van die verskillende plantlyne vegelyk is. In teenstelling, geen konsekwente tendense was sigbaar tussen Fru 2,6-P₂ en sukrose in die ODe plante nie.

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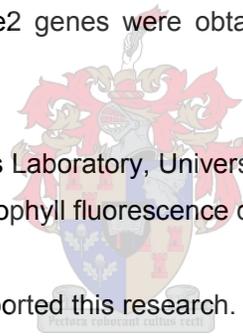


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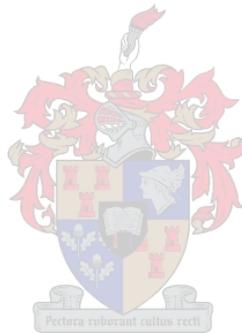
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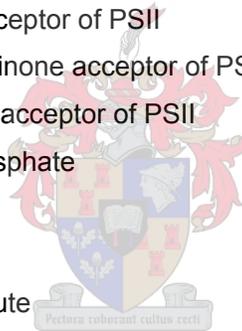
°C	: degrees centigrade
2,4-D	: 2,4-dichloro-phenoxyacetic acid
³² P	: phosphorus-32 (radio isotope)
35S-p	: 35S promoter of the CaMV
3PGA	: 3-phosphoglycerate
6PF2K	: 6-phosphofructo 2-kinase (EC 2.7.1.105)
ADP	: adenosine 5'-diphosphate
AGPase	: adenosine 5'-diphosphate-glucose pyrophosphorylase (EC 2.7.7.27)
AMP	: adenosine 5'-monophosphate
Amp ^R	: ampicillin resistance
ANOVA	: analysis of variance
ATP	: adenosine 5'-triphosphate
bp	: base pairs (nucleic acid)
BS	: bundle sheath
CAM	: crassulacean acid metabolism
cAMP	: cyclic AMP
CaMV	: cauliflower mosaic virus
CaMV-t	: CaMV poly adenylation sequence
chl	: chlorophyll
Ci	: curie
CWI	: cell wall invertase (EC 3.2.1.26)
Da	: Dalton
dCTP	: deoxycytidine 5'-triphosphate
ddH ₂ O	: double distilled water
DHAP	: dihydroxyacetone phosphate
DNA	: deoxyribonucleic acid
DNase I	: deoxyribonuclease I (EC 3.1.21.1)
dNTPs	: deoxynucleotide triphosphates
DTT	: 1,4-dithiothreitol
ECL	: enhanced chemiluminescence
EDTA	: ethylene diamine tetra-acetic acid
EGTA	: ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
FBPase1	: fructose 1,6-bisphosphatase (EC 3.1.3.11)
FBPase2	: fructose 2,6-bisphosphatase (EC 3.1.3.11)
Fru 1,6-P ₂	: fructose 1,6-bisphosphate



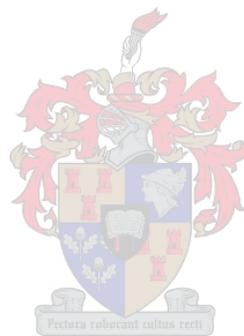
Fru 2,6-P ₂	: fructose 2,6-bisphosphate
Fru 6-P	: fructose 6-phosphate
FW	: fresh weight
g	: gram
G6PDH	: glucose 6-phosphate dehydrogenase (EC 1.1.1.49)
GDH	: glycerol 3-phosphate dehydrogenase (EC 1.1.1.8)
Glu 1,6-P ₂	: glucose 1,6-bisphosphate
Glu 6-P	: glucose 6-phosphate
GST	: glutathione-S-transferase
Hepes	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His ²⁵⁸	: histidine residue 258
HTC	: high transgene copy
IgG	: immunoglobulin G
IPTG	: isopropyl β-D-thiogalactoside
K _a	: activation constant
K _{eq}	: equilibrium constant
K _m	: Michaelis-Menten constant (substrate concentration producing half maximal velocity)
l	: litre
LB broth	: Luria-Bertani broth
LTC	: low transgene copy
m	: metre
M	: molar
mol	: mole
MOPS	: 3-[N-Morpholino]propanesulfonic acid
MS media	: Murashige-Skoog media
n	: sample size
NADH	: reduced β-nicotinamide adenine dinucleotide
NADP	: oxidised β-nicotinamide adenine dinucleotide phosphate
NADPH	: reduced β-nicotinamide adenine dinucleotide phosphate
NCBI	: national centre for biotechnology information
NPT II	: neomycin phosphotransferase II
OAA	: oxaloacetate
OCe plants	: 6PF2K transgenic sugarcane (variety NCo310) plants
OD	: optical density
ODe plants	: FB Pase2 transgenic sugarcane (variety NCo310) plants
Pa	: Pascal



PAGE	: polyacrylamide gel electrophoresis
pat	: phosphinothricin acetyl transferase
PCR	: polymerase chain reaction
PEA	: plant efficient analyzer
PEP	: phospho(enol)pyruvate
PEPC	: PEP carboxylase (EC 4.1.1.31)
PFK	: phosphofructokinase (EC 2.7.1.11)
PFP	: pyrophosphate: fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90)
PGI	: phosphoglucoisomerase (EC 5.3.1.9)
Pi	: inorganic phosphate
Pol	: polarisation
PPdK	: pyruvate orthophosphate dikinase (EC 2.7.9.1)
PPi	: inorganic pyrophosphate
PQH ₂	: reduced plastoquinone
PSII	: photosystem II
Q _A	: primary quinone acceptor of PSII
Q _A ⁻	: reduced primary quinone acceptor of PSII
Q _B	: secondary quinone acceptor of PSII
Rbu 1,5-P ₂	: ribulose 1,5-bisphosphate
RNA	: ribonucleic acid
RNase A	: ribonuclease A
rpm	: revolutions per minute
Rubisco	: ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39)
SASRI	: South African Sugar Research Institute
SDS	: sodium dodecyl sulphate
Ser ³²	: serine residue 32
SNI	: sugarcane neutral invertase (EC 3.2.1.26)
SPP	: sucrose-phosphate phosphatase (EC 3.1.3.24)
SPS	: sucrose-phosphate synthase (EC 2.4.1.14)
SSC	: saline sodium citrate
Stdev	: standard deviation
SuSy	: sucrose synthase (EC 2.4.1.13)
TBS-T	: Tris-buffered saline-Tween
TE buffer	: Tris-EDTA buffer
TPi	: triose phosphate isomerase (EC 5.3.1.1)
TPT	: triose phosphate/phosphate translocator
Tris	: 2-amino-2-(hydroxymethyl)-1,3-propanediol



U	: unit (enzyme)
UBI-p	: maize ubiquitin 1 promoter
UDP-glucose	: uridine 5'-diphosphoglucose
UV	: ultraviolet
v/v	: volume per volume
VAI	: vacuolar acid invertase (EC 3.2.1.26)
V_{\max}	: maximum velocity (of an enzyme)
W	: Watt
w/v	: weight per volume
xg	: gravitational force



CHAPTER 1

General introduction

Sugarcane (*Saccharum* hybrid) is a C₄ perennial grass that is an important crop due to its ability to store large quantities of stem / internodal sucrose (Moore and Maretzki, 1996). As for July 2005/2006, the South African sugar industry will produce an estimated 2 512 000 tons of saleable sugar from 21 492 000 tons of crushed cane. This will contribute approximately R2 billion to the country's foreign exchange earnings. The South African sugar industry provides employment (direct and indirect) for an estimated 350 000 people¹.

Increasing sucrose content in sugarcane through conventional breeding has reached a plateau even though current commercial yields are only attaining 50% of the potential physiological limit of sucrose storage (Grof and Campbell, 2001), i.e. 27% fresh weight (FW) (Bull and Glasziou, 1963). A plausible explanation for not improving stem sucrose content is the narrow gene pool used in the breeding of modern sugarcane varieties (Roach, 1989). In addition, sugarcane is a polyploid that makes breeding extremely difficult and time consuming – one new variety is the product of 600 crosses (180 000 seedlings) and approximately 15 years².

The more recent approach of genetic transformation is an attractive adjunct to conventional breeding. Genetic transformation is more specific than conventional breeding allowing the manipulation of only one gene at a time. This technology is also not limited to only native sugarcane genes. Genetic transformation has already improved crops such as tomato, canola, cotton, soybean and maize by enhancing agronomic traits including quality and resistance to herbicides, pathogens and abiotic stress (commercial releases of biotechnology companies such as Monsanto and Calgene; Stitt and Sonnewald, 1995; Birch, 1996). The first genetically modified (transgenic) sugarcane plants were generated in 1992 (Bower and Birch, 1992).

Fructose 2,6-bisphosphate (Fru 2,6-P₂) is a signal metabolite that allosterically regulates two cytosolic plant enzymes, i.e. fructose 1,6-bisphosphatase (FBPase1, EC 3.1.3.11) and pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) (Sabularse and Anderson, 1981b; Wong *et al.*, 1987; Stitt, 1990a). The *in vivo* concentration of Fru 2,6-P₂ is regulated by 6-phosphofructo 2-kinase (6PF2K, EC 2.7.1.105) and fructose 2,6-bisphosphatase (FBPase2, EC 3.1.3.46) that synthesise and hydrolyse Fru 2,6-P₂ respectively (Claus *et al.*, 1984). The activities of these enzymes are modulated by intermediates of photosynthesis and

¹ www.sasa.org.za

² South African Sugar Association Experiment Station Senior Certificate Course in Sugarcane Agriculture. Chapter 10. Varieties, Breeding.

sucrose metabolism (Cséke and Buchanan, 1983; Cséke *et al.*, 1983; Stitt *et al.*, 1984a; Larondelle *et al.*, 1986).

In all plants investigated, Fru 2,6-P₂ plays a key role in the feedforward and feedback regulation of photosynthetic sucrose metabolism via its inhibition of FBPase1 (Stitt *et al.*, 1983; Sicher *et al.*, 1986; Sicher *et al.*, 1987; Stitt, 1990a; Scott and Kruger, 1994; Trevanion, 2000). In contrast to photosynthetic tissues, the role of Fru 2,6-P₂ in non-photosynthetic plant tissues is less certain (Kruger and Scott, 1994; Nielsen *et al.*, 2004). In addition the regulating steps in sucrose metabolism in sugarcane internodal tissue are not clear (Birch, 1996; Moore and Maretzki, 1996; Grof and Campbell, 2001; Rohwer and Botha, 2001). Although the role of PFP in plants is not understood (Hajirezaei *et al.*, 1994; Kruger and Scott, 1994; Fernie *et al.*, 2001), work done on sugarcane showed a negative correlation between sucrose content and PFP activity in different genotypes (Whittaker and Botha, 1999) and therefore a possible regulatory function for Fru 2,6-P₂ in sucrose metabolism in sugarcane internodal tissue.

In this study, genetic transformation was used to study the role of Fru 2,6-P₂ in sucrose metabolism in photosynthetic (leaves) and non-photosynthetic tissues (internodes) of sugarcane. Recombinant rat 6PF2K and FBPase2 were expressed in a bacterial system to verify that they are catalytically active and to raise antibodies against them (chapter 3). Because the measurement of Fru 2,6-P₂ from plant tissue is often problematic (Stitt, 1990b), extraction and assay procedures for Fru 2,6-P₂ from sugarcane tissues were developed (chapter 4). The recombinant 6PF2K and FBPase2 genes were then transferred to sugarcane plants and the transgenic plants were analysed (chapter 5).

CHAPTER 2

Fructose 2,6-bisphosphate as a signal metabolite in plants

2.1 Introduction

Fructose 2,6-bisphosphate (Fru 2,6-P₂, figure 2.1) is signal metabolite common to all eukaryotes (Stitt, 1990a; Okar *et al.*, 2001). Fru 2,6-P₂ was only discovered in 1980 following work done on liver metabolism (Van Schaftingen *et al.*, 1980). In liver Fru 2,6-P₂ activates phosphofructokinase (PFK, EC 2.7.1.11) (*reaction 1*) and inhibits fructose 1,6-bisphosphatase (FBPase1, EC 3.1.3.11) (*reaction 2*) that catalyse key irreversible reactions in glycolysis and gluconeogenesis respectively (Van Schaftingen, 1987).



(Fru 6-P = fructose 6-phosphate, ATP = adenosine 5'-triphosphate, Fru 1,6-P₂ = fructose 1,6-bisphosphate, ADP = adenosine 5'-diphosphate, Pi = inorganic phosphate)

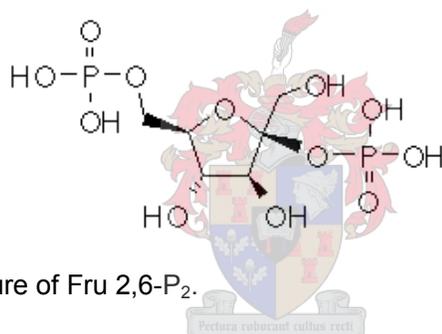
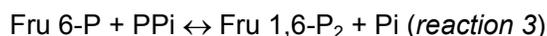


Figure 2.1. The structure of Fru 2,6-P₂.

This review will focus on plants, where for reasons that will be discussed later in this chapter the role of Fru 2,6-P₂ is less clear in comparison to animals. In plants Fru 2,6-P₂ inhibits cytosolic FBPase1, but PFK is not regulated by Fru 2,6-P₂ (Wong *et al.*, 1987). Fru 2,6-P₂ is however a potent activator of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) (*reaction 3*) in plants (Sabulase and Anderson, 1981b).



(PPi = inorganic pyrophosphate)

Apart from cytosolic FBPase1 and PFP, Fru 2,6-P₂ might also affect other plant enzymes. Plastidic PFK (but not the cytosolic isozyme) of castor oil seeds is activated by Fru 2,6-P₂ (activation constant (K_a) ~ 14 nM) (Miernyk and Dennis, 1982). This probably has no *in vivo* significance because Fru 2,6-P₂, the enzymes involved in its regulation and its targets are all confined to the cytosol in plants (Stitt *et al.*, 1983; Weiner *et al.*, 1987; Macdonald *et al.*, 1989; Stitt, 1990a).

Matic *et al.* (2004) reported that sucrose synthase (SuSy, EC 2.4.1.13) isoform 2 (SuSy2) of heterotrophic cultured tobacco cells is stimulated by micromolar concentrations of Fru 2,6-P₂. SuSy2 is the less abundant isoform in tobacco cells, it is activated by actin and probably involved in channelling uridine 5'-diphosphoglucose (UDP-glucose) for cell wall synthesis. The *in vivo* relevance of the activation of SuSy2 by Fru 2,6-P₂ has to be established.

In this chapter, the *in vivo* regulation of Fru 2,6-P₂ and its protein targets (cytosolic FBPase1 and PFP) are briefly discussed. The chapter focuses on the control Fru 2,6-P₂ has on plant carbohydrate metabolism through the modulation of cytosolic FBPase1 and PFP. The valuable contribution made by transgenic plants in our understanding of the function of Fru 2,6-P₂ in plants is emphasised.

2.2 Fru 2,6-P₂ metabolism

In leaves, the most dramatic changes in Fru 2,6-P₂ levels coincide with the transition from light to dark and *vice versa* (Stitt *et al.*, 1983; Sicher *et al.*, 1986; Sicher *et al.*, 1987; Scott and Kruger, 1994; Trevanion, 2000). In spinach leaves Fru 2,6-P₂ levels drop more than half within 5 min upon transition from dark to light (Stitt *et al.*, 1983). In maize Fru 2,6-P₂ increases approximately 12-times within 30 min upon transition from light to dark (Sicher *et al.*, 1987). Fru 2,6-P₂ levels are also not constant in non-photosynthetic tissues, in these tissues Fru 2,6-P₂ levels respond to conditions such as anoxia (Mertens *et al.*, 1990), wounding (Van Schaftingen and Hers, 1983) and exposure to ethylene (Stitt *et al.*, 1986a).

The *in vivo* concentration of Fru 2,6-P₂ is regulated by the relative activities of two enzymes: 6-phosphofructo 2-kinase (6PF2K, EC 2.7.1.11) (*reaction 4*) and fructose 2,6-bisphosphatase (FBPase2, EC 3.1.3.11) (*reaction 5*) that synthesise and hydrolyse Fru 2,6-P₂ respectively (Claus *et al.*, 1984).



In liver and plants, single bifunctional enzymes (6PF2K/FBPase2) catalyse these two activities (El-Maghrabi *et al.*, 1982; Van Schaftingen *et al.*, 1982a; Larondelle *et al.*, 1986), whereas separate proteins are found in yeast (Francois *et al.*, 1988). Several tissue specific isoforms of 6PF2K/FBPase2 have been identified in mammals, but molecular data suggest a single gene codes for 6PF2K/FBPase2 in plants (Nielsen *et al.*, 2004). An additional monofunctional FBPase2 is present in some plants such as mung bean (Avigad and Bohrer, 1984), castor bean (Kruger and Beevers, 1985), spinach and artichoke (Larondelle *et al.*, 1989). The bifunctional

FBPase2 has a much higher affinity for Fru 2,6-P₂ (K_m ~ 30 nM) than the monofunctional FBPase2 (K_m ~ 30 μM) (Larondelle *et al.*, 1986; Larondelle *et al.*, 1989).

The carboxy (COOH)-termini of plant 6PF2K/FBPase2 enzymes, contain the catalytic domains and are highly conserved (Draborg *et al.*, 1999; Villadsen *et al.*, 2000). This region (400 amino acids) of the *Arabidopsis* enzyme (National Centre for Biotechnology Information (NCBI) accession number = AF190739) shows 90%, 88% and 84% shared sequence identity to the potato (AF073830), spinach (AF041848) and maize (AF007582) enzymes respectively. The catalytic domains of the *Arabidopsis* and rat (J04197) enzymes show 90% shared sequence identity, but when the entire sequences of the *Arabidopsis* and rat enzymes are compared there is only 39% shared sequence identity. The amino (NH₂)-termini are more variable between plants species but short conserved motifs are present (Villadsen *et al.*, 2000).

The activities of 6PF2K and FBPase2 are modulated by metabolites representing intermediates of primary metabolism (Cséke and Buchanan, 1983; Cséke *et al.*, 1983; Stitt *et al.*, 1984a; Larondelle *et al.*, 1986; Villadsen and Nielsen, 2001) (table 2.1).

Table 2.1. The metabolites involved in the regulation of 6PF2K and FBPase2

Metabolite	6PF2K	FBPase2	
		Bifunctional	Monofunctional
Pi	Activator	Inhibitor	Inhibitor
Fru 6-P	Activator	Inhibitor	Inhibitor
DHAP	Inhibitor	No effect	No effect
3PGA	Inhibitor	No effect	No effect
PPi	Inhibitor	No effect	No effect
Fru 1,6-P ₂	No effect	No effect	Inhibitor
AMP	No effect	No effect	Inhibitor
PEP	Inhibitor	No effect	Nd
Pyruvate	Activator	Inhibitor	Nd
6-phospho gluconate	No effect	Inhibitor	Nd
Mg ²⁺	Cofactor	No effect	Inhibitor

Nd = not determined, DHAP = dihydroxyacetone phosphate, 3PGA = 3-phosphoglycerate, AMP = adenosine 5'-monophosphate, PEP = phospho(enol)pyruvate

The degrees to which Fru 6-P and Pi inhibit the bifunctional and monofunctional FBPase2 enzymes are converse. A Fru 6-P concentration of 0.2 mM produces 50% inhibition of the bifunctional FBPase2, whereas 4 mM Fru 6-P inhibits the activity of the monofunctional enzyme only 30%. For a 50% inhibition in activity of the bifunctional and monofunctional enzymes, 20 mM and 0.5 mM Pi are needed respectively (Larondelle *et al.*, 1986; Larondelle *et al.*, 1989). In addition Mg²⁺, Fru 1,6-P₂ (Larondelle *et al.*, 1989) and AMP (Macdonald *et al.*, 1989) inhibit only the monofunctional enzyme. Unlike the bifunctional FBPase2 from rat and plants (Larondelle *et al.*, 1986; Van Schaftingen, 1987), the monofunctional FBPase2 does not form a stable phosphoenzyme intermediate during catalysis (Macdonald *et al.*, 1987).

The activities of the liver and yeast 6PF2K/FBPase2 enzymes are also regulated by extracellular signals via cyclic adenosine 5'-monophosphate (cAMP) dependent protein phosphorylation. Phosphorylation at serine residue 32 (Ser³²) inhibits 6PF2K and activates FBPase2 (Van Schaftingen *et al.*, 1981; Van Schaftingen *et al.*, 1982a; Kurland *et al.*, 1992). There is evidence suggesting a role for protein phosphorylation in the regulation of 6PF2K/FBPase2 in plants as well (Stitt *et al.*, 1986b; Walker and Huber, 1987; Rowntree and Kruger, 1995). Further support for an additional regulatory mechanism in plants came from a study in which a full length and a truncated (the NH₂-terminus of 345 amino acids were deleted) 6PF2K/FBPase2 from *Arabidopsis* were expressed in *Escherichia coli* (Villadsen *et al.*, 2000). The 6PF2K/FBPase2 ratio of the full length and truncated enzymes were 3.3 and 1.4 respectively. The truncated enzyme showed similar 6PF2K activity but increased FBPase2 activity. The NH₂-terminus region of spinach and *Arabidopsis* contain 11 and 19 potential phosphorylation sites respectively (Villadsen *et al.*, 2000).

14-3-3 proteins are found in all eukaryotes where they bind to phosphorylated sites on numerous different target proteins thereby altering their activity (Tzivion and Avruch, 2002). Kulma *et al.* (2004) showed that 14-3-3 proteins bound to glutathione-S-transferase (GST)-*Arabidopsis* 6PF2K/FBPase2 phosphorylated by recombinant *Arabidopsis* calcium-dependent protein kinase isoform 3, rat liver mammalian AMP-activated protein kinase or an *Arabidopsis* cell extract. However they observed no effect on catalytic activities of the enzymes.

2.3 The enzymatic targets of Fru 2,6-P₂

2.3.1 FBPase1

The allosteric inhibition of cytosolic FBPase1 is central to the mechanism by which Fru 2,6-P₂ regulates carbohydrate metabolism in photosynthetic tissues (section 2.4.1). Cytosolic FBPase1 requires divalent metal ions (such as Mg²⁺) for catalytic activity and is weakly inhibited by AMP (Herzog *et al.*, 1984; Stitt *et al.*, 1985). Inhibition by Fru 2,6-P₂ decreases the affinity of FBPase1 for Fru 1,6-P₂ and increases its sensitivity to AMP. In addition Fru 2,6-P₂ increases sensitivity to product inhibition by Pi, but product inhibition by Fru 6-P is abolished (Herzog *et al.*, 1984; Stitt *et al.*, 1985).

The chloroplast FBPase1 is poorly inhibited by Fru 2,6-P₂. This enzyme is not inhibited by AMP and has a much lower affinity for Fru 1,6-P₂ and Mg²⁺ (Cséke *et al.*, 1982; Stitt *et al.*, 1982). The chloroplast FBPase1 is regulated by thioredoxin (Buchanan, 1980) and light (involving red light signalling) (Lee and Hahn, 2003).

2.3.2 PFP

In contrast to FBPase1, PFP is exclusively located in the cytosol of plants and its reaction (*reaction 3*) is reversible and close to equilibrium (K_{eq} of 3.3) (Edwards and ap Rees, 1986; Weiner *et al.*, 1987). Therefore, in theory PFP could catalyse a net flux of carbon in the direction of glycolysis (section 2.5.1) or gluconeogenesis (section 2.5.2).

PFP enzymes from most plants are heterotetramers of approximately 260 kilo Daltons (kDa) that consists of a larger α -subunit (about 66 kDa) that is involved in regulation and a smaller immunologically unrelated catalytic β -subunit (about 60 kDa) (Yan and Tao, 1984; Kruger and Dennis, 1987; Wong *et al.*, 1988; Botha and Botha, 1991). However a heterooctameric PFP was identified in potato tuber (Podesta *et al.*, 1994) and *Brassica nigra* (Theodorou and Plaxton, 1996). Apart from the heterotetrameric form, PFP comprising of only the two β -subunits was identified in wheat seedlings (Yan and Tao, 1984) and Pi-fed *B. nigra* cells (Theodorou *et al.*, 2004). A less active 130 kDa dimeric PFP was also observed in pea seedlings (Wu *et al.*, 1984). In addition PFP deaggregates into lower molecular mass forms when diluted in the absence of 1,4-dithiothreitol (DTT) (Podesta *et al.*, 1994).

The forward reaction (Fru 1,6-P₂ producing) of PFP is strongly inhibited by Pi (Krombrink *et al.*, 1984; Botha *et al.*, 1987; Stitt, 1989; Theodorou and Plaxton, 1996). On the other hand, the reverse reaction (Fru 6-P producing) is strongly inhibited by PPI (Bertagnolli *et al.*, 1986; Stitt, 1989; Theodorou and Plaxton, 1996). PFP from phosphate-starved *B. nigra* suspension cells showed inhibition in both directions by MgATP, MgADP and PEP, but at concentrations well in excess of their *in vivo* levels (Theodorou and Plaxton, 1996).

PFP requires a divalent ion (especially Mg²⁺) for catalytic activity (Krombrink *et al.*, 1984). Both the forward and reverse reactions of plant PFP enzymes are activated by nanomolar levels of Fru 2,6-P₂ ($K_a = 2$ to 50 nM) (Van Schaftingen *et al.*, 1982b). Upon activation, the affinity of PFP for Fru 6-P and Fru 1,6-P₂ increases (Krombrink *et al.*, 1984; Bertagnolli *et al.*, 1986; Van Schaftingen *et al.*, 1982b; Stitt, 1989). In the presence of Fru 2,6-P₂ the affinity of PFP for PPI increases only sometimes (Krombrink *et al.*, 1984; Theodorou and Plaxton, 1996; Stitt, 1990a). The inhibition caused by high concentrations (higher than 1 mM) of PPI on the forward reaction is relieved by Fru 2,6-P₂ (Cséke *et al.*, 1982; Krombrink *et al.*, 1984). Fru 2,6-P₂ however provides only some relieve caused by Pi inhibition (Krombrink *et al.*, 1984).

Redox active sulfhydryl groups (primarily on the α -subunit) are required for Fru 2,6-P₂ mediated activation of PFP (Kiss *et al.*, 1991). The inability of Fru 2,6-P₂ to activate tomato PFP treated with an oxidant (5,5'-dithiobis(2-nitrobenzoic acid)) was reversed by the addition of DTT. It was

proposed that binding of Fru 2,6-P₂ converts PFP from the dimeric to the tetrameric form that promotes activity in the glycolytic direction. On the other hand, PPI promotes the dimeric form of PFP and also activity in gluconeogenic direction (Dennis and Greyson, 1987). However activation of PFP by Fru 2,6-P₂ does not generally coincide with a change in molecular mass (Stitt, 1990a).

The degree to which Fru 2,6-P₂ activates PFP is greatly dependent on conditions: The affinity of PFP for Fru 2,6-P₂ is positively correlated with an increase in Fru 6-P or Fru 1,6-P₂, and decreases with an increase in Pi (Cséke *et al.*, 1982; Van Schaftingen *et al.*, 1982b; Kombrink *et al.*, 1984; Stitt, 1989). Several phosphorylated intermediates (Kombrink *et al.*, 1984), citrate (Van Praag *et al.*, 1998), and certain anions (Van Schaftingen *et al.*, 1982b; Kombrink *et al.*, 1984; Degli Agosti *et al.*, 1992) also decrease the affinity of PFP for Fru 2,6-P₂. The maximal fold activation of barley leaf PFP by Fru 2,6-P₂ (in the forward reaction) was observed at pH 6.9, although the pH optimum was 7.7 (Podesta and Plaxton, 2003): Fru 2,6-P₂ (5 μM) produced 14-fold and 2-fold activation at pH 6.9 and pH 7.7 respectively. The affinity of potato tuber PFP for Fru 2,6-P₂ decreases when the temperature was lowered from 25 °C to 2 °C, but the sensitivity of the activation was increased (5-fold compared to 50-fold) (Trevanion and Kruger, 1991).

The above surely has important implications: For example, Van Schaftingen *et al.* (1982b) reported a K_a (Fru 2,6-P₂) for potato PFP of 5.5 nM. A second study determined a K_a value that was 3.5-times lower for the same enzyme (Degli Agosti *et al.*, 1992). Van Schaftingen and co-workers measured PFP activity in 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl. This buffer contains 39 mM Cl⁻ (a competitive inhibitor of Fru 2,6-P₂), which explains the discrepancy in the reported K_a values (Degli Agosti *et al.*, 1992).

The most sensitive and widely used method for measuring Fru 2,6-P₂ relies on linear stimulation of potato tuber PFP by Fru 2,6-P₂ (Van Schaftingen *et al.*, 1982b). Because several metabolites in plant extracts influence the affinity of PFP for Fru 2,6-P₂, the activation pattern of PFP is unique for each sample. This necessitates the use of internal standards for each sample in which the endogenous Fru 2,6-P₂ is hydrolysed (acid treatment) (Van Schaftingen and Hers, 1983). The sample is then spiked with increasing amounts of Fru 2,6-P₂.

Apart from Fru 2,6-P₂, the most potent activator of PFP, other metabolite activators have also been identified. Previous work suggested that Fru 1,6-P₂ is not only a substrate for PFP but also a weak activator of PFP (Sabularse and Anderson, 1981a; Kombrink *et al.*, 1984; Botha *et al.*, 1986; Stitt, 1990a). Subsequent results showed that Fru 1,6-P₂ is actually a strong allosteric activator of PFP that competes with Fru 2,6-P₂ (Nielsen, 1995; Podesta and Plaxton, 2003). Barley leaf PFP is significantly activated by 5 to 25 μM Fru 1,6-P₂ and fully activated at 100 to

200 μM . This also explains why initial reports claimed that the reverse reaction of PFP was less stimulated by Fru 2,6-P₂ than the forward reaction (Stitt, 1990a): The reverse reaction typically contains 0.5 mM Fru 1,6-P (added as substrate) that is sufficient to activate PFP. In addition Wang and Shi (1999) demonstrated that Fru 1,6-P₂ (unlike Fru 2,6-P₂) protects the α -subunit of PFP against proteolysis.

PFP is also weakly stimulated by glucose 1,6-bisphosphate (Glu 1,6-P₂) (Sabulase and Anderson, 1981a; Van Schaftingen *et al.*, 1982b; Kombrink *et al.*, 1984). The forward reaction of potato PFP was activated to a similar degree with Glu 1,6-P₂ than with Fru 2,6-P₂ but at a 20 000-times higher concentration (Van Schaftingen *et al.*, 1982b).

The regulation of PFP might also be under hormonal control in certain tissues. It is known that hormones such as kinetin and gibberellic acid are derived from the radicle (Longo *et al.*, 1979; Bewley and Black, 1983). Increased PFP activity associated with the germination of *Citrullus lanatus* was largely dependent on the presence of the radicle (Botha and Botha, 1990). Kinetin, ethrel and gibberellic acid (to a lesser extent) compensated for the removal of the radicle.

Increased PFP activity is not necessarily due to the activation of existing PFP but might also be the result of an increase in the synthesis of the PFP protein (Botha *et al.*, 1989; Botha and Botha, 1990).

2.4 Fru 2,6-P₂ as a regulator of plant carbohydrate metabolism

The regulation of plant carbohydrate metabolism by Fru 2,6-P₂ through its enzymatic targets (FBPase1 and PFP) will be discussed next. The significance of Fru 2,6-P₂ in photosynthetic and non-photosynthetic tissues are discussed separately. The latter section especially contains relevant information on sugarcane which product, sucrose, is of worldwide economical importance.

2.4.1 Fru 2,6-P₂ as a regulatory molecule in photosynthetic tissues

2.4.1.1 Feedforward stimulation of photosynthetic sucrose synthesis

In all plants investigated, the most dramatic changes in diurnal Fru 2,6-P₂ levels occur with the transition from light to dark and *vice versa* and coincide with the onset of sucrose accumulation or mobilisation (Stitt *et al.*, 1983; Sicher *et al.*, 1986; Sicher *et al.*, 1987; Scott and Kruger, 1994; Trevanion, 2000).

The first product of C3 photosynthesis is 3PGA. This three-carbon molecule (hence the name C3 photosynthesis) is produced from ribulose bisphosphate (Rbu 1,5-P₂) and CO₂ by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) in the photosynthetic carbon

reduction cycle. Most of the triose phosphates produced in the photosynthetic carbon reduction cycle are utilised to regenerate Rbu 1,5-P₂, one-sixth of the triose phosphates are exported from the chloroplast to the cytosol via the triose phosphate/phosphate translocator (TPT) for the synthesis of sucrose (Flugge and Heldt, 1984) (figure 2.2). The Pi generated during sucrose synthesis is recycled to the chloroplast to maintain photosynthesis.

Cytosolic FBPase1 (section 2.3.1) is inhibited by Fru 2,6-P₂ until a certain triose phosphate concentration is reached (Gerhard *et al.*, 1987; Stitt *et al.*, 1987a; Stitt *et al.*, 1987b). Once this threshold level is exceeded, 6PF2K and FBPase2 are simultaneously inhibited and activated respectively. This results in a rapid drop in Fru 2,6-P₂ levels. Although the accumulation of both triose phosphates and 3PGA contribute to the decline in Fru 2,6-P₂ levels upon illumination, the crucial signal appears to be the rising 3PGA/Pi ratio (Neuhaus and Stitt, 1989; Stitt, 1990a). The decline in Fru 2,6-P₂ allows the activity of FBPase1 to increase and thus leads to the accumulation of sucrose.

Another key enzyme in sucrose synthesis, sucrose-phosphate synthase (SPS, EC 2.4.1.14) (figure 2.4) is activated (dephosphorylated) in the light in response to the increased supply of photosynthate (Stitt *et al.*, 1988). An increased in the glucose 6-phosphate (Glu 6-P)/Pi ratio also stimulates SPS activity (Doehlert and Huber, 1983; Stitt *et al.*, 1988).

Although the majority of higher plants perform C₃ photosynthesis, a number of species including sugarcane and maize, operate the C₄ photosynthetic pathway. Hatch and Slack (1966) first described the C₄ photosynthetic pathway following work done on sugarcane. Sugarcane photosynthetic tissues are arranged in concentric rings around the vascular bundles (known as Kranz anatomy) (figure 2.3). Unlike C₃ plants, compartmentation of photosynthesis occurs between the mesophyll and bundle sheath (BS) cells in C₄ plants. The BS cells are surrounded by a lamella that is highly resistant to the diffusion of CO₂. The rapid decarboxylation of the C₄ acids enables CO₂ concentrations of 27 μM to 70 μM in the BS of C₄ plants (4 to 9-times more than those found in the mesophyll of C₃ plants) (Jenkins *et al.*, 1989; Dai *et al.*, 1993). These high CO₂ concentrations suppress the oxygenase activity of Rubisco that prevents energy expensive photorespiration. Photorespiration is below detectable levels also in sugarcane (Moore and Maretzki, 1996). The inhibition of photorespiration allows the photosynthetic carbon reduction cycle to operate more efficiency. This adaptation to C₃ photosynthesis enables more efficient photosynthesis in hot climates with sporadic rainfall, typical the habitat of many C₄ plants.

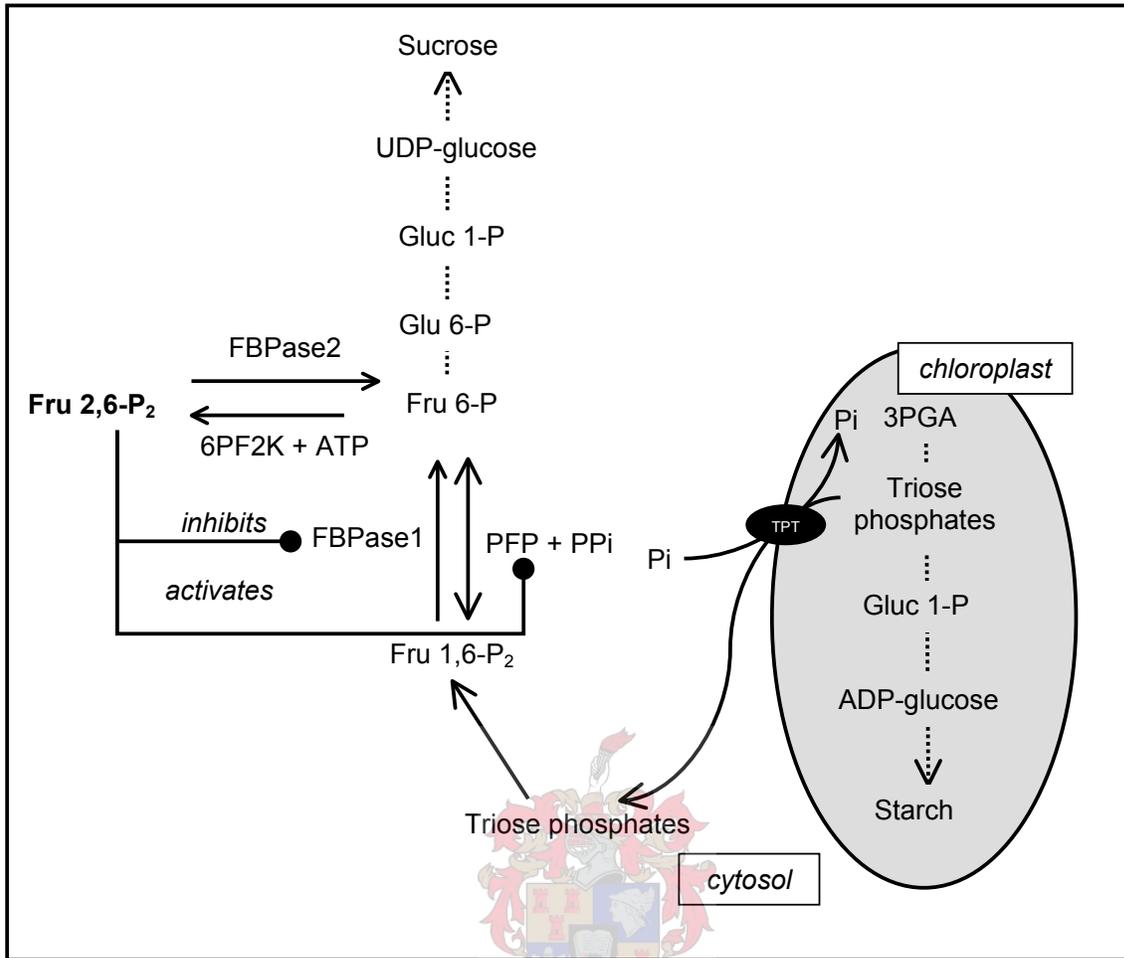


Figure 2.2. The role of Fru 2,6-P₂ in photosynthetic sucrose metabolism (see text for details).

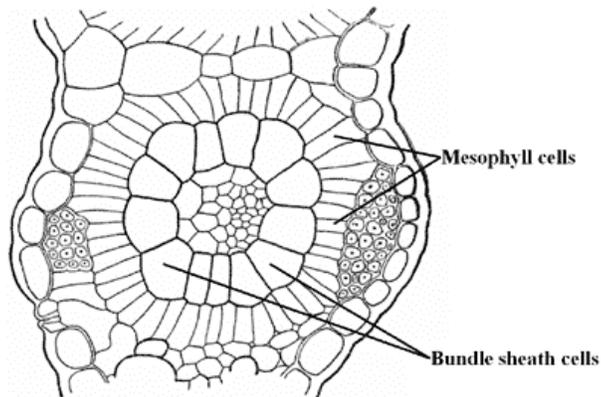


Figure 2.3. A transverse section through the leaf of a C4 grass. The layer of mesophyll cells can be seen surrounding the layer of BS cells (Kranz anatomy). Drawing by G. Haberlandt.

In sugarcane mesophyll cells PEP and CO₂ are converted by PEP carboxylase (PEPC, EC 4.1.1.31) to oxaloacetate (OAA). This four-carbon dicarboxylic acid (hence the name C₄ photosynthesis) is reduced to malate by malate dehydrogenase (EC 1.1.1.82) using reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Malate is transported to the BS cells where it is decarboxylated by NADP-malic enzyme (EC 1.1.1.40) to pyruvate and CO₂. The latter is fixed by Rubisco in the photosynthetic carbon reduction cycle to produce 3PGA as in C₃ photosynthesis. Rubisco is restricted to the BS cells in C₄ plants. Pyruvate could diffuse back to the mesophyll and is phosphorylated by pyruvate orthophosphate dikinase (PPdK, EC 2.7.9.1) using ATP as the phosphoryl donor to regenerate the carbon acceptor (PEP).

The site of photosynthetic sucrose synthesis in C₄ plants is still disputed (Lunn and Furbank, 1997) because it is often difficult to separate the mesophyll and BS cells. Nevertheless, it is generally accepted that sucrose is synthesised in the mesophyll in maize leaves. Some of the 3PGA therefore diffuses from the BS to the mesophyll – maize BS contains 3-times higher 3PGA concentrations than the mesophyll (Stitt and Heldt, 1985). In the mesophyll the 3PGA is reduced to triose phosphates and is exported to the cytosol for sucrose synthesis. Two-thirds of the triose phosphates must however return to the BS to maintain the pool of photosynthetic carbon reduction cycle intermediates (Stitt, 1985). As for 3PGA, this transfer relies on diffusion and high triose phosphate concentrations are therefore required in the mesophyll (Stitt, 1985). For this purpose higher 3PGA concentrations are needed to inhibit 6PF2K in maize mesophyll cells. In addition maize mesophyll FBPase1 has a 10-times higher K_m for Fru 1,6-P₂ and is less sensitive to Fru 2,6-P₂. The above adaptations allow DHAP concentrations as high as 10 mM in the maize mesophyll, whereas maize BS cells contain only 0.5 mM DHAP (Stitt and Heldt, 1985).

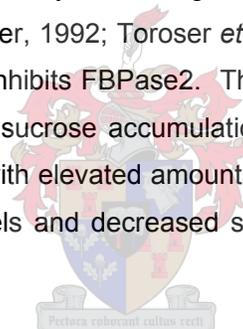
The belief that the mesophyll is the major site for sucrose synthesis in maize is supported by findings that most of the Fru 2,6-P₂ (Stitt and Heldt, 1985), 6PF2K and FBPase2 (Soll *et al.*, 1985) are restricted to mesophyll cells in maize leaves. Furthermore, maize leaf SPS is almost exclusively found in the mesophyll (Downton and Hawker, 1973). However Clayton *et al.* (1993) showed that maize BS cells could contain significant amounts of 6PF2K, FBPase2 and Fru 2,6-P₂. Furthermore, Lunn and Furbank (1997) found that up to 35% of the total leaf SPS activity could be present in the BS in C₄ plants.

Earlier studies on the role of Fru 2,6-P₂ in plants relied on indirect methods to manipulate Fru 2,6-P₂ levels. These included altering the rate of CO₂ fixation or sucrose accumulation (Stitt *et al.*, 1984b; Stitt *et al.*, 1984c), and manipulating enzymes not directly involved in Fru 2,6-P₂

metabolism such as phosphoglucose isomerase (PGI, EC 5.3.1.9) (Neuhaus *et al.*, 1989). The more recent approach of recombinant deoxyribonucleic acid (DNA) technology to produce metabolic mutants is more specific (Kruger and Scott, 1994). Results from transgenic plants including tobacco (Scott *et al.*, 1995; Scott *et al.*, 2000), *Kalanchoe daigremontiana* (Truesdale *et al.*, 1999), *Arabidopsis* (Draborg *et al.*, 2001) and potato (Rung *et al.*, 2004) showed unequivocally that Fru 2,6-P₂ indeed plays an integral role in the co-ordination of photosynthesis and sucrose synthesis and that its concentration does not merely change as a result of another stimulus.

2.4.1.2 Feedback inhibition of photosynthetic sucrose synthesis

In spinach and tobacco there is a gradual increase in Fru 2,6-P₂ and a decrease in sucrose synthesis during the photoperiod (Stitt *et al.*, 1983; Scott and Kruger, 1994). Feedback inhibition of sucrose synthesis occurs when the rate of sucrose synthesis exceeds the rate that sucrose is exported from the leaf or moved into the vacuole for storage. The feedback inhibition of sucrose synthesis is shared between SPS and FBPase1 (Neuhaus *et al.*, 1990b). In sucrose accumulating leaves SPS is inhibited by increasing Pi and deactivated by phosphorylation at serine residue 158 (Huber and Huber, 1992; Toroser *et al.*, 1999). This leads to an increase in Fru 6-P that activates 6PF2K and inhibits FBPase2. Thus Fru 2,6-P₂ levels increases, that will inhibit FBPase1 and subsequently sucrose accumulation (Sicher *et al.*, 1986; Gerhardt *et al.*, 1987). *Clarkia xantiana* mutants with elevated amounts of Fru 6-P due to reduced PGI activity also had increased Fru 2,6-P₂ levels and decreased sucrose levels (Kruckeberg *et al.*, 1989; Neuhaus *et al.*, 1989).



The feedback inhibition of sucrose synthesis exerted by both Fru 2,6-P₂ and SPS is more profound at low than high rates of photosynthesis (Kruckeberg *et al.*, 1989; Neuhaus *et al.*, 1989; Neuhaus *et al.*, 1990b). A possible explanation is that at low photosynthetic rates, sucrose synthesis must be controlled to prevent depletion of triose phosphates from the chloroplast that will otherwise inhibit photosynthesis. During high rates of photosynthesis the levels of 3PGA and triose phosphates mainly regulate the rate of sucrose synthesis (Neuhaus *et al.*, 1990b).

The role of Fru 2,6-P₂ in the feedback inhibition of sucrose synthesis in C₄ plants is not well investigated. In maize (Sicher *et al.*, 1987) and wheat (Trevanion, 2000) Fru 2,6-P₂ levels drop upon illumination but then remain constant throughout the light period. Therefore, Fru 2,6-P₂ appears not to be involved in the feedback inhibition of sucrose synthesis in these species. Fru 2,6-P₂ increases however slightly in *Lolium* leaves during the light period (Pollock *et al.*, 1989). The diurnal Fru 2,6-P₂ profile in sugarcane leaves is unknown.

2.4.1.3 Co-ordination of cytosolic and plastidic metabolism

A rise in Fru 2,6-P₂ during the photoperiod results in the feedback inhibition of sucrose synthesis (section 2.4.1.2) that decreases the release of Pi in the cytosol and thus the counter exchange of triose phosphates from the chloroplast via the TPT (Stitt *et al.*, 1983; Stitt *et al.*, 1987a). Subsequently, the 3PGA/Pi ratio in the chloroplast increases and activates ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27) (Preiss, 1982). This enzyme catalyses the synthesis of ADP-glucose and is the key enzyme in regulating starch synthesis. Photosynthate is now directed towards starch (Preiss, 1982; Preiss, 1988) (figure 2.2).

Results from transgenic plants including tobacco (Scott *et al.*, 1995; Scott *et al.*, 2000), *Kalanchoe daigremontiana* (Truesdale *et al.*, 1999) and *Arabidopsis* (Draborg *et al.*, 2001) confirmed the proposed role of Fru 2,6-P₂ in the co-ordination of the rates of photosynthesis and sucrose synthesis and its role in the partitioning of photosynthate between sucrose and starch (Stitt *et al.*, 1983; Stitt *et al.*, 1987a). It is crucial that enough Pi is recycled in the cytosol through sucrose synthesis to support the current rate of photosynthesis in the chloroplast. Elevated cytosolic Pi, as a result of a too high sucrose synthesis rate, will however deplete the pool of phosphorylated intermediates in the chloroplast and prevent the regeneration of Rbu 1,5-P₂ and inhibit photosynthesis (Stitt, 1990a).

Similar as for the feedback inhibition of sucrose synthesis, the contribution made by Fru 2,6-P₂ in carbon partitioning depends on conditions. Fru 2,6-P₂ regulates photosynthetic carbon partitioning more successfully under low than high light in both *Clarkia xantiana* (Neuhaus and Stitt, 1989) and spinach (Neuhaus *et al.*, 1990b) and has more effect on carbon partitioning at the beginning than the end of the photoperiod in tobacco (Scott *et al.*, 1995; Scott *et al.*, 2000). However Fru 2,6-P₂ played a similar role at the beginning and end of the photoperiod in *Arabidopsis* (Draborg *et al.*, 2001).

The above mentioned plant species, in which a role for Fru 2,6-P₂ in the partitioning of photosynthate was demonstrated, store leaf carbon mainly as starch. This role of Fru 2,6-P₂ in photosynthate partitioning is however not universal as evident from studies on barley (Sicher *et al.*, 1984), *Lolium temulentum* (Pollock *et al.*, 1995), and wheat (Trevanion, 2000; Trevanion, 2002). These plants store leaf carbon mainly as sucrose and there is little or no build-up of Fru 2,6-P₂ during the photoperiod. Because the capacity for starch synthesis is small in sucrose-storing plants, other strategies must be employed to prevent excess cytosolic Pi levels. These might include fructan synthesis or storage of photosynthate in other tissues such as internodes (Trevanion, 2002).

Apart from Fru 2,6-P₂, other factors are involved in the partitioning of photosynthate. Potato plants with reduced TPT activity accumulated more starch during the day and were able to compensate for limited triose phosphate export by a higher rate of starch mobilisation during the night (Reismeyer *et al.*, 1993). Also, *Arabidopsis* knock-out mutants that possessed TPT activity of below 5% of wild type plants compensated for the lack of the TPT activity by continuous accelerated starch turnover and the export of neutral sugars (rather than triose phosphates) to the cytosol for sucrose synthesis throughout the day (Schneider *et al.*, 2002), thus bypassing the key regulatory step catalysed by FBPase1 (section 2.4.1.1). Interestingly, Fru 2,6-P₂ dropped upon illumination in these mutants similar as in the wild type plants. The drop in Fru 2,6-P₂ probably occurred without the build-up of triose phosphates (and 3PGA) that is generally accepted as a signal in regulation Fru 2,6-P₂ levels (Stitt *et al.*, 1984a; Larondelle *et al.*, 1986). Transgenic tobacco expressing antisense TPT cDNA showed in comparison with sense plants a 20% increase in starch whereas total soluble sugars decreased by 20% (Gray *et al.*, 1995).

An increase in irradiance caused more photosynthate to be partitioned to starch at the expense of sucrose in maize (Lunn and Hatch, 1997). Increased light intensity also favours partitioning towards starch in wheat but has little effect on Fru 2,6-P₂ levels or the rate of sucrose synthesis (Trevanion, 2002).

2.4.2 Fru 2,6-P₂ as a regulatory molecule in non-photosynthetic tissues

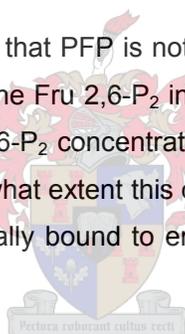
In contrast to the well documented role of Fru 2,6-P₂ as a signal metabolite in plant photosynthetic tissues (section 2.4.1), the function of this metabolite in non-photosynthetic plant tissue is only poorly understood (Nielsen *et al.*, 2004). One of the important reasons for this is that non-photosynthetic tissues often lack appreciable FBPase1 activity (Enwistle and ap Rees, 1990), the enzyme central in the mechanism of how Fru 2,6-P₂ regulates carbohydrate metabolism in photosynthetic tissues. If cytosolic FBPase1 is absent from non-photosynthetic tissues, this would imply that the Fru 2,6-P₂ mediated effects on metabolism in these tissues are attributed to PFP (or an undiscovered effect on another enzyme) (Stitt, 1990a; Scott and Kruger, 1994). But the function of PFP in plants remains controversial (Stitt, 1990a; Hajirezaei *et al.*, 1994; Paul *et al.*, 1995; Nielsen and Stitt, 2001, Nielsen *et al.*, 2004).

Possible roles of PFP in plants are discussed in section 2.5. That section includes examples of the importance of PFP in non-photosynthetic plant tissues. A possible role for PFP in sucrose accumulation in sugarcane internodal tissue is discussed in section 2.6.

2.5 Possible roles of PFP in plants

Understanding the role of PFP in higher plants is impeded by three factors: Firstly, unlike in other PFP-containing organisms investigated (except *Euglena gracilis* (Mertens, 1991), PFP co-exists with PFK and FBPase1 in plants – these two enzymes catalyse similar reactions (reactions 1 and 2) than PFP (reaction 3). Secondly, the compartmentation of plant metabolism between the cytosol and plastids prevents accurate quantification of metabolites in the cytosol (Stitt, 1990a). Thirdly, PFP appeared to be fully activated *in vivo* and therefore unlikely to respond to changes in the amount of Fru 2,6-P₂: *In vivo* Fru 2,6-P₂ concentrations in plants are between 0.21 and 300 μM (Theodorou and Kruger, 2001). In non-photosynthetic plant tissues the concentrations of Fru 2,6-P₂ are at the lower level of this range: 0.3 and 2.5 μM in potato tubers and carrot roots (Hajirezaei and Stitt, 1991) and 3 μM in sugarcane internodal tissue (Whittaker and Botha, 1999). Thus Fru 2,6-P₂ concentrations even in non-photosynthetic tissue are well in excess of PFP's K_a for Fru 2,6-P₂, i.e. 2 to 50 nM Fru 2,6-P₂ is required for half-maximal activation (Van Schaftingen *et al.*, 1982b).

However more recent results suggest that PFP is not necessarily fully activated *in vivo*: Hue *et al.* (1985) reported that over 90% of the Fru 2,6-P₂ in rat liver cells might be bound to cytosolic enzymes, arguing that the free Fru 2,6-P₂ concentration *in vivo* is much lower than the amount determined *in vitro*. It is unknown to what extent this occurs in plants but a significant proportion of Fru 2,6-P₂ might also be allosterically bound to enzymes in plants (Stitt, 1987; Nielsen and Wischmann, 1995).



In addition Nielsen and Wischmann (1995) demonstrated that in barley leaves the concentrations of PFP and Fru 2,6-P₂ are in the same order of magnitude. In fact, the concentration of PFP exceeds that of Fru 2,6-P₂ in the leaf base. Thus all the allosteric binding sites on PFP are not occupied, meaning that PFP is probably not fully activated. Although the concentration of Fru 2,6-P₂ exceeds that of PFP in the tip of barley leaves, a significant amount of FBPase1 is present in this photosynthetic active area of the leaf that will also bind Fru 2,6-P₂ and decrease its free concentration.

In the presence of physiological concentrations of Pi and various metabolic intermediates, the K_a (Fru 2,6-P₂) values of spinach leaf and potato tuber PFP are significantly higher than the *in vitro* determined values and corresponds to *in vivo* Fru 2,6-P₂ levels (Theodorou and Kruger, 2001).

2.5.1 A role for PFP in glycolysis and starch mobilisation

Because PFP is stimulated by Fru 2,6-P₂ in higher plants (Sabularse and Anderson, 1981b), changes in Fru 2,6-P₂ levels might correspond to changes in PFP activity. Fru 2,6-P₂ increases upon treatments known to stimulate glycolysis, however the time course of the increase in Fru 2,6-P₂ does not always correspond with the rise in respiration (Stitt, 1990a). A convincing role for Fru 2,6-P₂ in glycolysis was demonstrated in *Chenopodium* cell suspension cultures (Hatzfeld *et al.*, 1990) where the simultaneous addition of an uncoupler and hydroxyl ions resulted in a 3-fold increase in dark-respiration and O₂-uptake and correlated with a rise in Fru 2,6-P₂ and a decrease in Fru 6-P and PPI.

A role for PFP in glycolysis was also demonstrated in cell suspensions of *Phaseolus vulgaris* (Botha *et al.*, 1992). These cultures showed a high rate of respiration and the calculated PFK activity was insufficient to sustain the glycolytic flux. However no change in Fru 2,6-P₂ was observed in mature *Arum maculatum* spadix in spite of a dramatic rise of respiration (ap Rees *et al.*, 1985a).

Heterotrophic transgenic tobacco calli with elevated Fru 2,6-P₂ possessed decreased levels of Fru 6-P and Glu 6-P and higher amounts of 3PGA (Fernie *et al.*, 2001). Transgenic potato tubers with a 3-fold increase in Fru 2,6-P₂ also possessed lower levels of hexose phosphates and increased triose phosphates (Kruger and Scott, 1994). In both studies the changes in metabolites were attributed to the stimulation of PFP. In addition tubers in which PFP activity was decreased by anti-sense inhibition possessed less triose phosphates and more hexose phosphates (Hajirezaei *et al.*, 1994). The above argues that in these tissues PFP is a glycolytic enzyme.

Increased Fru 2,6-P₂ levels are associated with the dormancy-breaking process and germination in *C. lanatus* (Botha and Botha, 1993), Jerusalem artichoke tubers (Van Schaftingen and Hers, 1983), and apple (Bogatek, 1995). Only some dormancy-breaking chemicals rapidly increased embryo Fru 2,6-P₂ levels in red rice (Footitt and Cohn, 1995). Nevertheless, embryo Fru 2,6-P₂ levels were always highly correlated with the subsequent germination rate. The growth stimulant lepidimic acid increases Fru 2,6-P₂ in *Amaranthus* seedlings. Lepidimic acid treatment also increased Fru 1,6-P₂ and decreased Fru 6-P suggesting that PFP is stimulated (Kato-Noguchi *et al.*, 2001).

Scott and Kruger (1995) illustrated a role for Fru 2,6-P₂ in starch breakdown in tobacco leaves in the dark. Transgenic tobacco with increased Fru 2,6-P₂ levels showed a gradual accumulation of starch throughout their growth. Results implied that starch accumulated as a

result of a decrease in starch mobilisation in the dark, rather than an increase in starch synthesis during the photoperiod. The authors argued that the elevated Fru 2,6-P₂ stimulated PFP that caused an increased 3PGA/Pi ratio in the cytosol in the dark. If transmitted to the chloroplast, an increased 3PGA/Pi ratio would activate AGPase (Preiss, 1982) that explains the decrease in starch mobilisation observed in the dark (Scott and Kruger, 1995).

In contrast, a subsequent investigation (using calli derived from the above study) showed no relationship between Fru 2,6-P₂ and starch metabolism in the dark (Fernie *et al.*, 2001). A possible explanation provided by the authors was that the stimulation of PFP will not only increase 3PGA but also decrease hexose phosphates. The latter is an immediate precursor for starch synthesis (Keeling *et al.*, 1988). Therefore, although AGPase is stimulated there is also a decrease in its substrate. In addition the differences (increased sucrose and slightly lower starch) observed between wild type and transgenic *Arabidopsis* with decreased Fru 2,6-P₂ was eliminated in the dark even though significant differences in Fru 2,6-P₂ persisted (Draborg *et al.*, 2001). Thus, although Fru 2,6-P₂ could under certain conditions influence starch metabolism in the dark, it is apparently not directly involved.

The finding that hexose moieties rather than triose phosphates are imported for starch accumulation in non-photosynthetic plastids (Tyson and ap Rees, 1988; Stitt, 1990a), excludes PFP (if operating in the glycolytic direction) to be crucial in starch synthesis. PFP might however be indirectly involved in starch synthesis (section 2.5.2) by generating PPI (section 2.5.3) that is needed for sucrose synthase mediated mobilisation of sucrose to provide the hexose moieties for starch synthesis (Stitt, 1990a).

2.5.2 A role for PFP in gluconeogenesis and starch accumulation

The measured FBPase1 activity in the cotyledons of *Citrullus lantus* is insufficient to sustain gluconeogenesis (Botha and Botha, 1993). Here, Fru 2,6-P₂ increased during gluconeogenesis and coincided with an increase in PFP activity. The PFP activity was adequate to sustain the calculated gluconeogenetic flux.

Crassulacean acid metabolism (CAM) plants perform nocturnal fixation of CO₂ and the produced malate is stored in the vacuole. In a starch-storer CAM plant such as *Bryophyllum tubiflorum* malate is decarboxylated and the produced PEP is converted to starch in the chloroplast during the day. Alternatively, PEP is converted to soluble sugars extrachloroplastic as in *Ananas comosus* (pineapple) (Black *et al.*, 1982). This plant possesses high Fru 2,6-P₂ levels (500 pmol.g⁻¹ fresh weight (FW)) compared to *B. tubiflorum* (10 pmol.g⁻¹ FW) during deacidification (Fahrendorf *et al.*, 1987). In addition *A. comosus* contains 35-times higher PFP activity than *B. tubiflorum* (Fahrendorf *et al.*, 1987). Because FBPase1 is likely to be largely

inhibited by the high Fru 2,6-P₂ levels in *A. comusus*, PFP might play a gluconeogenic role during deacidification in this CAM species.

Tobacco transformed with a PFP gene from *Giardia lamblia* (that is insensitive to regulation by Fru 2,6-P₂), contained decreased leaf starch at the beginning and end of the photoperiod (Wood *et al.*, 2002). Hajirezaei *et al.* (1994) reported that antisense PFP potato tubers possessed 20 – 50% less starch than the wild type tubers. Another study on potato found no relationship between starch synthesis and Fru 2,6-P₂ levels (Morell and ap Rees, 1986). In addition the ratio between PFP and PFK decreased before starch accumulation started in maize endosperm (Doehlert *et al.*, 1988).

Members of *Alliaceae* store no starch or lipids and are therefore unlikely to conduct gluconeogenesis in their non-photosynthetic tissues. Nevertheless these plants contain appreciable PFP activity implying that gluconeogenesis is not the primary function of PFP (ap Rees *et al.*, 1985a; ap Rees *et al.*, 1985b).

PFP might play an important gluconeogenic role in the base of young tobacco leaves (Nielsen and Stitt, 2001). Interestingly, in contrast to the classical decrease in Fru 2,6-P₂ observed in leaves during the transition from dark to light, Fru 2,6-P₂ levels increase upon illumination in this area of the leaf. The increase in Fru 2,6-P₂ will inhibit FBPase1 and activate PFP suggesting that PFP rather than FBPase1 is involved in sucrose synthesis in the light in the leaf base. The maximum velocity (V_{max}) of PFP is 10-times higher than that of FBPase1 in this area of the tobacco leaf and it decreases towards the leaf tip (Nielsen and Stitt, 2001).

2.5.3 A role for PFP in PPI metabolism and metabolite cycling

Unlike PFK and most enzymes in metabolism, PFP and at least two other plant cytosolic enzymes, i.e. a PPI dependent proton pump (H⁺-pyrophosphatase, EC 3.6.1.1) located in the tonoplast (Rea and Sanders, 1987) and UDP-glucose pyrophosphorylase (EC 2.7.7.9) (ap Rees *et al.*, 1985b) utilise PPI instead of ATP as the phosphoryl donor.

PPI is produced during the synthesis of sucrose and macromolecules such as proteins, nucleic acids and polysaccharides. One reason for high plant cytosolic PPI concentrations (about 250 μM (Weiner *et al.*, 1987)) is that pyrophosphatase (catalyses the hydrolysis of PPI) is restricted to chloroplasts and the tonoplast in plants (Gross and ap Rees, 1986).

In contrast to adenine nucleosides PPI levels are unaffected by Pi deprivation (Dancer *et al.*, 1990b). It was proposed that PFP acts as an adenylate bypass for the PFK reaction under such stress conditions (Duff *et al.*, 1989). Fru 2,6-P₂ levels also increase in response to different

treatments that decrease the ATP/ADP ratio (Stitt, 1990a). In agreement with this proposal the increase in Fru 2,6-P₂ observed during prolonged oxygen deprivation in rice seedlings coincides with the synthesis of PFP but not of PFK (Mertens *et al.*, 1990).

Plant metabolism is characterised by rapid metabolite cycling in which metabolites are synthesised and degraded again. These include cycles between hexoses and sucrose (Wendler *et al.*, 1990), and between hexose monophosphates and triose monophosphates in which a role for PFP was indicated in *C. rubrum* (Hatzfeld and Stitt, 1990), potato (Hajirezaei *et al.*, 1994) and carrot (Krook *et al.*, 2000). It was postulated that the amount of sucrose in heterotrophic cell suspension cultures of *C. rubrum* (Dancer *et al.*, 1990a) and sugarcane (Wendler *et al.*, 1990) (section 2.6) is determined by the nett product of the cycle of sucrose synthesis and degradation. It was also postulated that the sucrose and triose phosphate cycles are coupled by their requirement for PPi (Dancer *et al.*, 1990a; Hatzfeld and Stitt, 1990). However results from tobacco calli indicated no obligatory link between these cytosolic cycles (Ferne *et al.*, 2001).

PFP might be involved in the regulation of PPi concentrations in the plant cytosol by participating in substrate cycles with PFK or FBPase1 to generate or remove PPi respectively (Neuhaus *et al.*, 1990a; Stitt, 1990a). However transgenic potato tubers with near 100% removal of their PFP activity contained similar PPi concentrations than the wild type plants (Hajirezaei *et al.*, 1994).

Costa dos Santos *et al.* (2003) demonstrated that the PPi used by H⁺-pyrophosphatase to create a proton gradient across the tonoplast in maize roots originates from Fru 1,6-P₂ cleavage catalysed by PFP in the reverse direction – PFP also regenerates the PPi that was hydrolysed. They showed that the addition of 20 mM Fru 6-P (thus driving the PFP reaction in the opposite direction) dissipated the pH gradient formed by the H⁺-pyrophosphatase.

It was also proposed that a key function of PFP is to generate PPi needed for sucrose synthase mediated breakdown of sucrose (ap Rees *et al.*, 1985a; Xu *et al.*, 1986). However results presented by Dancer and ap Rees (1989) argued that PFP is involved but not uniquely associated with sucrose breakdown. They studied starch accumulation in developing endosperm of wild type and *sh1* maize mutants that are deficient in sucrose synthase activity. Sucrose breakdown in the wild type occurred mainly via the sucrose synthase pathway whereas the mutants compensated for the lack of this enzyme by increased alkaline invertase and glucokinase activities. The activity of PFP and levels of PPi and Fru 2,6-P₂ were similar in the mutants and wild type plants.

Fru 2,6-P₂ increases the K_m of carrot root PFP for Fru 6-P (3-fold) (Wong *et al.*, 1988). This will prevent excess sucrose breakdown and retain hexoses for the resynthesis of sucrose, which might be an adaptation in tissues that store substantial amounts of sucrose (Wong *et al.*, 1988). However this is not a common feature of PFP' enzymes from sucrose-storing tissues because Fru 2,6-P₂ does not decrease the affinity of sugarcane PFP for Fru 6-P (personal communication J-H Groenewald³).

2.6 Sucrose accumulation in sugarcane

Commercial sugarcane varieties contain up to 25% stem FW sucrose (Bull and Glasziou, 1963). The sugarcane stem consists of internodes that are at different stages of development with the more mature internodes (highest sucrose content) at the bottom (Moore, 1995). Sucrose accumulation (ripening) increases after internode elongation has stopped and is inversely correlated with the availability of growth-promoting nutrients (Das, 1936; Veith and Komor, 1993). There is a rapid increase in the rate of sucrose accumulation between internodes 4 to 7, indicating that sucrose accumulation is not merely a function of time (Whittaker and Botha, 1997).

Although sucrose metabolism in plant photosynthetic tissues is well understood (section 2.4.1), the biochemical basis and the important controlling steps of sucrose accumulation in the sugarcane stem are still unclear (Moore and Maretzki, 1996; Grof and Campbell, 2001). Leaf sucrose content is not significantly correlated with stem sucrose (Grof and Campbell, 2001). Stalk biomass is also not significantly correlated with sucrose concentration (Zhu *et al.*, 1997).

The sucrose content of *Saccharum officinarum* and *S. spontaneum* (modern sugarcane varieties are multispecies hybrids of among other these two species) is 17.48% FW and 3.96% FW respectively (Bull and Glasziou, 1963). The rate of photosynthesis of *S. spontaneum* is nearly double that of *S. officinarum* (Irvine, 1975), which indicates that the difference in sucrose storage between these species is not regulated by source activity (Moore and Maretzki, 1996). However the fact that sucrose accumulation is slower during periods of rapid growth than during periods of slow growth implies that at least under certain conditions source activity influences the amount of sucrose allocated for storage in the stem (Moore and Maretzki, 1996). Increased source activity might improve sucrose accumulation in the internodes if it coincides with more photosynthate partitioned into storage than structural elements (Birch, 1996).

The rate of phloem loading and the translocation process of sucrose from source to sink tissues might be rate-limiting steps in sucrose accumulation (Moore and Maretzki, 1996). A comparison of sugar beet and fodder beet, related species that differ significantly in their sucrose storing

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abilities, showed that phloem loading is the determining factor for sucrose accumulation in the taproots of these plants (Heldt *et al.*, 1998). If phloem loading is limited, the build-up of sucrose in the leaf will deactivate SPS (Stitt, 1985; Stitt *et al.*, 1988), resulting in an increase in Fru 2,6-P₂ that will inhibit photosynthetic sucrose synthesis (Stitt *et al.*, 1983; Gerhardt *et al.*, 1987; Scott and Kruger, 1994). It will be interesting to see if this regulatory mechanism could be overridden in transgenic sugarcane plants with altered leaf Fru 2,6-P₂ levels and what the subsequent effect on stem sucrose content would be.

Arriving at the sink tissue the sucrose is unloaded into the apoplast and hydrolysed by acid invertase (EC 3.2.1.26) to produce hexoses (glucose and fructose) before entering the sink parenchyma cells (Sacher *et al.*, 1963; Hawker and Hatch, 1965). Alternatively sucrose could follow a symplastic route. This is probably mandatory in mature sugarcane internodes since the cell walls of the bundle sheath cells are suberised forming an apoplastic barrier (Jacobsen *et al.*, 1992). The abundance of plasmodesmata that connect the phloem to the storage parenchyma cells in matured internodes supports this view (Walsh *et al.*, 1996).

Inside the sink parenchyma cells, depending on the physiological demands of the plant, sucrose could be utilised for metabolism and respiration or moved to the vacuole for storage (Hawker *et al.*, 1991). The vacuole occupies up to 90% of the total volume of the sugarcane culm cell (Komor, 1994).

Cell suspension cultures are often used to study sucrose accumulation due to their simplicity, fast growth rate and a similar sucrose accumulation profile than intact internodes. When transferred to new medium these cultures are characterised by an initial period of fast growth followed by a slow growth phase during which sucrose is accumulated to a concentration of about 150 mM (Thom *et al.*, 1982; Veith and Komor, 1993). Although there is a decline in both nitrogen and phosphate before sucrose starts to accumulate (Thom *et al.*, 1982; Wendler *et al.*, 1990; Veith and Komor, 1993), only nitrogen depletion provokes sucrose storage (Veith and Komor, 1993).

Sucrose is simultaneously synthesised and degraded within the sugarcane storage tissue (Sacher *et al.*, 1963; Batta and Singh, 1986; Wendler *et al.*, 1990). About 30% of the sucrose that is synthesised is cleaved again (Whittaker and Botha, 1997). This phenomenon is referred to as “futile cycling” (Dancer *et al.*, 1990a) because it appears to be energetically expensive. However cycling allows the switch between rapid sucrose mobilisation and storage with only small changes enzyme and metabolite levels (Wendler *et al.*, 1990). In addition cycling results in the partitioning of osmotica among compartments that insures low turgor that promotes the translocation of sucrose from the source to sink while sucrose accumulates to high levels

(Moore, 1995). It is believed that the net product of these opposing cycles of sucrose synthesis and degradation determines the sucrose content in sugarcane (Wendler *et al.*, 1990). The enzymes involved in sucrose metabolism in the sugarcane stem are discussed next.

2.6.1 Enzymes involved in sucrose metabolism

The sucrolytic enzymes are SuSy and invertase (figure 2.4). Different isoforms of invertase that differ in pH optima, subcellular location and solubility have been characterised (Avigad, 1982; Hawker, 1985).

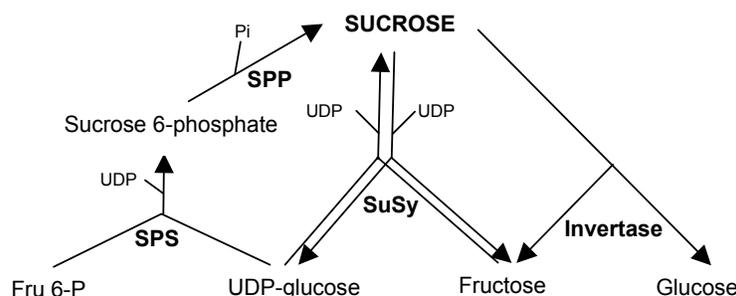


Figure 2.4. Enzymes directly involved in sucrose metabolism.

Vacuolar acid invertase (VAI) or soluble acid invertase is largely located in the vacuole (Leigh *et al.*, 1979) but is also present in the apoplastic cell wall space (Glasziou and Waldron, 1964). Sugarcane varieties that retain high VAI activity in mature internodes have a poor sucrose storing ability (Hatch and Glasziou, 1963). Varieties with low VAI activity however do not necessarily store high levels of sucrose. Transgenic sugarcane with reduced VAI activity did not store increased sucrose either (unpublished results of CPL Grof and FC Botha⁴). In contrast, sugarcane calli (liquid culture) transformed with a sugarcane antisense VAI gene showed a 65% reduction in VAI activity and a 2-fold increase in sucrose accumulation (Ma *et al.*, 2000).

Cell wall invertase (CWI) or insoluble acid invertase is localised in the apoplast and is ionically linked to the cell wall (Avigad, 1982; Hawker, 1985). There is no correlation between sucrose storage and extracellular invertase activity in sugarcane cell suspension cultures (Veith and Komor, 1993).

Soluble neutral invertase catalyses sucrose hydrolysis in the cytosol (Avigad, 1982; Hawker, 1985). The gene encoding the sugarcane neutral invertase (SNI) was isolated (Bosch *et al.*, 2003) and expression profiles from Northern and protein blot analysis were similar to the

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enzyme activity patterns reported by Voster and Botha (1999). SNI expression increases from internode 3 to 5 and then decreases towards the older internodes. In sucrose accumulating tissue SNI has a higher specific activity than VAI (Voster and Botha, 1999). SNI is a promising target for genetic manipulation according to the *in vitro* kinetic model developed by Rohwer and Botha (2001). This model predicts that a decrease in SNI could reduce futile cycling that might increase sucrose storage.

The reaction catalysed by SuSy is reversible. Although SuSy activity was 10 to 15-fold higher than the activity of SPS in sugarcane cell suspension cultures, it did not make any appreciable contribution to sucrose synthesis (Wendler *et al.*, 1990). Sucrose was exclusively synthesised by SPS (via sucrose 6-phosphate and sucrose-phosphate phosphatase (SPP, EC 3.1.3.24, figure 2.4). In young internodal tissue SuSy contributes to sucrose synthesis (Botha and Black, 2000) but despite comparable SuSy and SPS activities in mature internodal tissue (Zhu *et al.*, 1997; Botha and Black, 2000) SPS solely synthesises sucrose (Botha and Black, 2000).

Immunohistochemistry revealed that sugarcane SuSy is localised in both the vascular and storage parenchyma tissue in young and mature internodes (Schäfer *et al.*, 2004). Results from other higher plants demonstrated a role for SuSy in phloem unloading in sink tissues (Martin *et al.*, 1993) and suggested that SuSy activity is associated with an actively filling sucrose sink and therefore an indicator of sink strength (Clausen *et al.*, 1986; Sung *et al.*, 1989; Farrar, 1993). In sugarcane SuSy might be an indicator of sucrose import for respiration and other biosynthetic activities such as supplying UDP-glucose for cell wall synthesis in young actively growing tissue (Lingle and Smith, 1991). SuSy is not a good indicator of sink strength for sucrose accumulation however (Botha and Black, 2000). In addition previous work suggested a relationship between SuSy activity and sucrose accumulation (Lingle, 1996) and ripening in sugarcane (Lingle and Irvine, 1994), but subsequent results showed no correlation between sugar accumulation and SuSy activity (Zhu *et al.*, 1997; Lingle, 1999; Botha and Black, 2000).

Maximum SPS activity increases significantly during sucrose accumulation in cell suspension cultures (Wendler *et al.*, 1990) and during internode maturation (Lingle, 1999; Botha and Black, 2000). Botha and Black (2000) showed that both the specific and total activity of SPS are highly correlated with sucrose content in internodes of variety N19. Lingle (1999) found a correlation between sucrose content and SPS activity in developing internodes but not in matured internodes. Zhu *et al.* (1997) reported that SPS activity alone is not correlated with stem sucrose. However sucrose concentration was strongly correlated with the difference in the activities of SPS and VAI. Similar results showing a correlation between sucrose content and SPS activity minus acid invertase activity were obtained from sugarcane grown at optimum, below and above optimum temperatures (Ebrahim *et al.*, 1998).

2.6.2 A role for PFP in sucrose accumulation in sugarcane internodal tissue

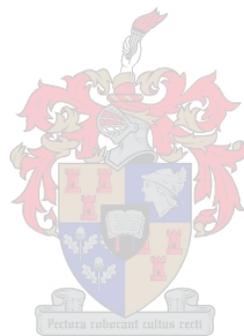
Sucrose accumulation in sugarcane coincides with the redirection of carbon from insoluble matter, amino acids, phosphorylated intermediates and respiration into sucrose as evident from research done on both cell suspension cultures (Veith and Komor, 1993) and internodal tissue (Moore, 1995; Whittaker and Botha, 1997; Whittaker and Botha, 1999). The decreased allocation of carbon to respiration corresponds with a decreased flux from hexoses (Whittaker and Botha, 1997), which might be attributed to the decline in PFP activity that is observed with internodal maturation (Whittaker and Botha, 1999).

Whittaker and Botha (1999) showed that PFP activity and stem sucrose are inversely correlated across sugarcane varieties. In contrast, no relationship was found between PFK activity and sucrose content. In the absence of Fru 2,6-P₂, sugarcane PFP and PFK have comparable activities. However in the presence of 1 μM Fru 2,6-P₂, PFP activity exceeds PFK activity several times (Lingle and Smith, 1991). Transgenic sugarcane plants with reduced expression of the endogenous PFP β-subunit (contains the catalytic site (Yan and Tao, 1984; Botha and Botha, 1991)) possessed increased sucrose in maturing internodes when compared to control plants (Groenewald and Botha, 2001). In contrast to the above studies, Lingle and Smith (1991) found no clear relationship between sucrose content and the activity of PFP in sugarcane internodes.

Although PFP and sucrose was inversely correlated in the study performed by Whittaker and Botha (1999), their results did not indicate a correlation between PFP activity and Fru 2,6-P₂ levels and also not between Fru 2,6-P₂ levels and sucrose content. Sugarcane PFP activity was however correlated with the protein content of the β-subunit. This demonstrates that sugarcane PFP is at least in part under transcriptional and / or translational coarse control.

The concentration of total extractable Fru 2,6-P₂ in sugarcane internode 7 is between 2.3 and 4.2 μM (Whittaker and Botha, 1999). This concentration is considerable more than the K_a of sugarcane PFP that is 69.3 nM and 82.2 nM for the forward and reverse reaction respectively (personal communication J-H Groenewald)⁵. Nevertheless, it is now accepted that PFP is not necessarily fully activated *in vivo* (section 2.4.2). Thus, allosteric regulation by Fru 2,6-P₂ is not excluded. Lingle and Smith (1991) compared Fru 2,6-P₂ and sucrose levels in three independent experiments. A significant negative correlation between Fru 2,6-P₂ and sucrose was observed in one experiment, indicating that under certain conditions Fru 2,6-P₂ might be involved in regulating sucrose metabolism in the sugarcane culm. In addition both PFP activity and Fru 2,6-P₂ levels decrease before sucrose storage commenced in cell suspension cultures

(Wendler *et al.*, 1990). Transgenic sugarcane plants with altered Fru 2,6-P₂ levels could be valuable in gaining insights in the function of Fru 2,6-P₂ in sucrose metabolism in non-photosynthetic tissue.



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CHAPTER 3

Expression of functional recombinant rat 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase in *Escherichia coli*

Abstract

In vivo levels of fructose 2,6-bisphosphate are controlled by the ratio between the activities of 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase. In this study, two plasmid constructs harbouring genes that encode for recombinant rat liver 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase respectively were prepared and introduced into *Escherichia coli*. Activity tests performed on the bacterially expressed glutathione-S-transferase fusion proteins showed that they are catalytically active. In addition antibodies were raised against the bacterially expressed proteins. The bacterial system was applied to verify that the genes encode functional enzymes before including them in a sugarcane transformation programme.

3.1 Introduction

Fructose 2,6-bisphosphate (Fru 2,6-P₂) is a regulator of carbohydrate metabolism and is common to all eukaryotes (Van Schaftingen, 1987; Okar *et al.*, 2001). The *in vivo* concentration of Fru 2,6-P₂ is regulated by 6-phosphofructo 2-kinase (6PF2K, EC 2.7.1.105) and fructose 2,6-bisphosphatase (FBPase2, EC 3.1.3.11), that synthesise and hydrolyse Fru 2,6-P₂ respectively (Claus *et al.*, 1984). 6PF2K and FBPase2 are mostly expressed by a single gene and located on a bifunctional enzyme (Pilkis *et al.*, 1987).

The rat (*Rattus norvegicus*) liver bifunctional enzyme is a homodimeric protein and each subunit is composed of a regulatory domain (residues 1 – 36), a kinase domain (residues 37 – 249) and a bisphosphatase domain (residues 250 – 470). Serine residue 32 (Ser³²) acts as a phosphorylation site. Phosphorylation is performed by a protein kinase that is activated by a cascade of reactions triggered by cyclic adenosine 5'-monophosphate (cAMP) in response to a hormonal (glucagon) signal. Phosphorylation inhibits 6PF2K and activates FBPase2 (Pilkis *et al.*, 1988). Histidine residue 258 (His²⁵⁸) is essential for FBPase2 activity (Tauler *et al.*, 1990).

A recombinant 6PF2K (or kinase-only mutant) was generated with site directed mutagenesis by changing Ser³² and His²⁵⁸ of the rat bifunctional enzyme both to alanine (Tauler *et al.*, 1989; Tauler *et al.*, 1990). Silent mutations were also introduced (figure 3.1A). An in-frame *Bgl*II deletion of 612 base pairs (bp) to the native rat gene subsequently removed amino acids 15 – 218 and resulted in a recombinant FBPase2 (or phosphatase-only mutant) (Colosia *et al.*, 1988) (figure 3.1B).

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A DNA 1 TCTCGAGAGATGGGAGAACTCACTCAAACCAGGTTACAGAAGATCTGGATTCCACACAGC 60
aa 1 S R E M G E L T Q T R L Q K I W I P H S 20
AGCAGT AGC TCC
61 AGTTCGAGCTCCGTGCTGCAACGGCGAAGGGGCGCATCCATACCACAGTTCACTAATTCT 120
21 S S S S V L Q R R R G A S I P Q F T N S 40
S S S S
121 CCCACGATGGTGATTATGGTGGGTTTACCAGCTCGAGGCAAGACCTACATCTCTACGAAG 180
41 P T M V I M V G L P A R G K T Y I S T K 60

181 CTCACACGCTATCTCAACTGGATAGGAACACCAACTAAAGTGTTAATTTAGGTCAGTAT 240
61 L T R Y L N W I G T P T K V F N L G Q Y 80

241 CGACGAGAGGCAGTGAGTTACAGGAACTATGAATTTCTTCGCCAGACAACACAGAGGCC 300
81 R R E A V S Y R N Y E F F R P D N T E A 100

301 CAGCTTATCAGGAAGCAGTGTGCTCTAGCAGCCCTAAAGGATGTCCATAAGTATCTCAGC 360
101 Q L I R K Q C A L A A L K D V H K Y L S 120

361 CGCGAGGAAGGTCATGTTGCGGTTTTTGATGCCACCAACTATACCAGAGAACGGAGATCG 420
121 R E E G H V A V F D A T N Y T R E R R S 140

421 TTGATTCTACAGTTTGCTAAGGAACATGGTTATAAGTTTTTTTTATTGAGTCTATTTGT 480
141 L I L Q F A K E H G Y K V F F I E S I C 160

481 AATGACCCCGAAATTATGCAGAAAACATCAAGCAAGTAAACTTGGTAGTCCTGATTAC 540
161 N D P E I I A E N I K Q V K L G S P D Y 180

541 ATAGACTGTGACCAAGAAAAGGTTTTGGAAGACTTTCTAAAGAGAATAGAGTGCTATGAG 600
181 I D C D Q E K V L E D F L K R I E C Y E 200

601 ATCAACTACCAACCTTTGGATGAGGAATGGACAGCCACCTGTCTACATCAAGATCTTC 660
201 I N Y Q P L D E E L D S H L S Y I K I F 220

661 GACGTGGGCACACGCTACATGGTAAATCGAGTGCAGGACCACGTTTCAGAGCCGTACAGCC 720
221 D V G T R Y M V N R V Q D H V Q S R T A 240

721 TACTACCTCATGAACATCCATGTCACACCTCGATCTATCTACCTATGCCGCCATGCTGGTGAG 780
241 Y Y L M N I H V T P R S I Y L C R A G E 260
H

781 AGTGAACTCAACCTTAGAGGCCGATTGGAGGTGACTCTGGCCTCTCAGCTCGGGGCAAG 840
261 S E L N L R G R I G G D S G L S A R G K 280

841 CAGTATGCCTATGCACTAGCCAACTTCATCCGGTCTCAAGGCATCAGCTCCCTGAAAAGTA 900
281 Q Y A Y A L A N F I R S Q G I S S L K V 300

901 TGGACTAGCCACATGAAGAGGACCATTAGACCCGCTGAAGCCCTAGGTGTCCCTATGAA 960
301 W T S H M K R T I Q T A E A L G V P Y E 320

961 CAGTGAAGGCCCTGAATGAGATTGATGCGGGTGTCTGTGAAGAGATGACCTATGAAGAA 1020
321 Q W K A L N E I D A G V C E E M T Y E E 340

1021 ATTACAGAACACTACCCTGAGGAATTTGCACTACGGGACCAGGATAAATATCGTTACCGC 1080
341 I Q E H Y P E E F A L R D Q D K Y R Y R 360

1081 TATCCGAAGGGAGAGTCCTATGAGGATCTGGTTCAGCGTCTTGAACCAGTTATAATGGAG 1140
361 Y P K G E S Y E D L V Q R L E P V I M E 380

1141 CTAGAACGGCAAGAAAATGTACTGGTGATCTGTCCAGGCTGTATGCGGTGCCTCCTG 1200
381 L E R Q E N V L V I C H Q A V M R C L L 400

1201 GCATACTTCTGGATAAAAAGTTTCAGATGAGCTGCCCTATCTCAAGTGTCTCTGCATACT 1260
401 A Y F L D K S S D E L P Y L K C P L H T 420

1261 GTGCTCAAACCTCACACCTGTGGCTTATGGCTGCAGAGTGGAGTCCATCTACCTGAATGTG 1320
421 V L K L T P V A Y G C R V E S I Y L N V 440

1321 GAGGCTGTGAACACACACCGGGACAAGCCTGAGAATGTGGACATCACCCGTGAAGCTGAG 1380
441 E A V N T H R D K P E N V D I T R E A E 460

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	1381	GAAGCCTTGGACACTGTACCTGCCATTA	1409
	461	E A L D T V P A H	470

B	DNA	1 TCTCGAGAGATGGGAGAAGTCACTCAAACAGGTTACAGAAGATCTTCGACGTGGGCACA	60
	aa	1 S R E M G E L T Q T R L Q K I F D V G T	20
		61 CGCTACATGGTAAATCGAGTGCAGGACCACGTTTCAGAGCCGTACAGCCTACTACCTCATG	120
		21 R Y M V N R V Q D H V Q S R T A Y Y L M	40
		121 AACATCCATGTCACACCTCGATCTATCTACCTATGCCGCCATGGTGAGAGTGAACCAAC	180
		41 N I H V T P R S I Y L C R H G E S E L N	60
		181 CTTAGAGGCCGATTGGAGGTGACTCTGGCCTCTCAGCTCGGGCAAGCAGTATGCCTAT	240
		61 L R G R I G G D S G L S A R G K Q Y A Y	80
		241 GCACTAGCCAACCTTCATCCGGTCTCAAGGCATCAGCTCCCTGAAAGTATGGACTAGCCAC	300
		81 A L A N F I R S Q G I S S L K V W T S H	100
		301 ATGAAGAGGACCATTGACACCGCTGAAGCCCTAGGTGTCCCTATGAACAGTGAAGGCC	360
		101 M K R T I Q T A E A L G V P Y E Q W K A	120
		361 CTGAATGAGATTGATGCGGGTGTCTGTGAAGAGATGACCTATGAAGAAATTCAGGAACAC	420
		121 L N E I D A G V C E E M T Y E E I Q E H	140
		421 TACCTGAGGAATTTGCACTACGGGACCAGGATAAATATCGTTACCGCTATCCGAAGGGA	480
		141 Y P E E F A L R D Q D K Y R Y R Y P K G	160
		481 GAGTCCTATGAGGATCTGGTTCAGCGTCTGAACAGTTATAATGAGCTAGAACGGCAA	540
		161 E S Y E D L V Q R L E P V I M E L E R Q	180
		541 GAAAAAGTACTGGTGATCTGTCAACAGGCTGTGATGCGGTGCCTCCTGGCATACTTCCTG	600
		181 E N V L V I C H Q A V M R C L L A Y F L	200
		601 GATAAAAGTTCAGATGAGCTGCCCTATCTCAAGTGTCTCTGCATACTGTGCTCAAACCTC	660
		201 D K S S D E L P Y L K C P L H T V L K L	220
		661 ACACCTGTGGCTTATGGCTGCAGAGTGGAGTCCATCTACCTGAATGTGGAGGCTGTGAAC	720
		221 T P V A Y G C R V E S I Y L N V E A V N	240
		721 ACACACCGGGACAAGCCTGAGAATGTGGACATCACCCGTGAAGCTGAGGAAGCCTTGGAC	780
		241 T H R D K P E N V D I T R E A E E A L D	260
		781 ACTGTACCTGCCATTAC	798
		261 T V P A H Y	266

Figure 3.1. The DNA and amino acid sequences of the recombinant 6PF2K (A) and FBPase2 (B). Regions of the recombinant 6PF2K as a result of site directed mutagenesis are highlighted in yellow (original rat sequence in red). The region in blue represents the *Bgl*III (recognition site: A/GATCT) deletion to the original rat sequence. Religation of the remaining sequence resulted in the recombinant FBPase2.

These recombinant 6PF2K and FBPase2 genes were used in tobacco leaves to alter the levels of Fru 2,6-P₂ (Scott *et al.*, 1995; Scott *et al.*, 2000). After verifying that the recombinant genes are intact and that they encode for functional enzymes we will use a similar genetic engineering approach to manipulate the levels of Fru 2,6-P₂ in sugarcane.

In this study the recombinant rat 6PF2K and FBPase2 genes were expressed in a bacterial system as glutathione-S-transferase (GST) fusion proteins. Activity tests performed on these

enzymes indicated that they are functional. In addition antibodies were raised against the recombinant proteins.

3.2 Materials and Methods

3.2.1 Plasmid constructs

General molecular biology techniques (Sambrook *et al.*, 1989) were applied for the preparation of the plasmids. All deoxyribonucleic acid (DNA) modifying enzymes were from Promega (Madison, AL, USA). Plasmids (pKK 233-2_PK2 and pF26-2P) harbouring the recombinant rat liver 6PF2K and FBPase2 genes were obtained from Dr NJ Kruger (University of Oxford, Oxford, UK).

The recombinant 6PF2K gene was digested from pKK 233-2_PK2 with restriction endonuclease *HindIII*. This 1425 bp fragment was then blunt-ended with the Klenow fragment of DNA polymerase I and cloned in-frame with the initiation codon of the GST gene into pGEX 4T1 (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) (digested with *AvaI*, blunt-ended and dephosphorylated with shrimp alkaline phosphatase). This recombinant 6PF2K bacterial expression vector was designated pBF2K 4T1.

The recombinant FBPase2 gene was digested from pF26-2P with restriction endonucleases *XhoI* and *NotI*. This 849 bp fragment was cloned in-frame with the initiation codon of the GST gene into pGEX 4T1 (digested with *SalI* and *NotI*). This recombinant FBPase2 bacterial expression vector was designated pBF2P 4T1.

3.2.2 Bacterial expression of the recombinant 6PF2K and FBPase2 enzymes

The GST Gene Fusion System (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) in combination with a method described by Tauler *et al.* (1988) were applied for the bacterial expression of the recombinant 6PF2K and FBPase2 genes. Bacterial cells (*Escherichia coli*, BL21) harbouring either pGEX 4T1, pBF2K 4T1 or pBF2P 4T1 were grown at 37 °C in 200 ml Luria-Bertani (LB) broth supplemented with 100 µg.ml⁻¹ ampicillin. Once an optical density at 600 nm (OD)₆₀₀ of 0.5 was reached, fusion protein expression was induced by the addition of 0.25 mM isopropyl β-D-thiogalactoside (IPTG). The pGEX 4T1 vector contains the *tac* promoter (a hybrid of the *E. coli trp* and *lac* promoters) that is IPTG inducible. The bacterial cells were allowed to grow for a further 2.5 h at 37 °C (for activity tests). The induction period for proteins expressed for antibody production was 72 h at room temperature. The cells were harvested by centrifugation for 20 min at 3000 *xg*. The pellet was resuspended in 5 ml of extraction buffer containing (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.5), 100 mM KCl, 1 mM 2,4-dithiothreitol (DTT), 2 mM ethylene diamine tetra-acetic acid (EDTA), 5 mM potassium phosphate, 1 mg.ml⁻¹ lysozyme, and Complete miniTM protease inhibitor tablets

(Roche, Mannheim, Germany) at 1 tablet per 10 ml. The suspension was incubated for 30 min on ice. The cells were then subjected to three freeze-thaw cycles in dry ice / ethanol. 10 mM MgSO₄ and 0.1 mg.ml⁻¹ deoxyribonuclease I (DNase I, EC 3.1.21.1) were added to the disrupted cells and the cells were incubated for 1 h at 4 °C. The lysate was centrifuged twice at 16 000 xg for 10 min at 4 °C and the supernatant clarified by filtration through a 0.45 µm filter. The binding of the fusion protein to the glutathione sepharose 4B affinity column and the subsequent elution was performed according to a GST Gene Fusion System manual. Following the addition of 10% (v/v) glycerol the bacterial protein extracts were frozen in liquid nitrogen and stored at -80 °C until further use.

3.2.3 Activity test for 6PF2K

A pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) coupled assay procedure was applied for the measurement of 6PF2K activity (Stitt, 1990b). This method relies on the positive correlation between PFP activity and Fru 2,6-P₂ (its activator) concentration.

The potato tuber PFP was purified according to Van Schaftingen *et al.* (1982b). All other enzymes were from Roche (Mannheim, Germany).

Commercial preparations of fructose 6-phosphate (Fru 6-P) contain traces of Fru 2,6-P₂ (Kruger *et al.*, 1983) that was removed by an acid treatment: 0.1 volumes of 2 M HCl was added to the Fru 6-P solution and incubated for 30 min at room temperature. The solution was then neutralised with the same volume of 2 M NaOH and Tris-Acetate (pH 7.8) was added to a concentration of 12.5 mM.

In the first part of the assay (figure 3.2A), 10 µl of the bacterial protein extract from pBF2K 4T1- or pGEX 4T1-harboured cells (negative control) was incubated in a kinase assay buffer containing 50 mM Tris-Acetate (pH 7.8), 5 mM magnesium acetate, 5 mM KH₂PO₄ and 5.6 mM acid-treated Fru 6-P (final volume of 180 µl). Activity of 6PF2K was initiated by the addition of 2.8 mM adenosine 5'-triphosphate (ATP). Immediately and at certain time points thereafter, 20 µl aliquots were removed and alkalinised (to stop the reaction) in 30 µl 250 mM KOH.

In the second part of the assay (figure 3.2B) the amount of Fru 2,6-P₂ produced in the aliquots was quantified through the stimulation of potato tuber PFP (Van Schaftingen *et al.*, 1982b). Assay buffer (200 µl) containing 25 mM Tris-Acetate (pH 7.8), 2 mM magnesium acetate, 1 mM Fru 6-P (acid-treated), 0.24 mM reduced β-nicotinamide adenine dinucleotide (NADH), 0.1 units (U) aldolase (EC 4.1.2.13), 1.6 U triose phosphate isomerase (TPI, EC 5.3.1.1), 4.5 U glycerol 3-phosphate dehydrogenase (GDH, EC 1.1.1.8) and 2.7 mU potato tuber PFP, was added to

the alkalinised aliquots and to Fru 2,6-P₂ (0 – 3 pmol) standards. The reaction was initiated by the addition of 0.8 mM inorganic pyrophosphate (PPi).

The activity of PFP was measured by following the oxidation of NADH at 340 nm in a microtiter plate reader (PowerwaveX, Biotek Instruments Inc., Winooski, VT, USA). The amount of Fru 2,6-P₂ in the sample was calculated using a standard curve for PFP activity against Fru 2,6-P₂ concentration prepared from the Fru 2,6-P₂ standards.

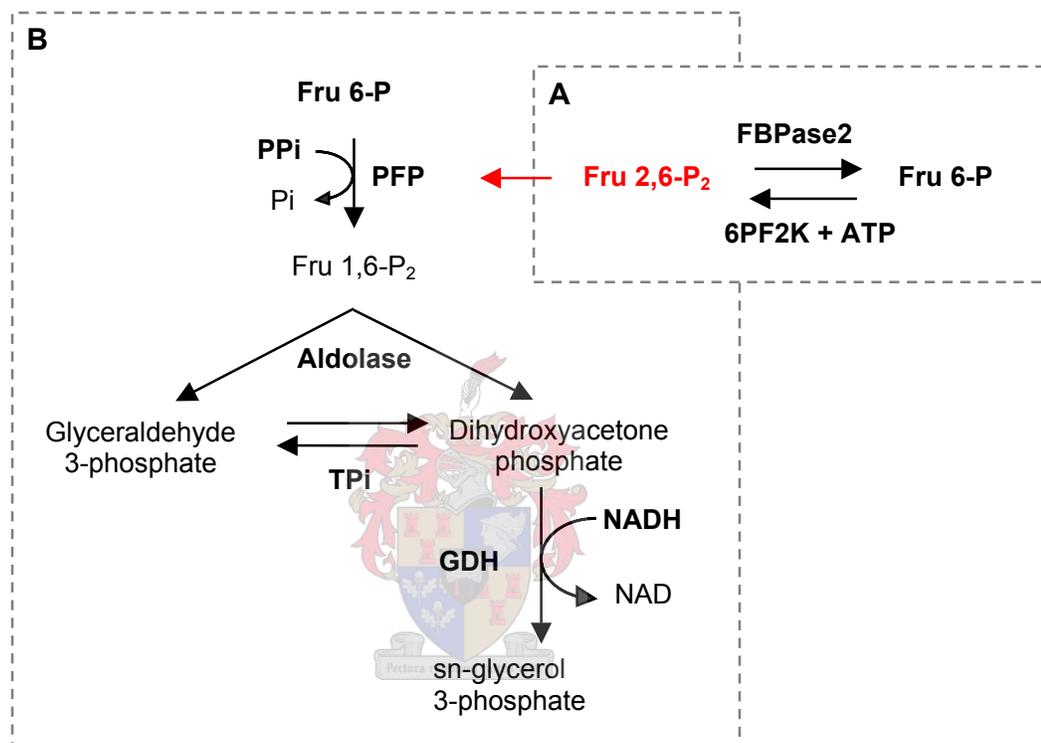


Figure 3.2. The PFP-coupled assay for the measurement of 6PF2K and FBPase2 activities. The amount of Fru 2,6-P₂ produced or hydrolysed after incubation with 6PF2K or FBPase2 respectively (A) is quantified through the stimulation of potato PFP (B). The activity of PFP was determined by the oxidation of NADH (measured at 340 nm). Abbreviations as in text.

3.2.4 Activity tests for FBPase2

Activity of FBPase2 was measured by two methods. Firstly, by a PFP-coupled assay based on the method described by (Stitt, 1990b). Secondly, by measuring Fru 6-P production from either Fru 2,6-P₂ or Fru 1,6-P₂ as dephosphorylation substrate.

3.2.4.1 PFP-coupled FBPase2 activity test

In the first part of the assay (figure 3.2A) bacterial protein extracts (10 μl) from cells that had harboured pBF2P 4T1 or pGEX 4T1 (negative control) were incubated in a phosphatase assay buffer (final volume of 140 μl) containing 50 mM Tris-Acetate (pH 7.8), 5 mM magnesium

acetate and 1 mM EDTA. Activity of FBPase2 was initiated by the addition Fru 2,6-P₂. Immediately and at certain time points thereafter, 20 µl aliquots (containing 3 pmol at the start of the reaction) were removed and alkalinised in 30 µl 250 mM KOH.

In the second part of the assay (figure 3.2B) the amount of Fru 2,6-P₂ remaining in these aliquots were quantified through the stimulation of potato tuber PFP (Van Schaftingen *et al.* 1982b) in a procedure similar as for the 6PF2K activity test.

3.2.4.2 Measurement of Fru 6-P production FBPase2 activity test

The assay was performed in a microtiter plate in a final volume of 250 µl. The bacterial protein extracts (10 µl) from pBF2P 4T1- or pGEX 4T1-harboured cells (negative control) were incubated in an assay buffer containing 100 mM Tris-Acetate (pH 7.8), 0.1 mM Fru 1,6-P₂ or Fru 2,6-P₂, 5 mM magnesium acetate, 0.4 mM oxidised β-nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and phosphoglucosomerase (PGI, EC 5.3.1.9).

The change in absorbance caused by the reduction of NADP to NADH was measured spectrophotometrically at 340 nm. The amount of NADH produced (a change in absorbance of 0.02532 = 1 nmol NADPH (1 cm path length)) is stoichiometric to the amount of Fru 6-P.

3.2.5 SDS-PAGE and Antibody production

One volume of loading buffer (8 M urea, 250 mM Tris-HCl (pH 6.8), 40% (v/v) glycerol, 100 mM DTT, 8% (w/v) sodium dodecyl sulphate (SDS), 1% (w/v) bromophenol blue) was added to 3 µg of the bacterially expressed 6PF2K and FBPase2 proteins. The suspension was incubated at room temperature for 15 min before the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (4% stacking gel, 12% resolution gel) (Laemmli, 1970). The proteins were visualised by Coomassie blue (staining solution: 0.5 mg.l⁻¹ Coomassie blue R-250, 8% (v/v) acetic acid and 25% (v/v) ethanol; destaining solution: 4.5% (v/v) acetic acid and 30% (v/v) ethanol).

The partially purified GST fusion 6PF2K and FBPase2 proteins (1 mg each) were given to Prof. D Bellstedt (Department of Biochemistry, University of Stellenbosch) to raise polyclonal antibodies against these proteins in rabbit (Bellstedt *et al.*, 1987). Sera were drawn at days 42 and 82 post-injection, respectively.

3.3 Results

3.3.1 Plasmid constructs

The recombinant rat liver 6PF2K and FBPase2 genes were cloned into a bacterial expression vector (pGEX 4T1) producing pBF2K 4T1 and pBF2P 4T1 respectively (figure 3.3). Restriction enzyme analysis confirmed that the plasmids were constructed successfully (figure 3.4, table 3.1). In addition DNA sequencing of pBF2K 4T1 and pBF2P 4T1 verified that the recombinant genes they harbour were in-frame with the initiation codon of the GST gene (results not shown).

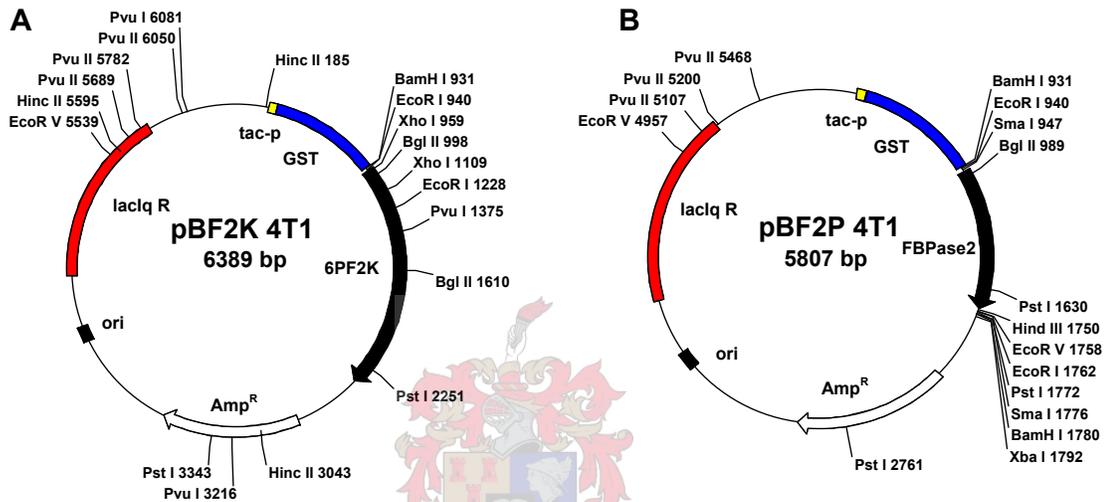


Figure 3.3. The bacterial expression vectors pBF2K 4T1 (A) and pBF2P 4T1 (B), harbouring the recombinant rat liver 6PF2K and FBPase2 genes respectively. Amp^R = ampicillin resistance, *lacIq R* = *lacIq* repressor gene, *ori* = bacterial origin of replication.

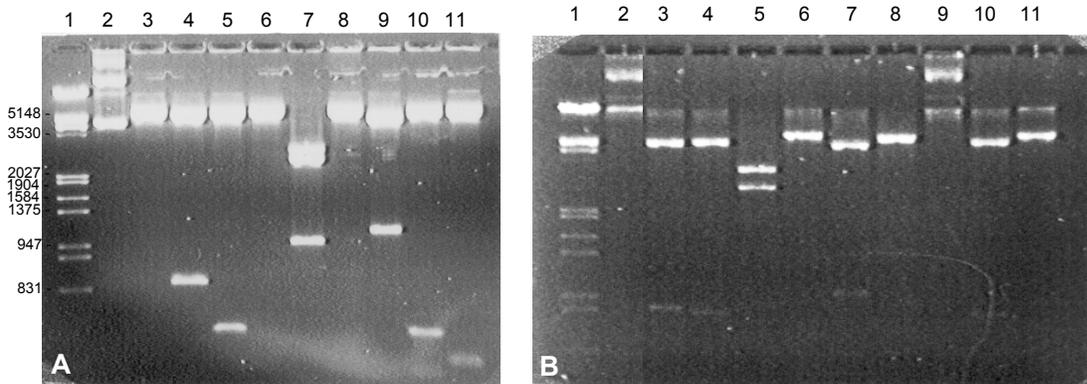


Figure 3.4. Ethidium bromide-stained agarose gels showing the fragments that were obtained after pBF2K 4T1 (A) and pBF2P 4T1 (B) were digested with restriction enzymes (see table 3.1).

Table 3.1. Restriction enzyme analysis of pBF2K 4T1 (A) and pBF2P 4T1 (B) (see figure 3.4).

A		
Lane		Expected fragments (bp)
1	DNA Marker III (Roche, Mannheim, Germany)	
2	pBF2K 4T1 (undigested)	
3	<i>Bam</i> HI	6389
4	<i>Bg</i> II	5777, 612
5	<i>Eco</i> RI	6101, 288
6	<i>Eco</i> RV	6389
7	<i>Hinc</i> II	2858, 2552, 979
8	<i>Hind</i> III	6389
9	<i>Pst</i> I	5297, 1092
10	<i>Pvu</i> II	6028, 268, 93
11	<i>Xho</i> I	6239, 150
B		
1	DNA Marker III (Roche, Mannheim, Germany)	
2	pBF2P 4T1 (undigested)	
3	<i>Bam</i> HI	4957, 850
4	<i>Eco</i> RI	4984, 823
5	<i>Eco</i> RV	3196, 2611
6	<i>Hind</i> III	5807
7	<i>Pst</i> I	4681, 984, (142)
8	<i>Pvu</i> I	5446, (268), (93)
9	<i>Sal</i> I	no recognition site
10	<i>Sma</i> I	4978, 829
11	<i>Xba</i> I	5807

3.3.2 Activity tests for 6PF2K and FBPase2

Activity tests performed on the bacterially expressed GST fusion proteins indicated that they are functional (figure 3.5). The recombinant 6PF2K produced 300 pmol Fru 2,6-P₂·min⁻¹·mg⁻¹ protein and FBPase2 hydrolysed 2 nmol Fru 2,6-P₂·min⁻¹·mg⁻¹ protein.

Substrate specificity of the bacterially expressed FBPase2 was also investigated. With Fru 2,6-P₂ as the dephosphorylation substrate protein extracts from pBF2P 4T1-harboured bacterial cells generated 5.2-times more Fru 6-P compared to those had harboured pGEX 4T1 (figure 3.6). Slightly more Fru 6-P was produced from fructose 1,6-bisphosphate (Fru 1,6-P₂) by the pGEX 4T1 protein extracts compared to the pBF2P 4T1 protein extracts.

Because the genes encoding the recombinant 6PF2K and FBPase2 proteins were originally part of a single gene encoding a bifunctional enzyme, the bacterially expressed enzymes were tested for residual bisphosphatase and kinase activity respectively. The bisphosphatase activity of 6PF2K was only 5.6% of its kinase activity (figure 3.7A). No residual kinase activity was detected for the recombinant FBPase2 (figure 3.7B).

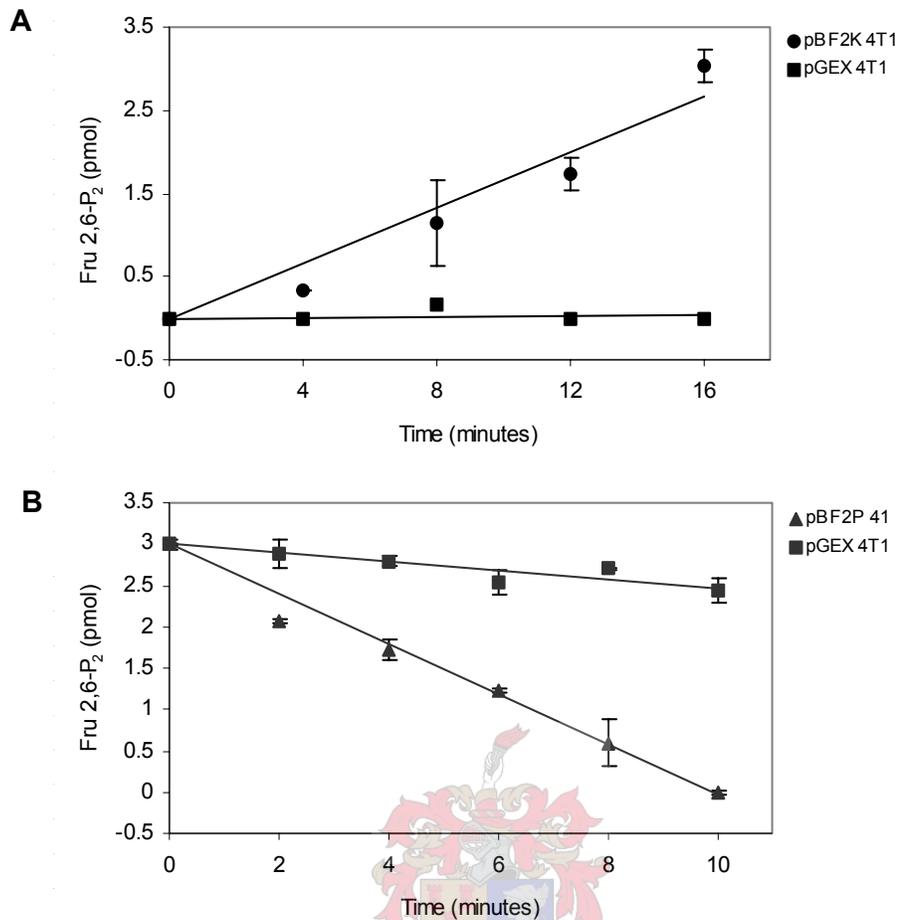


Figure 3.5. Changes in Fru 2,6-P₂ levels over time catalysed by bacterial protein extracts from cells that had harboured pBF2K 4T1 (A) and pBF2P 4T1 (B). Also showing results obtained with pGEX 4T1-harboured extracts (negative control). Each data point is the average of three readings. Error bars represent standard error.

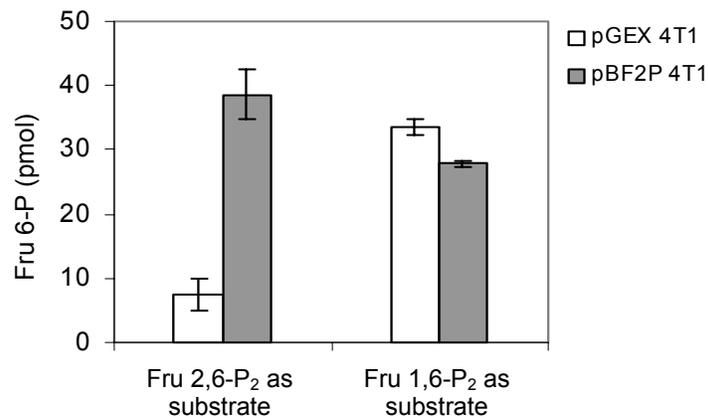


Figure 3.6. The amount of Fru 6-P generated from either Fru 2,6-P₂ or Fru 1,6-P₂ as substrate by bacterial protein extracts from pBF2P 4T1- or pGEX 4T1-harboured cells (negative control). Each data point is the average of three readings. Error bars represent standard error.

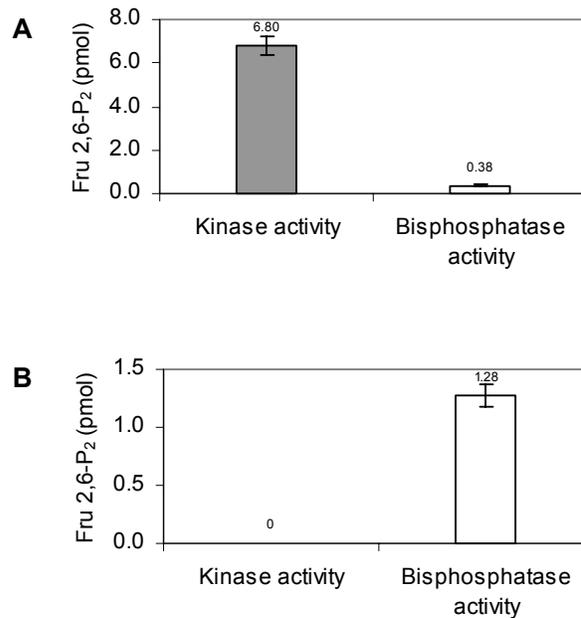


Figure 3.7. A comparison of the kinase and bisphosphatase activity (the amount of Fru 2,6-P₂ synthesised or hydrolysed respectively) by bacterial protein extracts from cells that had harboured pBF2K 4T1 (A) and pBF2P 4T1 (B). Each data point is the average of three readings. Error bars represent standard error.

3.3.3 Antibody production

The bacterially expressed 6PF2K (58 kilo daltons (kDa), band C) and 6PFase2 (82 kDa, band E) were partially purified and visualised on a SDS-PAGE gel stained with Coomassie blue (figure 3.8). Apart from the GST fusion recombinant proteins, the protein extracts also contained GST (27 kDa, band D) and a protein of approximately 62 kDa (band F).

Sufficient amounts of recombinant proteins (1 mg each) were only obtained after extending the standard 2 to 6 h induction period (see materials and methods) at 37 °C, to 72 h and at room temperature. The partially purified proteins were injected into rabbits to raise antibodies against them.

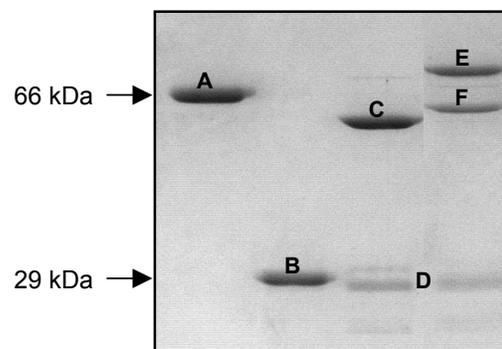


Figure 3.8. The bacterially expressed GST fusion recombinant FBPase2 (58 kDa, band C) and 6PF2K (82 kDa, band E). Also showing 66 kDa (band A) and 29 kDa (band B) protein molecular markers, GST (27 kDa, band D) and an additional protein of approximately 62 kDa (band F). Proteins were visualised by Coomassie blue after SDS-PAGE.

3.4 Discussion

Genes encoding recombinant rat 6PF2K and FBPase2 enzymes were cloned into a bacterial expression vector (pGEX 4T1) producing pBF2K 4T1 and pBF2P 4T1 respectively. *E. coli* was transformed with these plasmids and the recombinant enzymes were expressed. Activity tests performed on partially purified enzymes demonstrated that they are functional in synthesising and hydrolysing Fru 2,6-P₂ respectively.

The following observations argues that pBF2P 4T1 harbours a gene that encodes for a Fru 2,6-P₂ specific phosphatase: The amount of Fru 6-P produced from Fru 2,6-P₂ by the bacterial protein extracts from pBF2P 4T1-harboured cells was 5.2-times more compared to those that had harboured pGEX 4T1. Similar amounts of Fru 6-P were produced from Fru 1,6-P₂ as substrate by the pBF2P 4T1 and pGEX 4T1 protein extracts. The amount of Fru 6-P generated from Fru 1,6-P₂ by protein extracts from the pBF2P 4T1- and especially pGEX 4T1-harboured cells were much higher than anticipated. This is most likely the result of bacterial enzyme contamination such as fructose 1,6-bisphosphatase (FBPase1, EC 3.1.3.11) and / or non-specific phosphatases. The small amount of Fru 2,6-P₂ degradation observed with the pGEX 4T1-harboured extract probably also refers to non-specific bacterial phosphatases since *E. coli* does not contain FBPase2 (Okar *et al.*, 2001).

The above emphasises that although a bacterial expression system is a rapid and simple method to verify the activities of heterologous proteins, possible interference of bacterial contaminants should be kept in mind. It was therefore important to verify that no NADH oxidation was detected in the absence of PPI in the PFP-coupled assays for 6PF2K and FBPase2 (refer to figure 3.2), thus making sure that the conversion of Fru 6-P to Fru 1,6-P₂ was catalysed by PFP and not a bacterial contaminant such as phosphofructokinase (PFK, EC 2.7.1.11). PFK catalyses the same reaction but uses ATP as the phosphoryl donor. Although Fru 2,6-P₂ is absent in prokaryotes, it does stimulate bacterial PFK (Okar *et al.*, 2001).

Sufficient amounts of recombinant proteins (1 mg each) for antibody production were only obtained after extending the recombinant protein expression period to 72 h and at room temperature. Although not investigated, the reason for poor yield might have been that a large component of the expressed recombinant proteins was enclosed in insoluble inclusion bodies. This is often the problem when foreign proteins are expressed in *E. coli* (Marston, 1986). The antibodies that were raised against the recombinant enzymes will be used to detect these proteins in the transgenic sugarcane plants. FBPase2 forms a phosphoenzyme intermediate (Pilkis *et al.*, 1983), thus an alternative approach for detecting the recombinant FBPase2 could be incubation with [2-³²P] Fru 2,6-P₂ followed by SDS-PAGE and autoradiography.

CHAPTER 4

Fructose 2,6-bisphosphate levels in sugarcane leaves

Abstract

Fructose 2,6-bisphosphate is an important regulatory molecule in carbohydrate metabolism within eukaryotes. It is well known that extraction efficiencies can vary between different species and also within tissues from the same species. It is therefore important to ensure that the extraction and subsequent measurement methods are reliable. In this study a chloroform/methanol method was optimised for the extraction of fructose 2,6-bisphosphate from sugarcane leaves. The protocol presented here resulted in a $93 \pm 4\%$ recovery of fructose 2,6-bisphosphate. This is the first report on the diurnal changes in the levels of fructose 2,6-bisphosphate in sugarcane. Results suggest a role for fructose 2,6-bisphosphate in photosynthetic sucrose metabolism and in the partitioning of carbon between sucrose and starch.

4.1 Introduction

Fructose 2,6-bisphosphate (Fru 2,6-P₂) is an important regulator of carbohydrate metabolism in eukaryotes (Van Schaftingen, 1987; Stitt, 1990a; Okar *et al.*, 2001). Fru 2,6-P₂ plays a role in the co-ordination of the rate of sucrose synthesis with the rate of photosynthesis via its allosteric regulation of cytosolic fructose 1,6-bisphosphatase (FBPase1, EC 3.1.3.11) (Stitt *et al.*, 1983; Stitt *et al.*, 1987a; Stitt *et al.*, 1987b). Triose phosphates and 3-phosphoglycerate (3PGA) that are produced during photosynthesis together with the subsequent decrease in inorganic phosphate (Pi) inhibits 6-phosphofructo 2-kinase (6PF2K, EC 2.7.1.11) and simultaneously activates fructose 2,6-bisphosphatase (FBPase2, EC 3.1.3.11) (Larondelle *et al.*, 1986; Macdonald *et al.*, 1989). These enzymes synthesise and hydrolyse Fru 2,6-P₂ respectively, thus causing a rapid drop in Fru 2,6-P₂ levels upon illumination that abolishes the inhibition of FBPase1 and allows sucrose to accumulate. In the dark Fru 2,6-P₂ levels rise and sucrose accumulation is inhibited.

The rise in Fru 2,6-P₂ during the photoperiod results in the feedback inhibition of sucrose synthesis and directs photosynthate towards starch synthesis in some plant species (Stitt *et al.*, 1983; Stitt *et al.*, 1987a; Neuhaus *et al.*, 1989; Scott *et al.*, 1995; Truesdale *et al.*, 1999; Scott *et al.*, 2000; Draborg *et al.*, 2001).

The extraction of Fru 2,6-P₂ from plant tissues is often difficult because Fru 2,6-P₂ is extremely susceptible to hydrolysis by non-specific phosphatases. Fru 2,6-P₂ is also acid labile, therefore excluding the use of conventionally acid-based methods for the extraction of this metabolite (Stitt, 1990b).

The chloroform/methanol method (Stitt *et al.*, 1982) is traditionally used for the non-acidic extraction of Fru 2,6-P₂ from plant tissues. The effectiveness of this method differs greatly between plant species: Recoveries of 80% to 100% were reported for potato and carrot tubers, *Arum* spadix (Stitt, 1990b), pea, maize and onion leaves (Sicher *et al.*, 1987) and sugarcane internodal tissue (Whittaker *et al.*, 1997). However using the same method recoveries of 65%, 50%, 20%, 10% and 0% were obtained with *Lolium temulentum* (Pollock *et al.*, 1989), almond, stinging nettle leaves (Stitt, 1990b), unripe bananas (Ball and ap Rees., 1988) and tobacco leaves (Scott and Kruger, 1994) respectively.

The most widely used and sensitive method for measuring Fru 2,6-P₂ is through the stimulation of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) (Van Schaftingen *et al.*, 1982b) (chapter 3, figure 3.2). As little as 0.1 pmol Fru 2,6-P₂ could be measured with this bioassay, which relies on linear standard curves for PFP activity against increasing Fru 2,6-P₂ levels (prepared in extracts from which the endogenous Fru 2,6-P₂ is removed) (Stitt, 1990b). A standard curve is necessarily for each extract because the activation of PFP by Fru 2,6-P₂ is greatly influenced by many compounds and is therefore unique for each sample (Van Schaftingen *et al.*, 1982a; Kombrink *et al.*, 1984). These standard curves are then used to calculate the amount of Fru 2,6-P₂ in each extract. Linear standard curves are not always possible with all plant extracts, making accurate measurements impossible for these plants (Trevanion, 2000).

One of the main aims of our research programme is to genetically modify the Fru 2,6-P₂ levels in sugarcane. Currently no data are available on Fru 2,6-P₂ in sugarcane leaves and its role in sink tissues (such as sugarcane internodal tissue) is unclear. Reliable extraction and assay methods are a prerequisite for investigating *in vivo* changes in Fru 2,6-P₂.

Here we describe an efficient chloroform/methanol Fru 2,6-P₂ extraction method for sugarcane leaves. In addition diurnal changes in sugarcane leaf Fru 2,6-P₂ levels were compared with changes in sucrose and starch levels to investigate the role of Fru 2,6-P₂ in the metabolism of these photosynthetic products.

4.2 Materials and methods

4.2.1 Plant material

Sugarcane plants of the variety N19 were grown in a controlled environment cabinet (Convion CMP 3023, Winnipeg Manitoba, Canada). The plants were subjected to a 12-h light/12-h dark cycle with rapid light/dark transitions. The light intensity was 350 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and the relative humidity was 60%. The light and dark temperatures were 28 °C and 18 °C respectively. The plants were approximately 2 months post germination when their leaves were harvested.

Internodal tissue was obtained from sugarcane variety NCo310 grown in the field at Welgevallen (Stellenbosch, South Africa). Internodes 1 to 9 were pooled.

4.2.2. Extraction of Fru 2,6-P₂

The two most upright unfolded leaves (normally leaf 0 and +1) were harvested and immediately frozen in liquid nitrogen. Leaf +1 is the first unfolded leaf with a visible dewlap (van Dillewijn, 1952). The mid rib was removed and the remaining leaf material was powdered in liquid nitrogen using a mortar and pestle. The metabolites were extracted with chloroform/methanol based on the method described by Stitt (1990b). Important changes made to the original protocol are discussed in the results section (4.3.1).

The frozen tissue (300 mg) was added to 4.2 ml extraction medium (0.6 ml buffer containing 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol⁺ (Tris) (pH > 10), 20 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA) and 50 mM NaF; 1.2 ml chloroform and 2.4 ml methanol).

The tubes were covered in foil to protect the light-sensitive chlorophyll. The suspension was mixed and incubated for 30 min at 4 °C. Double distilled water (ddH₂O) (4 ml) was added, the suspension was mixed by vortexing for 20 s and centrifuged for 15 min at 6000 xg at 4 °C. The upper aqueous/methanol phase was transferred to a new tube and kept on ice. Most of the bottom chloroform phase (containing the chlorophyll) was also removed and kept for chlorophyll measurements. The suspension in the original tube was re-extracted with 1 ml 100 mM Tris⁺ (pH > 10), and after centrifugation (15 min at 10500 xg) the upper aqueous/methanol phase was removed and combined with the supernatant obtained above. The metabolite solution was dried down in a vacuum evaporator (Speed Vac[®], Savant, Holbrook, New York, USA) overnight at low temperature and then dissolved in 1 ml ddH₂O.

Phenolic and other compounds that could interfere with later spectrophotometric measurements were removed by the addition of 20 mg activated charcoal. The suspension was mixed by vortexing for 20 s and incubated on ice for 10 min. After centrifugation for 5 min at 16000 xg, the clear supernatant containing the metabolites was removed. Aliquots were frozen in liquid nitrogen and stored at -80 °C until further use.

The extraction of Fru 2,6-P₂ from internodal tissue was similar as for leaves but 3 g tissue was used and the volumes of solutions were doubled. Furthermore, the metabolite pellet was finally dissolved in only 500 μl ddH₂O.

For recovery experiments 50 pmol (about 100 pmol.mg⁻¹ chl) and 300 pmol (100 pmol.g⁻¹ FW) Fru 2,6-P₂ were added prior to extraction to the leaf and internodal material respectively. The amount of Fru 2,6-P₂ in these extracts, compared to extracts without additional Fru 2,6-P₂ revealed the amount of Fru 2,6-P₂ recovered.

4.2.3 *Extraction of sucrose and starch*

Leaf sucrose was extracted simultaneously with Fru 2,6-P₂ (section 4.2.2). The recovery of sucrose was 90 ± 2%. Starch was extracted based on the method of Beutler (1984) by adding 1 ml buffer (10 mM Tris-HCl (pH 8) and 70% (v/v) ethanol) to 100 mg leaf tissue (the same powdered material that was used for the extraction of Fru 2,6-P₂ and sucrose). The suspension was mixed and incubated for 30 min at 65 °C. The suspension was centrifuged for 10 min at 16 000 xg. The supernatant was removed, the insoluble matter (containing the starch) washed with 1 ml ddH₂O to remove all soluble sugars and then centrifuged for 15 min at 16 000 xg. This procedure was repeated another four times. Thereafter ddH₂O was added to a final volume of 1 ml. The suspension was autoclaved to liquefy the starch. The solution was mixed and centrifuged for 15 min at 16 000 xg to pellet residual insoluble tissue debris. The supernatant was removed and used for the measurement of starch.

4.2.4 *Measurement of metabolites*

Metabolite levels were determined enzymatically in a microtiter plate. The oxidation of reduced β-nicotinamide adenine dinucleotide (NADH) (assay for Fru 2,6-P₂) or reduction of oxidised β-nicotinamide adenine dinucleotide phosphate (NADP) (assays for sucrose and starch) were measured at 340 nm using a plate reader (PowerwaveX, Biotek Instruments Inc., Winooski, VT, USA). All coupling enzymes were from Roche (Mannheim, Germany).

4.2.4.1 *Measurement of Fru 2,6-P₂*

The amount of Fru 2,6-P₂ was measured through the stimulation of potato tuber PFP based on the method described by Stitt (1990b). The procedure was similar as described in chapter 3 (section 3.2.3, figure 3.2) with the following exceptions: The PFP reaction was initiated with 0.4 mM inorganic pyrophosphate (PPi) in an assay volume of 230 μl. Only after 3 min in the plate reader a volume of 20 μl (see preparation below) that effectively contained 2 μl leaf, 6 μl internodal extract or internal standards was added.

Internal standards refer to metabolite extracts from which the endogenous Fru 2,6-P₂ was hydrolysed by the addition of 0.1 volumes of 2M HCl, followed by an incubation period of 15 min at room temperature. The extracts were then neutralised by the addition of an equal volume (than the HCl) of 2M NaOH. The solution was kept on ice for 2 min and 10 mM Tris-Acetate (pH 7.8) was added to ensure an alkaline pH. These extracts were then spiked with authentic Fru

2,6-P₂ (0, 0.5 and 1 pmol). Standard curves for PFP activity against Fru 2,6-P₂ concentration were prepared for each sample and used to calculate the amount of Fru 2,6-P₂. The plant extracts in which Fru 2,6-P₂ levels were measured contained the same amount of HCl and NaOH but pre-mixed, i.e. neutralised.

4.2.4.2 Measurement of sucrose

Sucrose was measured according to the method of Bergmeyer and Bernt (1974). The sucrose in the extract (10 µl extract 6-times diluted) was hydrolysed to glucose and fructose in 50 µl buffer (100 mM citrate (pH 5), 5 mM MgCl₂ and 10 units (U) β-fructosidase (EC 3.2.1.26)). The mixture was incubated for 20 min at room temperature. The amount of glucose released from the sucrose cleavage is stoichiometric to the amount of sucrose. The sucrose concentration was calculated by subtracting the amount of glucose in the extract not treated with β-fructosidase from the amount of glucose in the β-fructosidase-treated extract. Glucose was measured by converting glucose to glucose 6-phosphate by adding 1 U hexose kinase (EC 2.7.1.1) and 1 U *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase (EC 1.1.1.49) in 200 µl buffer (150 mM Tris-HCl (pH 8.1), 5 mM MgCl₂, 1.1 mM adenosine 5'-triphosphate (ATP) and 0.3 mM NADP). A change in absorbance at 340 nm of 0.02532 = 1 nmol NADPH (1 cm path length) reflects the amount of glucose 6-phosphate and is stoichiometric to the amount of glucose.

4.2.4.3 Measurement of starch

Sodium acetate (pH 4.8) (final concentration of 3 mM) was added to the liquefied starch solution. Two 100 µl aliquots was removed and 1.5 U amyloglucosidase (EC 3.2.1.3) was added to one of these aliquots, mixed and incubated for 2 h at 56 °C. Amyloglucosidase cleaves α1,4- or α1,6-linked terminal glucose monomers of starch. The amount of starch was determined by subtracting the amount of glucose present in the aliquot without amyloglucosidase from the glucose content in the amyloglucosidase-treated aliquot. Glucose was determined as in section 4.2.4.2 (15 µl of each aliquot was used).

4.2.5 Measurement of chlorophyll

The chlorophyll solution was diluted in absolute ethanol to 4% (v/v) and measured spectrophotometrically according to the formula of Lichtenthaler and Wellburn (1983): chlorophyll *a+b* (µg) = (13.95A₆₆₅ - 6.88A₆₄₉) + (24.96A₆₄₉ - 7.32A₆₆₅).

4.3 Results

4.3.1 Optimisation of Fru 2,6-P₂ extraction and measurement

The chloroform/methanol method described for the extraction of Fru 2,6-P₂ from spinach leaves (Stitt *et al.*, 1983) gave good recoveries (higher than 80%) for sugarcane internodal tissue (Whittaker and Botha, 1997). However we experienced that this method was not suitable for the extraction of Fru 2,6-P₂ from sugarcane leaves.

A similar protocol, but containing EGTA (to chelate Ca²⁺ that could otherwise precipitate out with metabolites) and NaF (to inhibit phosphatases) (Stitt, 1990b) were therefore tested. Initial metabolite extracts prepared with this method had an acidic pH and since Fru 2,6-P₂ is hydrolysed within minutes at pH 3.5 (Stitt, 1990b), these extracts contained non-detectable amounts of Fru 2,6-P₂.

A recovery of 93 ± 4% (n = 4) of Fru 2,6-P₂ added to the tissue prior to extraction was obtained when the buffer capacity was increased from 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 8.5) to 100 mM Tris+ (pH > 10). In addition this method provided a Fru 2,6-P₂ recovery of 85 ± 5% (n = 4) for sugarcane internodal tissue.

The bioassay involving the activation of potato PFP (Van Schaftingen *et al.*, 1982b; Stitt 1990b) was optimised for the measurement of Fru 2,6-P₂ levels in sugarcane leaves. The initial acid treatment of 1 volume of 100 mM HCl for 15 min was insufficient to hydrolyse all the endogenous Fru 2,6-P₂. This residual Fru 2,6-P₂ resulted in a higher basal PFP activity (without any additional Fru 2,6-P₂ added) that caused non-linear standard curves. A more stringent acid treatment (0.1 volumes of 2 M HCl for 15 min) gave a linear response for PFP activity against Fru 2,6-P₂ concentration up to 2 pmol (figure 4.1).

A larger activation of PFP by Fru 2,6-P₂ was obtained when the concentration of PPi was reduced from 2 mM to 0.4 mM (figure 4.1). Using the above conditions a linear relationship between PFP activity and extract volume was observed (figure 4.2).

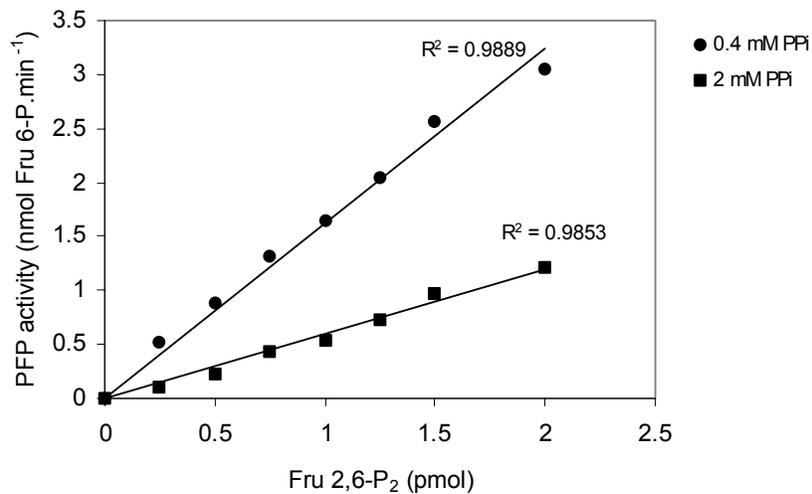


Figure 4.1. Standard curves for PFP activity against Fru 2,6-P₂ concentration in assays containing 5 μ l metabolite extract (endogenous Fru 2,6-P₂ removed). Also showing the effect of PPI concentration on the activation of PFP.

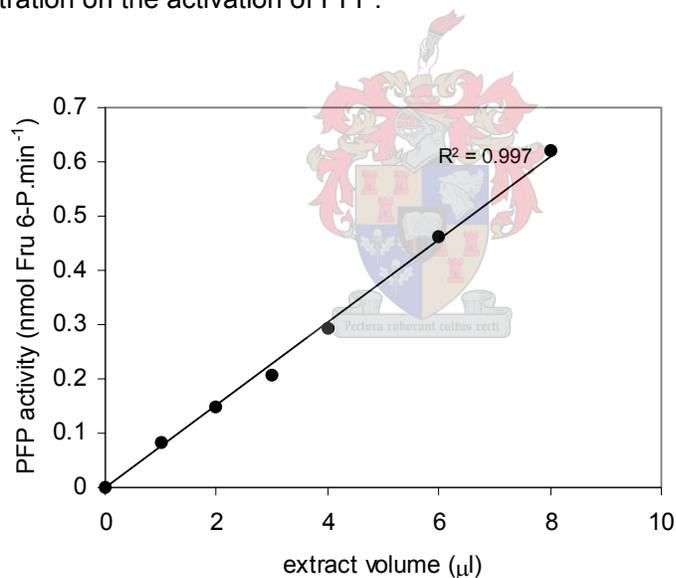


Figure 4.2. PFP activity against sugarcane leaf extract volume.

4.3.2 Diurnal changes in Fru 2,6-P₂

The most dramatic changes in sugarcane leaf Fru 2,6-P₂ levels were observed with the transition from dark to light and *vice versa* (figure 4.3). The levels of Fru 2,6-P₂ dropped 1.6-times within the first 30 min of illumination. Sugarcane leaf Fru 2,6-P₂ levels during the light (on average 158 pmol.mg⁻¹ chl) are in the range reported for other monocotyledons plants such as barley (Sicher *et al.*, 1986), maize (Sicher *et al.*, 1987), *Lolium* (Pollock *et al.*, 1989) and wheat (Trevanion, 2000) that accumulate leaf reserves predominantly as sucrose. The lowest Fru 2,6-

P₂ levels (126 pmol.mg⁻¹ chl) were measured at the end of the 12-h light period. The highest Fru 2,6-P₂ (264 pmol.mg⁻¹ chl) was measured 2 h into the subsequent dark period.

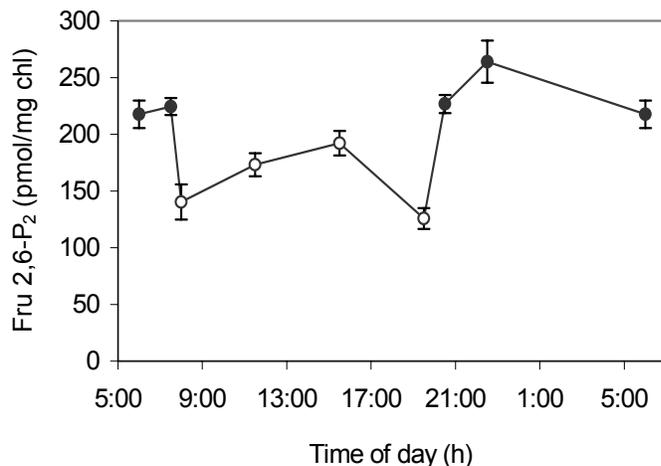


Figure 4.3. Diurnal changes in sugarcane leaf Fru 2,6-P₂. Open and closed circles represent light and dark time points respectively (light period = 07:30 to 19:30). Each data point is the average of duplicate measurements on three different plants. Error bars represent standard error.

4.3.3 Diurnal changes in sucrose and starch

Both sucrose and starch showed a gradual increase during the photoperiod (figure 4.4). Under the conditions in this study, photosynthate was primarily incorporated into sucrose. Nevertheless starch accounted for 17.7% of the accumulating carbohydrates. The sucrose/starch ratio declined from 22.8 at the end of the dark period to 6.2 at the end of the photoperiod.

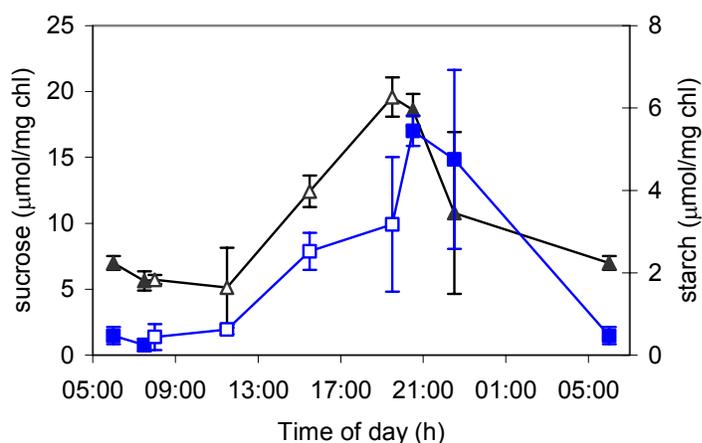


Figure 4.4. Diurnal changes in sugarcane leaf sucrose (▲) and starch (■). Open and closed shapes represent light and dark time points respectively (light period = 07:30 to 19:30). Each data point is the average of duplicate measurements on three different plants. Error bars represent standard error.

4.4 Discussion

Ball and ap Rees (1988) demonstrated the importance of recovery experiments by referring to the apparent rise in Fru 2,6-P₂ during the respiratory climacterium in banana (Mertens *et al.*, 1987). Careful analyses illustrated that this phenomenon was merely the result of an unreliable extraction method. Recoveries of Fru 2,6-P₂ from the unripe banana fruit were poor (only 10%) whereas recoveries from ripe fruit exceeded 90%.

High recoveries of Fru 2,6-P₂ added to the sugarcane leaf tissue prior to extraction was only obtained after the buffer capacity in the extraction buffer was increased from 20 mM Hepes (pH 8.5) to 100 mM Tris⁺ (pH > 10). Sicher *et al.* (1987) also used a higher buffer concentration (50 mM Tris⁺ (pH > 10) and dissolved the metabolites after vacuum evaporation in 100 mM Tris-HCl (pH 8) for the extraction of Fru 2,6-P₂ from maize leaves. Photosynthesis in both sugarcane and maize are characterised by the formation of C₄ organic acids, which might explain the need for a higher buffer concentration when extracting Fru 2,6-P₂ from these plants. The higher buffer concentration probably also explains the need for a more severe acid treatment to hydrolyse all the endogenous Fru 2,6-P₂ (for the preparation of internal standards).

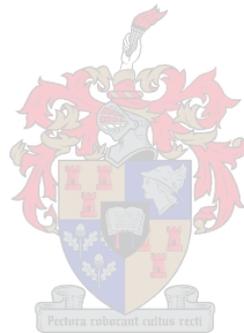
The larger activation of PFP by Fru 2,6-P₂ that was obtained with 0.4 mM PPI than 2 mM is consistent with previously findings showing that the PPI optimum of PFP changes from approximately 2 mM in the presence of high Fru 2,6-P₂ concentrations (1 μM) to around 0.5 mM in the absence of this activator (Kombrink *et al.*, 1984).

The most dramatic changes in sugarcane leaf Fru 2,6-P₂ were observed with the transition from light to dark and *vice versa*. These rapid alterations in Fru 2,6-P₂ coincided with the onset of sucrose accumulation and mobilisation. The above agrees with the accepted role of Fru 2,6-P₂ in photosynthetic sucrose synthesis through its action on FBPase1 (Stitt *et al.*, 1983; Stitt *et al.*, 1987a).

Plants such as spinach (Stitt *et al.*, 1983; Stitt *et al.*, 1987a), tobacco (Scott *et al.*, 1995; Scott *et al.*, 2000), *Clarkia xantiana* (Neuhaus *et al.*, 1989), *Kalanchoë daigremontiana* (Truesdale *et al.*, 1999) and *Arabidopsis* (Draborg *et al.*, 2001), where a role for Fru 2,6-P₂ in carbon partitioning between sucrose and starch was identified, all store leaf carbon as starch. Fru 2,6-P₂ is not involved in carbon partitioning in barley (Sicher *et al.*, 1986), wheat (Trevanion, 2000) and *Lolium temulentum* (Pollock *et al.*, 1995) where leaf carbon is stored as sucrose. However maize stores leaf carbon mainly as starch but Fru 2,6-P₂ levels remains constant throughout the photoperiod (Sicher *et al.*, 1987). It therefore appears that the stored carbon source is not the only criterion determining a role for Fru 2,6-P₂ in photosynthetic carbon partitioning.

Sugarcane stores leaf reserves mainly as sucrose (Sugarcane Botany: A Brief View, J D Miller and RS Lentini⁶; results presented in the present study). The decline in the sucrose/starch ratio, together with the rise in Fru 2,6-P₂ during the early photoperiod suggest that Fru 2,6-P₂ might be involved in photosynthetic carbon partitioning between sucrose and starch during that period in sugarcane leaves. However the drop in Fru 2,6-P₂ towards the end of the photoperiod did not result in a concurrent increase in the sucrose/starch ratio. Despite an increase in Fru 2,6-P₂ throughout the photoperiod in tobacco carbon partition was strongly influenced only in the early photoperiod (Scott and Kruger, 1994; Scott *et al.*, 1995).

It is evident from the results presented in this study that the optimised methods for Fru 2,6-P₂ extraction and measurement are reliable for sugarcane leaves (and internodes). These methods will be used to analyse transgenic sugarcane plants with possible altered Fru 2,6-P₂ levels (chapter 5).



⁶ <http://edis.ifas.ufl.edu/SC034>

CHAPTER 5

Expression of recombinant rat fructose 2,6-bisphosphatase and 6-phosphofructo 2-kinase in sugarcane

Abstract

Fructose 2,6-bisphosphate is a regulatory metabolite in plants. The levels of fructose 2,6-bisphosphate are regulated by the relative activities of 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase that synthesise and hydrolyse fructose 2,6-bisphosphate respectively. Transgenic sugarcane plants expressing either a recombinant rat fructose 2,6-bisphosphatase (ODe lines) or 6-phosphofructo 2-kinase (OCe lines) were generated as a strategy to alter the levels of fructose 2,6-bisphosphate. The ODe transgenic lines contained decreased leaf fructose 2,6-bisphosphate levels but increased internodal fructose 2,6-bisphosphate levels compared to the control plants. The effect of altered fructose 2,6-bisphosphate levels on metabolism was investigated by sugar measurements and chlorophyll fluorescence. The ODe transgenic lines contained higher leaf sucrose and reducing sugars (glucose and fructose) than the control plants. The transgenic lines contained decreased internodal sucrose and increased reducing sugars compared to the control plants. Opposite trends were observed for fructose 2,6-bisphosphate and sucrose when leaves, internodes 3+4 or internodes 7+8 of the different plant lines were compared. This suggests a role for fructose 2,6-bisphosphate in sugar metabolism in both photosynthetic (leaves) and non-photosynthetic tissue (internodes) of sugarcane. In contrast, no consistent trends were observed between fructose 2,6-bisphosphate and sucrose in the OCe transgenic lines.

5.1 Introduction

Fructose 2,6-bisphosphate (Fru 2,6-P₂) is a regulatory metabolite common to all eucaryotes (Van Schaftingen, 1987; Stitt, 1990a; Okar *et al.*, 2001). The *in vivo* concentration of Fru 2,6-P₂ is regulated by 6-phosphofructo 2-kinase (6PF2K, EC 2.7.1.11) that synthesises and fructose 2,6-bisphosphatase (FBPase2, EC 3.1.3.11) that hydrolyses Fru 2,6-P₂ (Claus *et al.*, 1984).

In leaves Fru 2,6-P₂ contributes to the co-ordination of sucrose synthesis with the rate of photosynthesis and in some plant species in the partitioning of fixed carbon between sucrose and starch (Stitt *et al.*, 1983; Sicher *et al.*, 1987; Stitt *et al.*, 1987a; Stitt, 1990a). Compared to control plants transgenic tobacco plants with reduced Fru 2,6-P₂ levels showed an increase in leaf sucrose accumulation and a decrease in starch accumulation over the initial phase of the light period (Scott *et al.*, 1995). In agreement opposite results were obtained with transgenic tobacco with elevated leaf Fru 2,6-P₂ levels (Scott *et al.*, 2000). Central to the proposed mechanism by which Fru 2,6-P₂ regulates carbohydrate metabolism in leaves is its allosteric inhibition of fructose 1,6-bisphosphatase (FBPase1, EC 3.1.3.11). FBPase1 catalyses the

irreversible conversion of fructose 1,6-bisphosphate (Fru 1,6-P₂) to fructose 6-phosphate (Fru 6-P).

In contrast to leaves the function of Fru 2,6-P₂ in non-photosynthetic plant tissues is not well understood. These tissues lack appreciable FBPase1 activity (Enwistle and ap Rees, 1990) implying that all Fru 2,6-P₂ mediated effects on metabolism must be attributed to pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) that is strongly activated by Fru 2,6-P₂ (Sabularse and Anderson, 1981b). Despite many studies in the past the role of PFP in plant metabolism is still unclear (Kruger and Scott, 1994; Stitt, 1998; Fernie *et al.*, 2001).

Sugarcane (*Saccharum* hybrid) is a C4 perennial grass that is an important economical crop due to its ability to store large quantities of stem (internodal) sucrose (Moore and Maretzki, 1996). Sucrose storage in the sugarcane internodal parenchyma is complex and not fully understood (Birch, 1996; Moore and Maretzki, 1996; Grof and Campbell, 2001). Sucrose storage is probably regulated within the translocation system and / or in the sink itself where sucrose cycling (continuous sucrose synthesis and breakdown) plays a crucial role (Wendler *et al.*, 1990; Moore and Maretzki, 1996).

A negative correlation exists between PFP activity and sucrose content in sugarcane internodes (Whittaker and Botha, 1999). In addition transgenic sugarcane with an 80% reduction in PFP activity contained increased sucrose in maturing internodes (Groenewald and Botha, 2001). However Lingle and Smith (1991) found no clear relationship among sucrose content and PFP activity in sugarcane internodes and the correlation between Fru 2,6-P₂ and sucrose was not consistent.

Here we report on transgenic sugarcane lines expressing either a recombinant rat FBPase2 (ODe lines) or 6PF2K (OCe lines), which were generated as a strategy to alter Fru 2,6-P₂ levels and to study the subsequent effect on sugar (especially sucrose) metabolism. The ODe transgenic lines contained decreased leaf Fru 2,6-P₂ but increased internodal Fru 2,6-P₂ levels. Results obtained from sugar measurements and chlorophyll fluorescence performed on the ODe transgenic plants suggest a role for Fru 2,6-P₂ in sugar metabolism in both photosynthetic (leaves) and non-photosynthetic (internodal) tissues of sugarcane. However no relationship between Fru 2,6-P₂ and sucrose was apparent in the ODe transgenic lines.

5.2 Materials and methods

5.2.1 Plasmid constructs

General molecular biology techniques (Sambrook *et al.*, 1989) were applied for the preparation of the plant expression plasmids. All deoxyribonucleic acid (DNA) modifying enzymes were from Promega (Madison, AL, USA). The recombinant FBPase2 gene was cloned as an 822 base pair (bp) *EcoRI* fragment from pBF2P 4T1 (chapter 3, figure 3.3B) into the *EcoRI* site of vector pUBI 510. This recombinant FBPase2 plant expression plasmid was designated pEF2P 510.

The recombinant 6PF2K gene was digested from pKK233-2_PK2 (obtained from Dr NJ Kruger, University of Oxford, Oxford, UK) with restriction endonuclease *HindIII*. This 1425 bp fragment was blunt-ended with the Klenow fragment of DNA polymerase I and cloned into the *SmaI* site of vector pUBI 510. This recombinant 6PF2K plant expression plasmid was designated pEF2K 510.

5.2.2 Transformation of sugarcane

Sugarcane (variety NCo310) Type 3 embryogenic calli (Ho and Vasil, 1983; Taylor *et al.*, 1992) were transformed with pEF2K 510 or pEF2P 510 using a Particle Inflow Gun (Finer *et al.*, 1992). Tungsten particles (M17, Bio-Rad Laboratories, Hercules, CA, USA) (25 μl ; 100 $\text{mg}\cdot\text{ml}^{-1}$) was added to 5 μg plasmid DNA (2.5 μg pEF2K 510 or pEF2P 510 and 2.5 μg pUbi Km). The latter plasmid harbours the neomycin phosphotransferase II (NPT II) gene of transposon Tn5 from *Escherichia coli* (Beck *et al.*, 1982) driven by the maize ubiquitin 1 promoter (UBI-p). This gene encodes for geneticin resistance that was used for positive selection of transformants. Calcium chloride (25 μl ; 2.5 M), spermidine (10 μl ; 100 mM) and double distilled water (ddH₂O) to a volume of 80 μl were added to the DNA suspension. The suspension was vortexed and incubated for 5 min on ice. The supernatant was discarded and the pellet resuspended in 15 μl of absolute ethanol. The suspension was sonicated three times for 3 s at 8 W with a Virsonic 60 ultrasonic processor (The Virtis Company, Gardiner, NY, USA). The calli were placed 12 cm from the point of outlet and bombarded with 3 μl DNA-coated tungsten with a pressure of 1200 kPa using discharged helium. The chamber vacuum pressure was 90 kPa and the solenoid time setting was 50×10^{-6} s.

After bombardment the calli were incubated in the dark at 28 °C on MS3 media (1x Murashige-Skoog (MS) basal salts and vitamins (Murashige and Skoog, 1962) (Sigma, St. Louis, MO, USA), 30 $\text{mg}\cdot\text{l}^{-1}$ sucrose, 1 $\text{g}\cdot\text{l}^{-1}$ casein acid hydrolysate, 3 $\text{mg}\cdot\text{l}^{-1}$ 2,4-dichloro-phenoxyacetic acid (2,4-D) and 2 $\text{g}\cdot\text{l}^{-1}$ gelrite (pH 6)). The calli were transferred to MS3 media supplemented with 30 $\text{mg}\cdot\text{l}^{-1}$ geneticin G418 (Roche Molecular Biochemical, Mannheim, Germany) after 7 days. Calli were subcultured every two weeks on this media. Once proliferated embryos were visible

the calli were transferred to the light onto media from which 2,4-D was replaced with 1 mg.l^{-1} kinetin (Sigma, St. Louis, MO, USA). Germinated embryos were transferred onto media from which kinetin was omitted and that contained half the concentration of geneticin. Putative transgenic plantlets with well established roots were hardened off.

5.2.3 Southern blot

5.2.3.1 Isolation of genomic DNA

Genomic DNA was extracted based on a method described by Dellaporta *et al.* (1983). Leaf material (6 g) was powdered in liquid nitrogen with a mortar and pestle. Extraction buffer (35 ml; 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl (pH 8), 500 mM NaCl, 50 mM ethylene diamine tetra-acetic acid (EDTA) (pH 8) and 0.2% (v/v) β -mercaptoethanol) was added and the suspension was vortexed. Sodium dodecyl sulphate (SDS) (3.5 ml; 20% (w/v)) was added and the suspension was incubated at 70°C for 20 min. Following the addition of 7 ml (5 M) potassium acetate the suspension was mixed and incubated on ice for 20 min. The cell debris and proteins were removed by centrifugation at $10500 \times g$ for 10 min at 4°C . The supernatant was filtered through muslin cloth (pre-wetted in extraction buffer) and 1 volume ice-cold isopropanol was carefully added to precipitate the DNA. The genomic DNA was spooled out with a glass Pasteur pipette hook and air-dried in a laminar flow cabinet. The genomic DNA was resuspended in 1 ml Tris-EDTA (TE) buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8)) overnight at 37°C . Contaminating ribonucleic acid (RNA) was degraded by the addition of Ribonuclease A (RNase A) to a concentration of $10 \mu\text{g.ml}^{-1}$ and the DNA mixture was incubated at 37°C for 1 h. The genomic DNA was quantified with a fluorometer (DyNA Quant 200, Hoefer Pharmacia Biotech, Buckinghamshire, England).

5.2.3.2 DNA membrane preparation

The genomic DNA ($15 \mu\text{g}$) was completely digested with restriction enzyme BamH I as recommended by the manufactures (Promega, Madison, AL, USA). The genomic DNA fragments were precipitated and resuspended in TE buffer. Loading buffer (30% (v/v) glycerol and 0.25% (w/v) bromophenol blue) was added to the DNA and the DNA was incubated at 65°C for 20 min. The DNA fragments were size fractionated on a 0.8% (w/v) agarose gel at 3 V.cm^{-1} until the bromophenol blue migrated 8 cm. The gel was incubated in denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 10 min to denature the DNA. The gel was rinsed with ddH_2O and incubated in neutralisation solution (1 M Tris-HCl (pH 7.4) and 1.5 M NaCl) for 15 min. The gel was equilibrated for 30 min in 10x Saline Sodium Citrate (SSC) (1.5 M NaCl and 0.15 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$). The DNA was then transferred to a positively charged nylon membrane (Roche Molecular Biochemical, Mannheim, Germany) using the downward transfer method of Chomczynski and Mackey (1994). The DNA was cross-linked to the membrane with ultraviolet (UV) light (120 mJ.cm^{-2}) for 1 min in a cross-linker (Ultra Lum, Carson, CA, USA).

5.2.3.3 Probe preparation, hybridisation and visualisation

The 822 bp *Eco*RI recombinant FBPase2 fragment from pEF2P 510 (figure 5.1A) was used as probe for both the FBPase2 and 6PF2K transgenes. The probe was labelled with 25 μ Ci [α - 32 P] deoxycytidine 5'-triphosphate (dCTP) (Amersham Pharmacia Biotech, Buckinghamshire, England) using the Prime-It[®] Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA). Unincorporated deoxynucleotide triphosphates (dNTPs) were removed with spin columns (QIAquick[®] polymerase chain reaction (PCR) Purification Kit, Qiagen, Hilden, Germany).

The membrane was pre-hybridised for 4 h in Rapid-hyb[™] hybridisation buffer (Amersham Pharmacia Biotech, Buckinghamshire, England) at 65 °C. The labelled probe was boiled for 5 min, added to the hybridisation buffer and incubated overnight at 65 °C. Non-specific binding to the membrane was removed by the following treatments all at 65 °C: The membrane was rinsed in 2x SSC and 0.1% (w/v) SDS; washed for 20 min in the same solution, washed twice for 20 min in 1x SSC and 0.1% (w/v) SDS and finally in 0.1x SSC and 0.1% (w/v) SDS for 20 min. The blot was visualised with the Cyclone[™] Storage Phosphor System (Packard Instrument Company, Meriden, CT, USA) after an overnight exposure of a Multipurpose[™] storage phosphor screen to the hybridised membrane.

5.2.4 Northern blot

5.2.4.1 Isolation of RNA

Total RNA was extracted based on a method described by Bugos *et al.* (1995). Homogenisation buffer (10 ml; 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA (pH 8), 1% (w/v) SDS and 0.2% (v/v) β -mercaptoethanol) together with 10 ml phenol:chloroform:isoamyl alcohol (25:24:1) was added to 3 g powdered tissue and the suspension was vortexed for 1 min. Sodium acetate (700 μ l; 3 M (pH 5.2)) was added to the suspension, mixed and incubated on ice for 20 min. After centrifugation at 10500 xg for 15 min at 4 °C the supernatant was transferred to a new tube. An equal volume of isopropanol was added to the suspension, gently mixed and incubated at -20 °C for 30 min. The precipitated nuclei acids were recovered by centrifugation at 10500 xg for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in 750 μ l ddH₂O. Lithium chloride (to a concentration of 2 M) was added to the nuclei acids, mixed and incubated at 4 °C overnight to selectively precipitate the RNA. The RNA pellet obtained after centrifugation (16000 xg for 20 min at 4 °C) was washed with 70% (v/v) ethanol, air-dried and resuspended in 250 μ l ddH₂O. The RNA was quantified with a plate reader (PowerwaveX, Biotek Instruments Inc., Winooski, VT, USA) where an absorbance at 260 nm of = 1 = 40 μ g.ml⁻¹ RNA (1 cm path length). The RNA was stored at -80 °C.

5.2.4.2 RNA membrane preparation

Two volumes of RNA sample buffer (2 ml 5x 3-[N-Morpholino]propanesulfonic acid (MOPS) buffer containing 0.2 M MOPS, 50 mM sodium acetate and 5 mM EDTA (pH 8); 10 ml deionised formamide; 3.5 ml 37% formaldehyde) was added to 10 µg RNA consisting of 3.3 µg RNA from three different plants of the same clone. Loading buffer (30% (v/v) glycerol and 0.25% (w/v) bromophenol blue) was added to the RNA solution and incubated for 15 min at 65 °C. The RNA was size fractionated on a 1.2% agarose gel at 2.5 V.cm⁻¹ until the bromophenol blue migrated 8 cm. The RNA was transferred and cross-linked to a positively charged nylon membrane as described in section 5.2.3.

5.2.4.3 Probe preparation, hybridisation and blot visualisation

The RNA membrane was pre-hybridised for 4 h in ULTRAhyb™ hybridisation buffer (Ambion Inc., Austin, TX, USA) at 42 °C. The labelled probe (section 5.2.3) was boiled for 5 min, added to the hybridisation buffer and incubated overnight at 42 °C. Non-specific hybridisation was removed by washing the membrane as in section 5.2.3 but at 42 °C. The blot was visualised with the Cyclone™ Storage Phosphor System as in section 5.2.3.

5.2.5 Western blot

5.2.5.1 Extraction of proteins

Five volumes of extraction buffer (100 mM Tris-HCl (pH 8), 2 mM EDTA (pH 8), 0.5% (v/v) β-mercaptoethanol, Complete™ protease inhibitor tablets (Roche Molecular Biochemical, Mannheim, Germany) at 1 tablet per 50 ml and 10% (v/v) glycerol) was added to 1 g powdered sugarcane tissue. The suspension was vortexed for 20 s and incubated for 10 min on ice before it was centrifuged at 12000 xg for 20 min at 4 °C. The supernatant containing the proteins was transferred to a new tube and the proteins were quantified according to the method of Bradford (1976) using Bio-Rad protein assay dye concentrate (Bio-Rad Laboratories, Hercules, CA, USA) and immunoglobulin G (IgG) as standard.

5.2.5.2 Protein membrane preparation

One volume of protein loading buffer (250 mM Tris-HCl (pH 6.8), 8 M urea, 100 mM 1,4-dithiothreitol (DTT), 8% (w/v) SDS, 1% (w/v) bromophenol blue and 40% (v/v) glycerol) was added to 50 µg protein consisting of 16.7 µg protein from three different plants of the same clone. The protein mixture was incubated at room temperature for 15 min before the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (4% stacking gel, 12% resolution gel) (Laemmli, 1970). Following electrophoresis the gel was equilibrated in transfer buffer (48 mM Tris-base, 39 mM glycine, 20% (v/v) methanol and 0.0375% (w/v) SDS). The proteins were transferred to a nitrocellulose membrane (Hybond™ enhanced chemiluminescence (ECL)™, Amersham Pharmacia Biotech, Buckinghamshire, England) with a

semi-dry electrophoretic transfer cell (Trans-Blot[®] SD; Bio-Rad Laboratories, Hercules, CA, USA). The membrane was stained with Ponceau-S to verify the transfer of the proteins and to mark the position of the protein molecular markers. The membrane was destained with Tris-buffered saline-Tween (TBS-T) (20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween-20).

5.2.5.3 Antibody binding and blot visualisation

The ECL[™] Western blotting system (Amersham Pharmacia Biotech, Buckinghamshire, England) was used for the detection of the recombinant FBPase2 protein. All solutions and antibody dilutions were prepared with TBS-T. The membrane was incubated with 5% (w/v) blocking reagent overnight at 4 °C. The following procedure was followed for all washes: The membrane was rinsed and then washed with TBS-T for 15 min, followed by two 5 min washes in the same buffer.

The membrane was incubated for 4 h with Day 82 anti-rat FBPase2 serum (1:500 diluted) (chapter 3, section 3.2.5). The membrane was washed and incubated with the secondary antibody (anti-rabbit IgG linked with horseradish peroxidase) (1:1000 diluted) for 1 h. The membrane was washed and exposed to X-ray film (BioMax MR single emulsion, Kodak, Rochester, NY) for 1 h as described in the ECL Western blotting manual. The film was developed with Polycon developer (Champion Photochemistry, Brentwood, Essex, UK) for about 2 min, rinsed in stop solution (3% (v/v) glacial acetic acid) and fixed with Perfix fixer (Champion Photochemistry, Brentwood, Essex, UK).

5.2.6 Characterisation of the transgenic sugarcane lines

Plants were grown at Stellenbosch (Western Cape, South Africa). Control plants refer to sugarcane (variety NCo310) plants that were *in vitro* propagated but not bombarded. Plants were harvested in the morning and immediately frozen in liquid nitrogen. Leaf 1 was defined as the first unfolded leaf with a visible dewlap and is attached to internode 1 (van Dillewijn, 1952). The mid rib of the leaves were removed but the internodes' rinds not. The tissue was powdered with an analytical mill (IKA A10, Labortechnik, Staufen, Germany).

The ODe and control plants were of second ratoon. The plants were 18 months old when harvested and the same material was used for the Northern blot, Western blot and metabolite measurements. It is important to mention that the plants were subjected to low winter temperatures before harvested. Chlorophyll fluorescence measurements were performed on greenhouse grown plants 9 months post germination.

The OCe transgenic plants were approximately twelve months old when analysed. Only internodal Fru 2,6-P₂ and sucrose were measured.

5.2.6.1 Extraction of metabolites

Fru 2,6-P₂ and sugars (sucrose, glucose and fructose) from sugarcane leaves and internodal Fru 2,6-P₂ were extracted according to the chloroform/methanol methods described in chapter 4 (section 4.2.2).

Internodal sugars were extracted by the addition of 1 ml extraction buffer (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.8), 70% (v/v) ethanol and 20 mM MgCl₂) to 100 mg powdered tissue. The suspension was mixed and incubated overnight at 70 °C. The suspension was centrifuged for 10 min at 16000 *xg* before the supernatant containing the dissolved sugars was transferred to a new tube.

5.2.6.2 Measurement of metabolites

Fru 2,6-P₂, sucrose and glucose were measured as in chapter 4 (section 4.2.4). When the reaction for measuring glucose was completed, 0.5 units (U) phosphoglucose isomerase (PGI, EC 5.3.1.9) was added. The change in absorbance reflected the amount of fructose 6-phosphate and is stoichiometric to the amount of fructose.

5.2.6.3 Chlorophyll fluorescence measurements

Chlorophyll fluorescence signals were recorded with a high time-resolution fluorometer (Plant Efficient Analyzer (PEA), Hansatech, King's Lynn, Norfolk, UK). The measurements were performed in the afternoon over three consecutive days on leaf 1. The leaves were dark-adapted with a clip for 20 min before a spot of 4 mm was illuminated with 600 W.m⁻² light (650 nm).

The JIP-test analysis program, Biolyzer version 1.25.10.99⁷, was used to calculate the JIP-test parameters and the OJIP average curves.

5.2.6.4 Field trial

The ODe lines were also propagated from setts at the South African Sugar Research Institute (SASRI, Mount Edgecombe, KwaZulu Natal, South Africa). The plants were 10 months post germination upon harvesting. The mill processed 1.5 kg material per sample that equated to six stalk-tops (internodes 1 to 6) or three stalk-bottoms (internodes 7 to 10). Polarisation (pol) was measured in the cane juice with a polarimeter.

5.2.7 Statistical analysis

Analysis of variance (ANOVA) was performed with the data analysis software, Statistica version 6 (StatSoft, Inc.⁸). A p-value of ≤ 0.05 was considered as significant.

⁷ <http://www.unige.ch/sciences/biologie/bioen/jipsoftware.html>

5.3 Results

5.3.1 Transformation of sugarcane with recombinant FBPase2 and 6PF2K genes

The recombinant rat liver FBPase2 and 6PF2K genes were cloned into a plant expression vector producing pEF2P 510 and pEF2K 510 respectively (figure 5.1). We showed previously with a bacterial expression system that the protein products of these genes are functional in hydrolysing and synthesising Fru 2,6-P₂ respectively (chapter 3). After the correctness of the constructs was verified with restriction enzyme digestions (figure 5.2, table 5.1) sugarcane calli (variety NCo310) were transformed with either pEF2P 510 or pEF2K 510.

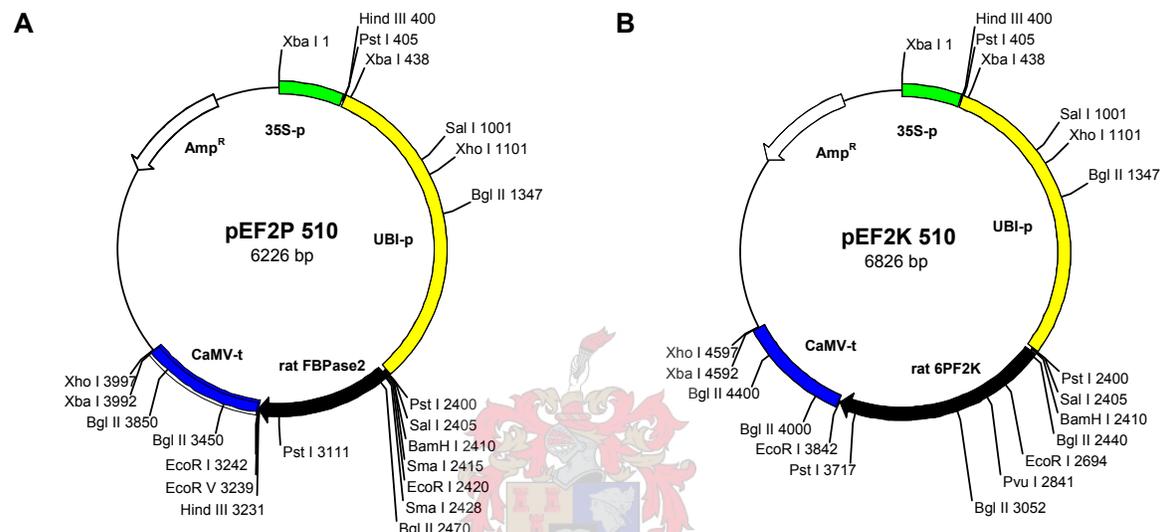


Figure 5.1. Plasmid map of pEF2P 510 (A) and pEF2K 510 (B) harbouring the recombinant rat FBPase2 and 6PF2K genes respectively. 35S-p = 35S promoter from the cauliflower mosaic virus (CaMV), UBI-p = maize ubiquitin 1 promoter, CaMV-t = CaMV poly adenylation sequence, Amp^R = ampicillin resistance encoded by the *bla* gene (β -lactamase).

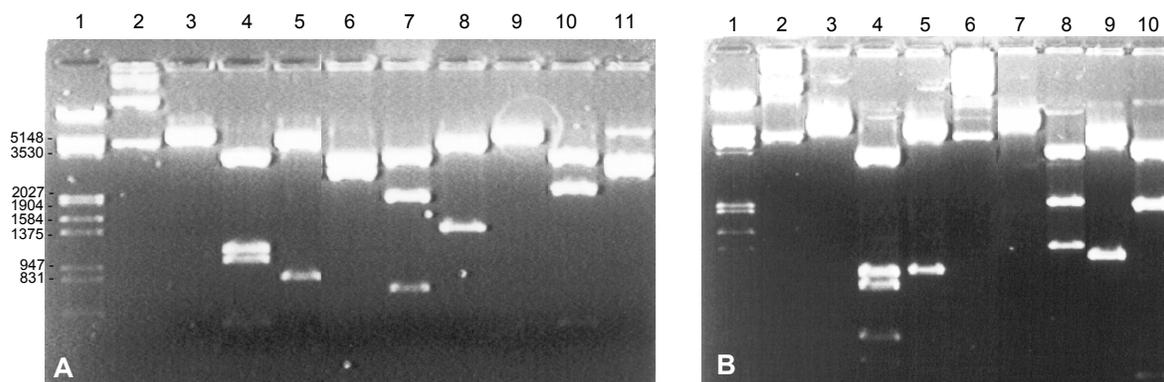


Figure 5.2. Ethidium bromide-stained agarose gels showing the fragments that were obtained after pEF2P 510 (A) and pEF2K 510 (B) were digested with restriction enzymes (see table 5.1).

Table 5.1. Restriction enzyme analysis of pEF2P 510 (A) and pEF2K 510 (B) (see figure 5.2).

A		
Lane	Restriction enzyme	Fragment size (bp)
1	DNA Marker III (Roche, Mannheim, Germany)	
2	pEF2P 510 (undigested)	
3	<i>Bam</i> HI	6226
4	<i>Bg</i> II	3723, 1123, 980, 400
5	<i>Eco</i> RI	5404, 882
6	<i>Hind</i> III	3395, 2831
7	<i>Pst</i> I	3520, 1995, 711
8	<i>Sal</i> I	4822, 1404
9	<i>Sma</i> I	6213, (13)
10	<i>Xba</i> I	3554, 2235, 437
11	<i>Xho</i> I	3330, 2896
B		
1	DNA Marker III (Roche, Mannheim, Germany)	
2	pEF2K 510 (undigested)	
3	<i>Bam</i> HI	6389
4	<i>Bg</i> II	3773, 1093, 948, 612, 400
5	<i>Eco</i> RI	5678, 1148
6	<i>Eco</i> RV	no recognition site
7	<i>Hind</i> III	6389
8	<i>Pst</i> I	3514, 1995, 1317
9	<i>Sal</i> I	5422, 1404
10	<i>Xba</i> I	4154, 2235, 437

Two clones with stable integration of the FB Pase2 transgene (designated the ODe lines) were identified with Southern blot analysis (figure 5.3). Plants ODe107, ODe113 and ODe119 had a low transgene copy (LTC) number, whereas plants ODe110 and ODe116 had a higher transgene copy (HTC) number. In addition two clones with stable integration of the 6PF2K transgene (designated the OCe lines) were identified (figure 5.4).

Transgene expression was driven by an in tandem arrangement of the CaMV 35S-p (Odell *et al.*, 1985) and UBI-p (Christensen *et al.*, 1992). The UBI-p expressed constitutively in all tissues of maize seedlings at 25 °C (Christensen *et al.*, 1992). The FB Pase2 transgene was expressed in both leaves and internodes of the LTC ODe lines as evident from Northern blot analysis (figure 5.5). The signal was more intense in leaves than internodes. The recombinant FB Pase2 transcript was present at substantial higher levels in the LTC ODe plants than the HTC plants. Recombinant transcript was only detected in leaves of the HTC plants. In agreement the recombinant FB Pase2 protein was detected only in the LTC ODe plants (leaves and internodes) as indicated with ECL Western blot analysis (figure 5.6). The plants were of second ratoon, which verified the stability of the transgene integration and expression.

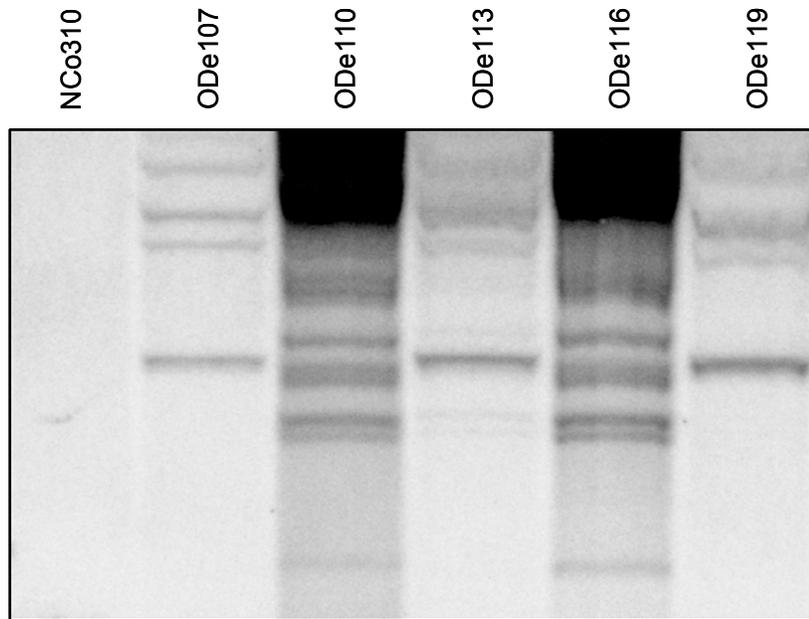


Figure 5.3. Southern blot analysis of the FBPase2 transgene in control sugarcane plants (variety NCo310) and the ODe transgenic lines.

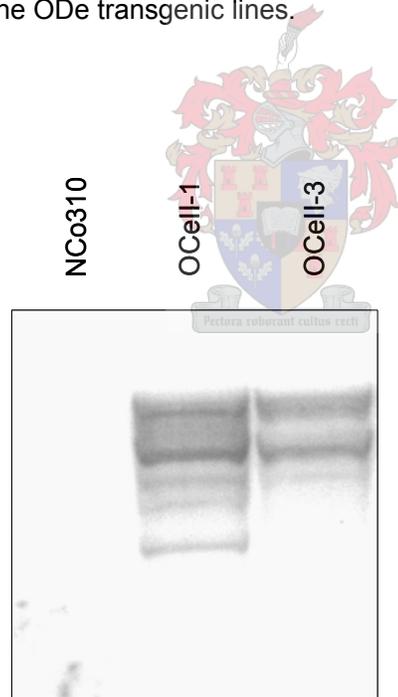


Figure 5.4. Southern blot analysis of the 6PF2K transgene in control sugarcane plants (variety NCo310) and the OCe transgenic lines.

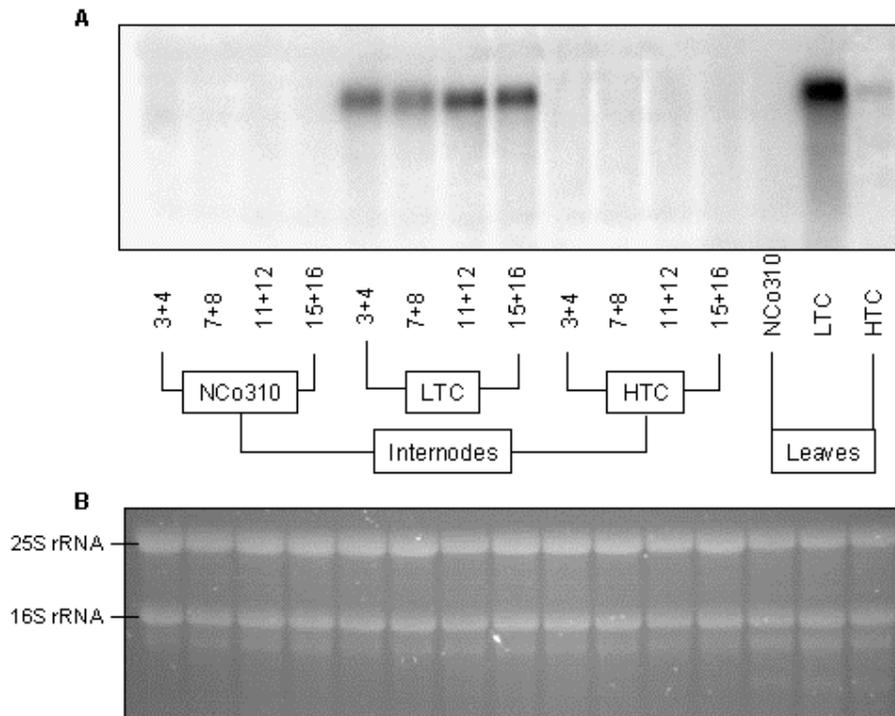


Figure 5.5. Northern blot analysis of the recombinant FBPase2 transcript in leaves and internodes of control sugarcane plants (NCo310) and the ODe transgenic lines (LTC and HTC) (A). Also showing RNA loading of individual lanes (B).

Northern blot analysis showed that the ODe transgenic lines expressed the recombinant 6PF2K (figure 5.7). RNA from a previously identified ODe plant was included in this blot. The probe, recombinant FBPase2 gene, hybridised to both recombinant FBPase2 and 6PF2K mRNA. This result was expected because the recombinant 6PF2K was derived from the native rat 6PF2K/FBPase2 by site directed mutagenesis (Tauler *et al.*, 1989; Tauler *et al.*, 1990). The recombinant FBPase2 is a 612 bp inframe deletion of the native rat 6PF2K/FBPase2 gene (Colosia *et al.*, 1988). This explains why a smaller signal was observed for the FBPase2 transcript than the 6PF2K transcript (figure 5.7). The recombinant sequences are discussed in more detail in chapter 3.

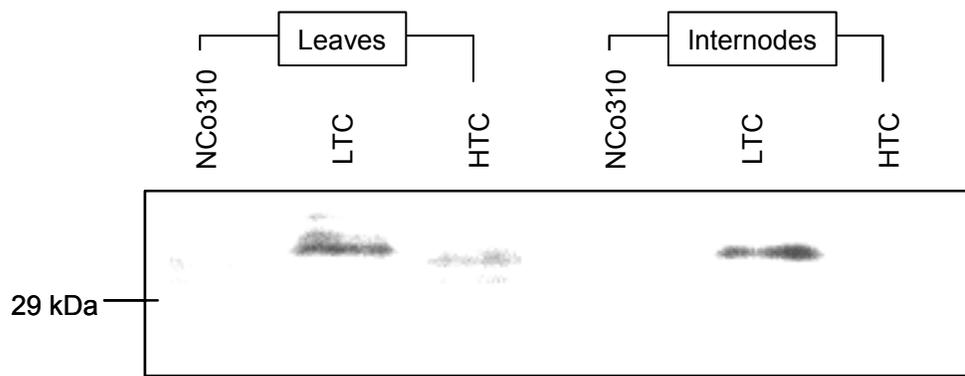


Figure 5.6. Western blot analysis of the recombinant FBPase2 protein (31 kDa) in leaves and internodes 11+12 of control sugarcane plants (NCo310) and the ODe transgenic lines (LTC and HTC).

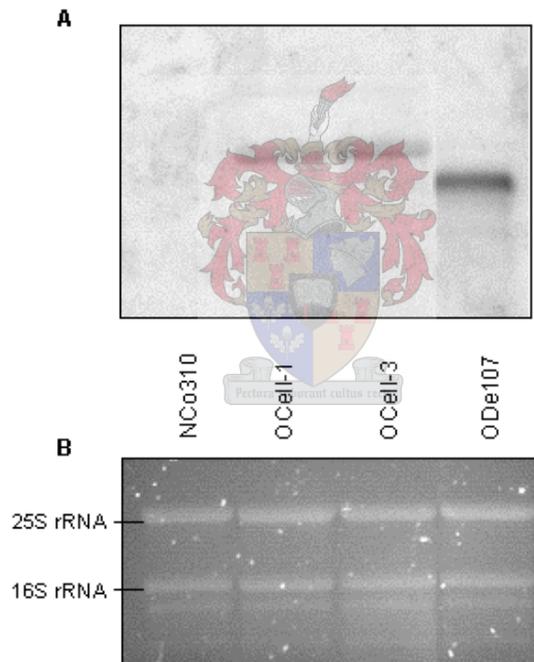


Figure 5.7. Northern blot analysis of the recombinant 6PF2K and FBPase2 transcripts in leaves of the control (NCo310), the OCe lines and ODe 107 (A). Also showing RNA loading of individual lanes (B).

5.3.2 Characterisation of the ODe transgenic plants

5.3.2.1 Fru 2,6-P₂ and sugar levels

Matured ODe transgenic lines and control plants were harvested and metabolites were extracted to determine firstly, whether the expression of the recombinant FBPase2 had altered the levels of Fru 2,6-P₂. Secondly, to determine whether altered Fru 2,6-P₂ levels had affected the levels of sucrose and the reducing sugars (glucose and fructose).

Differences in metabolite levels between the control and transgenic sugarcane plants were generally not significant. The variation within plants of the same clone was also quite high, which is not uncommon in glasshouse-grown sugarcane plants (personal experience). Nevertheless mean values showed consistent differences between the control and the ODe transgenic plants.

The LTC and HTC ODe lines contained an 11% and 18% decrease in leaf Fru 2,6-P₂ levels compared to the control plants respectively (figure 5.8A). In contrast the LTC and HTC ODe lines contained on average a 30% and 96% increase in internodal Fru 2,6-P₂ levels compared to the control plants respectively (figure 5.8B). Both the transgenic lines and the control plants showed an increase in Fru 2,6-P₂ levels from immature to mature internodes. The average Fru 2,6-P₂ content in internodes were 36, 24, and 15-times lower than in leaves for the control, LTC and HTC plants respectively. For this comparison there was assumed that 1 g fresh weight (FW) leaves contained 0.5 mg chlorophyll.

The LTC and HTC ODe transgenic lines contained 24% and 55% higher leaf sucrose levels than the control plants respectively (figure 5.9A). The average internodal sucrose content of the LTC ODe plants was similar as the control plants (102% of the control) (figure 5.9B). However the HTC ODe transgenic lines contained significant lower internodal sucrose levels than the control plants (88% of the control) (figure 5.9B). Our results again illustrate that leaf sucrose content and stem sucrose is not necessarily positive correlated in sugarcane (Grof and Campbell, 2001). Opposite trends were observed for Fru 2,6-P₂ and sucrose when leaves, internodes 3+4 or internodes 7+8 of the three plant lines were compared (figure 5.10).

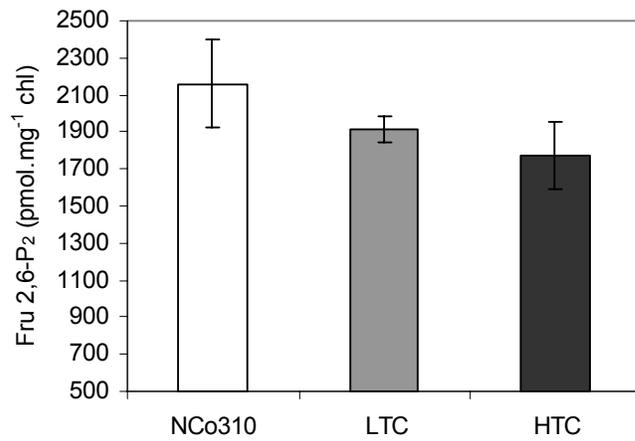
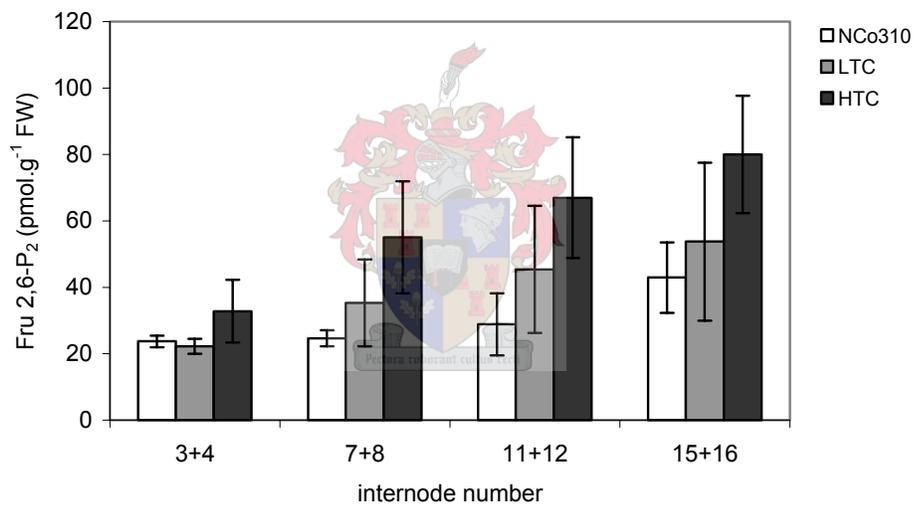
A**B**

Figure 5.8. Leaf (A) and internodal (B) Fru 2,6-P₂ levels in the control sugarcane plants (NCo310) and the ODe transgenic lines (LTC and HTC). Each data point is the mean of duplicate measurements on three extracts from different plants. Error bars represent standard error.

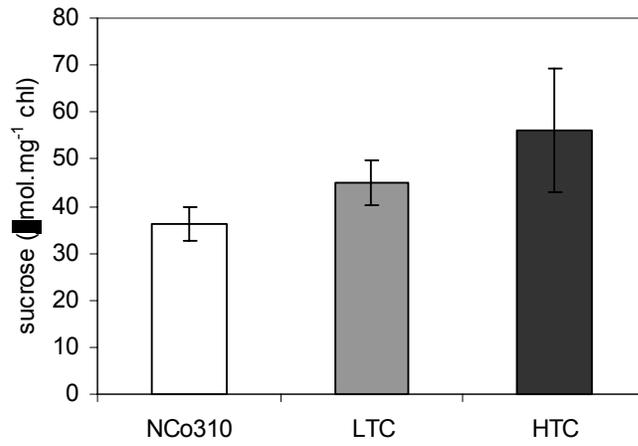
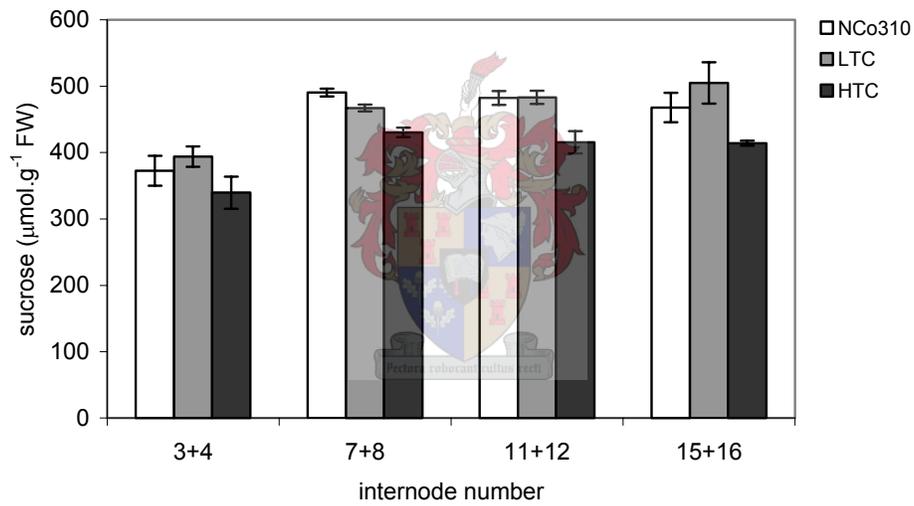
A**B**

Figure 5.9. Leaf (A) and internodal (B) sucrose levels in the control sugarcane plants (NCo310) and the ODe transgenic lines (LTC and HTC). Each data point is the mean of duplicate measurements on three extracts from different plants. Error bars represent standard error.

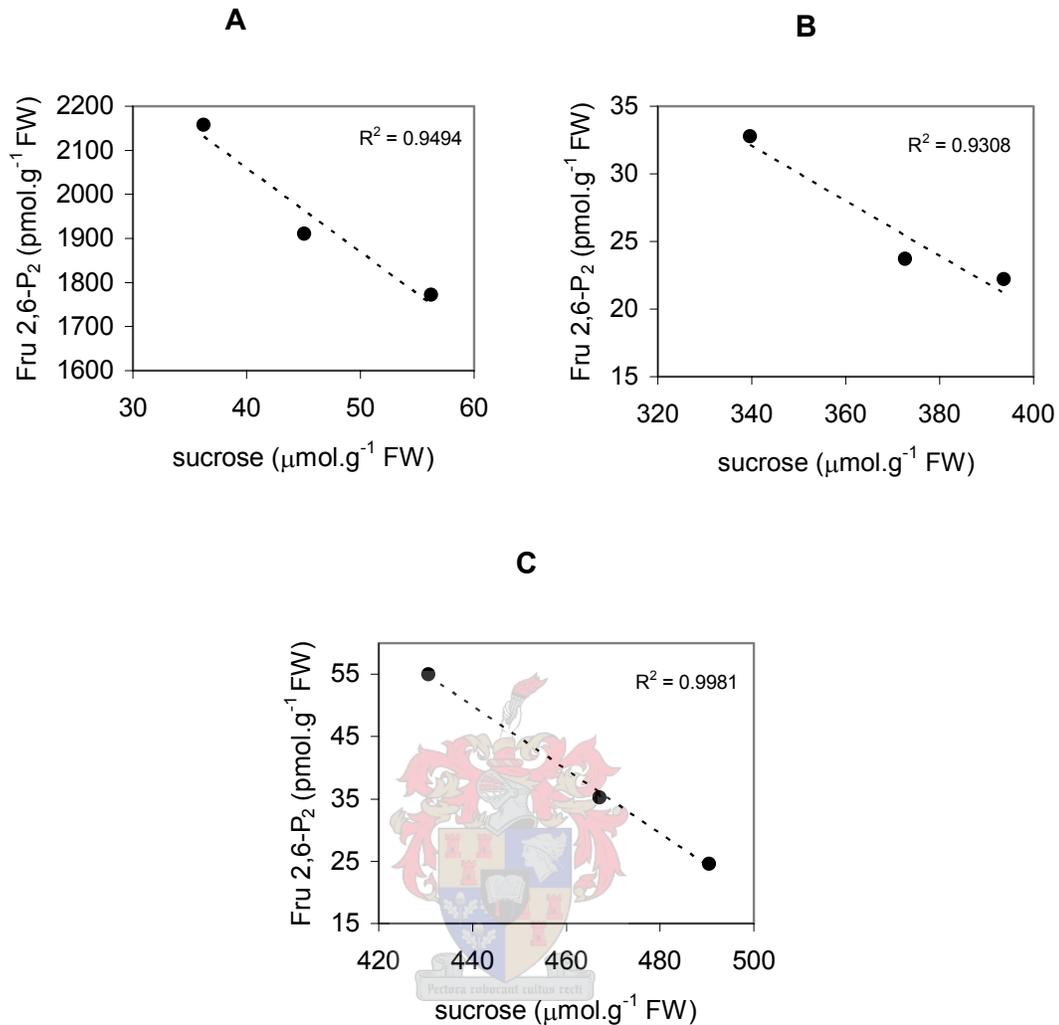


Figure 5.10. The relationship between Fru 2,6-P₂ and sucrose in leaves (A), internodes 3+4 (B) and internodes 7+8 (C) of the control (NC0310) and the ODe transgenic lines (LTC and HTC). Data points are means from figures 5.8 and 5.9.

The typical linear increase in sucrose from immature to matured internodes (Whittaker and Botha, 1997; Zhu *et al.*, 1997) was not evident in the present study. This is a characteristic of fully ripened cane, probably the result of the age of the cane and the cold temperatures the plants were subjected to before it was harvested. It is known that ripening is inversely correlated with growth rate. Conditions such as cold or depletion of growth-promoting nutrients will induce ripening (Das, 1936; Legendre, 1975).

The reducing sugars levels in leaves of the LTC and HTC ODe transgenic lines were 135% and 228% of that of the control plants respectively (figure 5.11A). The LTC ODe transgenic plants contained similar levels of reducing sugars in the internodes than the control plants (on average

104% of control) (figure 5.11B). The internodal reducing sugars levels of the HTC ODe plants were on average 360% of the control plants (figure 5.11B).

Internodal sucrose and reducing sugars were negatively correlated (figure 5.12), which is consistent with changes associated with internode maturation where reducing sugars are converted into sucrose (Lingle and Smith, 1991).

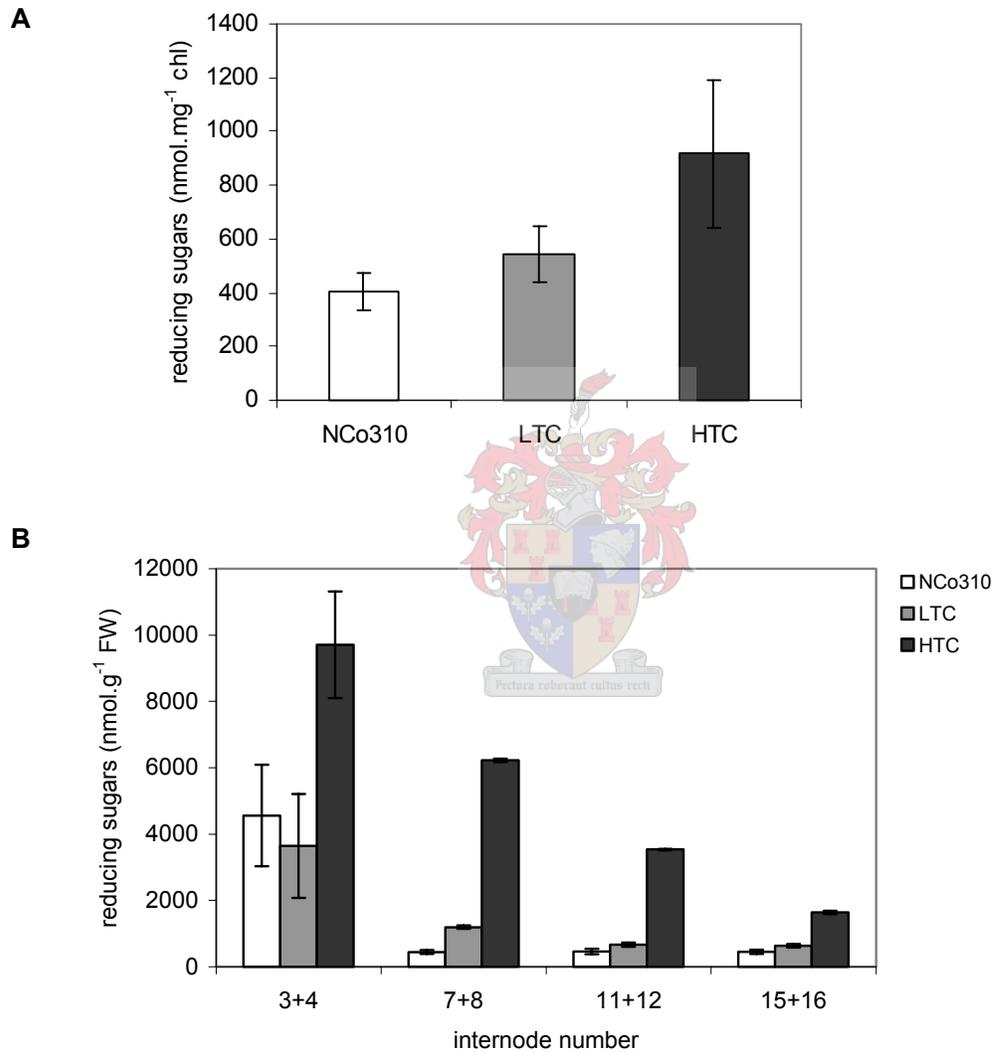


Figure 5.11. Leaf (A) and internodal (B) reducing sugars levels in the control sugarcane plants (NCo310) and the ODe transgenic lines (LTC and HTC). Each data point is the mean of duplicate measurements on three extracts from different plants. Error bars represent standard error.

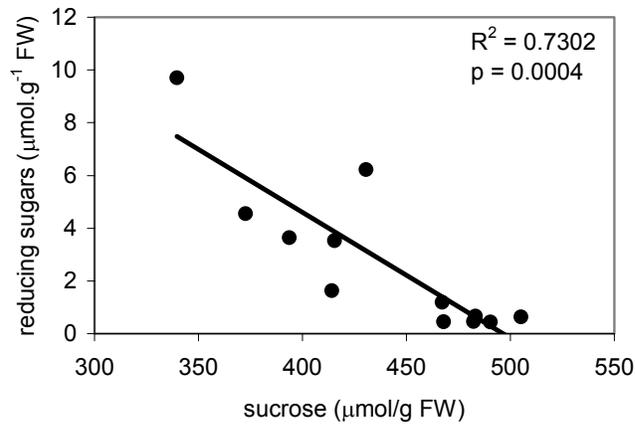


Figure 5.12. The relationship between internodal sucrose and reducing sugars (glucose and fructose). Data points are from figures 5.9B and 5.11B.

5.3.2.2 Chlorophyll fluorescence

Chlorophyll fluorescence is a powerful non-invasive technique to study the behaviour of the photosynthetic apparatus (Kautsky and Hirsch, 1931; Krause and Weiss, 1991; Strasser *et al.*, 2000). Chlorophyll fluorescence of the ODe transgenic lines and control plants were compared because it is known that Fru 2,6-P₂ plays a key role in the co-ordination of the rates of sucrose synthesis and CO₂ fixation in leaves (Stitt *et al.*, 1983; Stitt, 1990a). In agreement, the ODe transgenic lines showed increased sucrose levels compared to the control plants (figure 5.9A).

The basic principles of chlorophyll fluorescence are discussed in appendix A. During photosynthesis under normal conditions at moderate temperatures the fluorescence rise from phases O, J, I to P refers to the energy fluxes through photosystem II (PSII) and more specific the reduction of the primary quinone electron acceptor (Q_A). The average OJIP fluorescence transients are shown in figure 5.13. It is evident from the spider plot (figure 5.14), presenting the JIP-test parameters (table 5.2), that the HTC ODe plants varied most from the control plants. The most striking significant ($p \leq 0.05$) differences were a lower performance index on absorption basis (PI(ABS)), a lower photochemical rate constant (k_p), lower sum k (non-photochemical rate constant (k_n) plus k_p), and higher dissipation (DI).

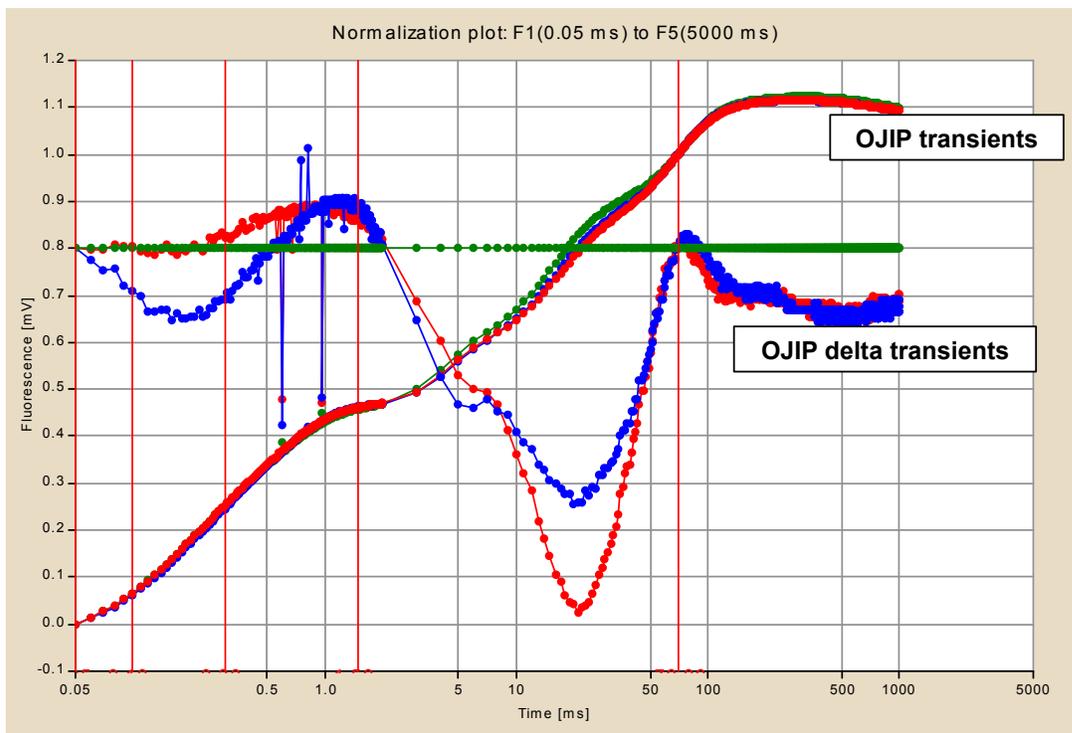


Figure 5.13. Average OJIP transients and delta transients recorded in the afternoon on the control (NCo310 (green)) and the ODe transgenic lines (LTC (blue) and HTC (red)).

Table 5.2. The JIP-test parameters calculated from afternoon chlorophyll fluorescence recordings on the control (NCo310) and the ODe transgenic lines (LTC and HTC). Each value is the mean of approximately 24 recordings per plant line; \pm is standard deviation.

	NCo310	LTC	HTC	p-value
PI(ABS)	13.823 \pm 2.050	14.062 \pm 2.634	11.974 \pm 4.075	0.02503
PI(CSo)	8121.500 \pm 1456.421	8423.622 \pm 1707.601	7676.540 \pm 2197.916	0.26850
ETo/ABS	0.424 \pm 0.015	0.419 \pm 0.024	0.403 \pm 0.031	0.01787
ETo/CSo	248.766 \pm 21.587	250.655 \pm 22.310	264.265 \pm 29.208	0.06363
ETo/RC	1.200 \pm 0.041	1.140 \pm 0.050	1.177 \pm 0.109	0.01380
ETo/TRo	0.574 \pm 0.029	0.562 \pm 0.033	0.559 \pm 0.028	0.40699
Dlo/ABS	0.259 \pm 0.031	0.255 \pm 0.011	0.280 \pm 0.042	0.00102
Dlo/CSo	151.175 \pm 15.039	152.274 \pm 10.221	187.896 \pm 54.579	0.00003
Dlo/RC	0.736 \pm 0.114	0.695 \pm 0.060	0.841 \pm 0.243	0.00055
RC/CSo	207.713 \pm 20.983	220.034 \pm 18.098	225.288 \pm 22.988	0.05048
Kn	0.448 \pm 0.085	0.428 \pm 0.033	0.425 \pm 0.036	0.31759
Kp	1.270 \pm 0.102	1.250 \pm 0.072	1.114 \pm 0.184	0.00003
sum k	1.718 \pm 0.165	1.678 \pm 0.097	1.540 \pm 0.189	0.00014

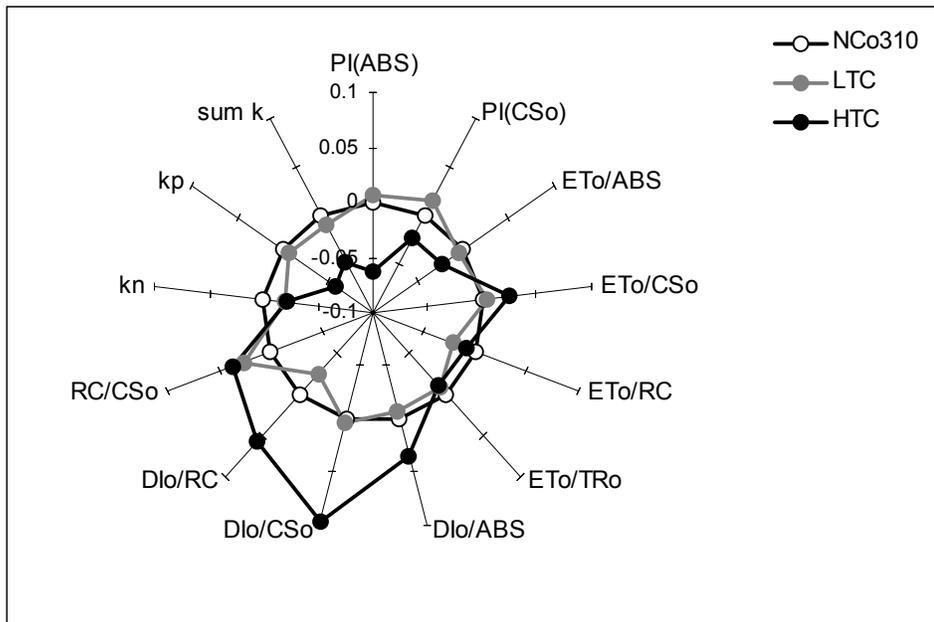


Figure 5.14. A spider plot showing the JIP-test parameters (table 5.2) for the control (NCo310) and the ODe transgenic lines (LTC and HTC).

The negative L-band ($50 - 200 \times 10^{-6}$ s) that was observed for the delta transient of the LTC plants suggests a slight increase in grouping between the light-harvesting complexes of PSII (figure 5.13). The rest of the delta transients for the HTC and LTC ODe transgenic lines had similar trends. The positive J-band ($1 - 2 \times 10^{-3}$ s) suggests a lower ETo/TRo that refers to the efficiency with which a trapped exciton moves an electron in the electron transport chain further than the reduced primary quinone acceptor (Q_A^-) of PSII. The negative I-band (5×10^{-3} s) and H-band (25×10^{-3} s) indicate a decrease in the reduction of the secondary quinone acceptor (Q_B to Q_B^- and Q_B^{2-}). The negative G-band ($70 - 1000 \times 10^{-3}$ s) probably refers to a decrease in the formation of reduced plastoquinone (PQH_2).

5.3.2.3 Field trial

The ODe transgenic lines and control plants were also propagated from setts of the glasshouse plants at the South African Sugar Research Institute (SASRI, Mount Edgecombe, KwaZulu Natal, South Africa) to investigate how the plants perform under agricultural climatic conditions. The plants were analysed in the millroom for polarisation (pol). Pol refers to the total amount of sugars in the cane juice. Pol is for most practical purposes the same as sucrose especially in matured internodes where sucrose levels exceed the reducing sugars levels by 20-times (internode 10 of variety NCo376, Voster and Botha, 1999). The LTC and HTC ODe lines contained on average 11% and 12.6% increased pol compared to the tissue culture and 5.2% and 6.8% increased pol compared to the wild type control plants respectively (figure 5.15).

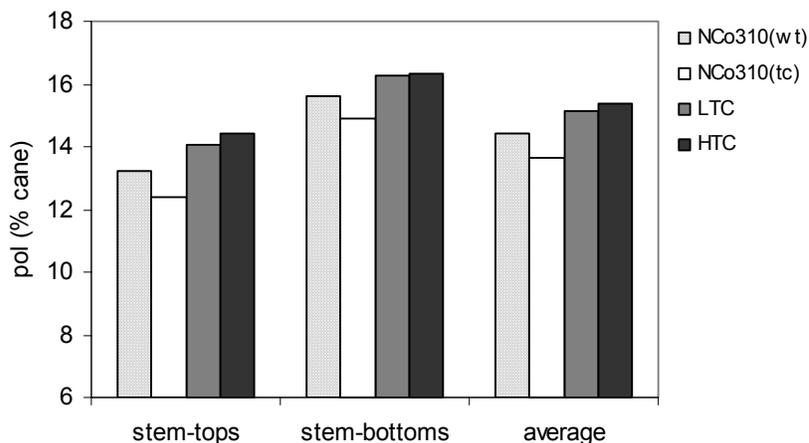


Figure 5.15. Polarisation measured in stem-tops (internodes 1 to 6) and stem-bottoms (internodes 7 to 10) in a field trial for control plants (wild type (NCo310wt) and tissue culture (NCo310tc)) and the ODe transgenic lines (LTC and HTC). Each value is for 3 stem-bottoms or 6 stem-tops for control plants, and 6 stem-bottoms or 12 stem-tops for the transgenic lines.

5.3.3 Characterisation of the ODe transgenic plants

Internodal Fru 2,6-P₂ (figure 5.16) and sucrose (figure 5.17) levels of the ODe transgenic plants expressing a recombinant 6PF2K did not differ significantly from the control plants. In addition no consistent trends between Fru 2,6-P₂ and sucrose were observed. Although internodes 3+4 of line OCell-1 contained Fru 2,6-P₂ levels 206% of the control plants the levels of sucrose were 106% of that of the control plants. Line OCell-3 contained on average 23% less internodal sucrose compared to the control plants but although internodes 3+4 and 11+12 also obtained less Fru 2,6-P₂ levels (89% and 46% of the control respectively), internodes 7+8 had similar (104% of the control) Fru 2,6-P₂ levels than the control plants.

As for the ODe transgenic plants the ODe plants lacked the typical dramatic difference in sucrose levels between the immature and matured internodes. The reason for this is uncertain but most likely not the result of ripening because the sucrose levels are much lower than in ripened cane.

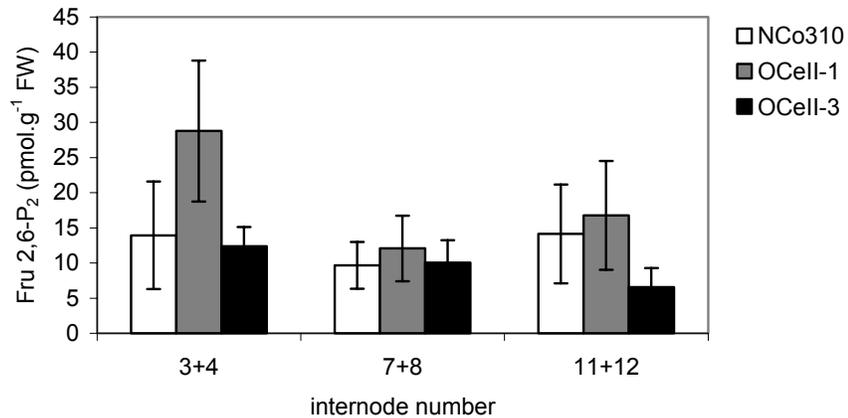


Figure 5.16. Internodal Fru 2,6-P₂ levels in the control sugarcane plants (NCo310) and the OCe transgenic lines. Each data point is the mean of duplicate measurements on four extracts from different plants. Error bars represent standard error.

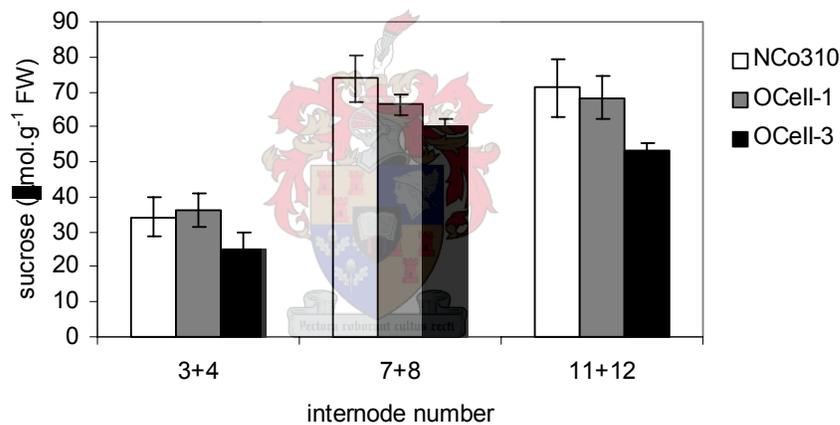


Figure 5.17. Internodal sucrose levels in the control sugarcane plants (NCo310) and the OCe transgenic lines. Each data point is the mean of duplicate measurements on four extracts from different plants. Error bars represent standard error.

5.4 Discussion

Recombinant FB Pase2 and 6PF2K genes, encoding enzymes that hydrolyse and synthesise Fru 2,6-P₂ respectively, were expressed in sugarcane as a strategy to alter the levels of Fru 2,6-P₂ and to study the subsequent effect on sugar metabolism. Higher recombinant FB Pase2 transcript and protein expression were detected in the transgenic plants with a low transgene copy number (LTC ODe plants) than in plants with a higher transgene copy number (HTC ODe plants). Previous work has suggested that multiple transgene insertions triggers silencing that might explain the poor transgene expression in the HTC ODe plants (Matzke *et al.*, 1994). However a correlation between transgene copy number and the level of expression is not always evident (Hansom *et al.*, 1999). In agreement transgene silencing was reported in

sugarcane plants harbouring one copy of the phosphinothricin acetyl transferase (*pat*) gene but not in plants with nine copies of that gene (Snyman, 2002).

The opposite trends for leaf Fru 2,6-P₂ and sucrose are consistent with the proposed role of Fru 2,6-P₂ in photosynthetic tissue. A reduction in the levels of Fru 2,6-P₂ releases the inhibition of FBPase1 that favours sucrose synthesis (Stitt *et al.*, 1983; Stitt *et al.*, 1987a). Results from chapter 4 also showed a role for Fru 2,6-P₂ in photosynthetic sucrose metabolism in sugarcane.

The ODe transgenic plants contained a dramatic increase in the levels of reducing sugars compared to the control plants. Potato plants with reduced leaf Fru 2,6-P₂ also showed a large (6-times) increase in hexose levels and it was argued that sucrose hydrolysis was induced to minimise or compensate for the increase in sucrose accumulation (Rung *et al.*, 2004).

Although CO₂ assimilation was not measured in the present study, the chlorophyll fluorescence parameter PI(ABS) compared well with the CO₂ assimilation rate in soybean (van Heerden *et al.*, 2003). As with CO₂ assimilation measurements PI(ABS) takes both biophysical and biochemical performances of the photosynthetic apparatus in account (Strasser *et al.*, 2000). Interestingly transgenic tobacco plants with decreased leaf Fru 2,6-P₂ levels contained increased sucrose levels and showed a decline in the rate of CO₂ assimilation (Scott *et al.*, 2000). It was suggested that the decreased Fru 2,6-P₂ before the start of the light period resulted in the super-optimal activation of FBPase1 upon illumination that caused high levels of Pi to be regenerated during cytosolic sucrose synthesis. This resulted in the excessive removal of triose phosphates from the chloroplast via the triose phosphate/phosphate translocator (TPT) that might have restricted the auto-catalytic build-up of photosynthetic carbon reduction cycle intermediates and therefore inhibited photosynthesis (Scott *et al.*, 2000). Transgenic potato with decreased Fru 2,6-P₂ (and increased sucrose) levels also had a lower photosynthetic rate than the control plants (Rung *et al.*, 2004) that might have been the result of sugar related repression of photosynthetic genes (Stitt *et al.*, 1991; Pego *et al.*, 2000).

In contrast to leaves the ODe lines contained higher internodal Fru 2,6-P₂ levels than the control plants. This result is unexpected for plants that overexpress an enzyme that hydrolyses Fru 2,6-P₂. It is known that increased Fru 2,6-P₂ levels are associated with different stress conditions such as low temperatures in winter and spring wheat (Van Praag and Degli Agosti, 1997), water stress in sorghum and wheat (Reddy, 1996; Reddy, 2000), upon wounding in tubers of Jerusalem artichoke (Van Schaftingen and Hers, 1983), anoxia in rice seedlings (Mertens *et al.*, 1990) and osmotic stress in barley leaves (Rung *et al.*, 2004). Although no physiological data were gathered to indicate that the plants in the present study were stressed, the leaf canopies showed signs of necrosis and buds germinated from the bottom (matured)

internodes of some of the stalks. These stress symptoms were probably caused by low temperatures. The plants were grown outside the normal agricultural region and were subjected to a severe winter. In addition the plants were grown in small pots that might have contributed to stress. The internodal Fru 2,6-P₂ levels in the control plants for the ODe lines were on average 2-times higher than the control plants in the OCe trial. The OCe plants showed no symptoms of stress.

The ODe transgenic plants might have been more sensitive to stress due to a longer tissue culture period, which could explain the increased internodal Fru 2,6-P₂ levels compared to the control plants. Tremblay *et al.* (1999) showed that somaclonal variation in tissue culture plants is a factor of time in maintenance. Somaclonal variation could affect the degree to which a plant is resistant to disease (Bajaj, 1990). The higher transgene expression in leaves than internodes (if higher transcription also reflects higher enzyme activity) might have resulted in a decrease in Fru 2,6-P₂ levels in leaves of the transgenic lines even under stress conditions. Interestingly, Rung *et al.* (2004) found that it is less effective to reduce Fru 2,6-P₂ levels in sink tissues than in source tissues of transgenic plants.

In the present study Fru 2,6-P₂ levels increased from immature to mature internodes in both the control and ODe transgenic lines. This contradicts with work done by Whittaker and Botha (1997) where they showed a decrease in Fru 2,6-P₂ levels from immature to mature internodes. Another study also reported highest Fru 2,6-P₂ levels in the most immature internodes (internodes 1 and 2) but no pattern in Fru 2,6-P₂ levels were found in the subsequent internodes down the stem (Lingle and Smith, 1991).

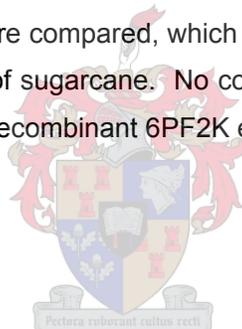
The conflicting internodal Fru 2,6-P₂ profiles between those reported by Whittaker and Botha (1997) and the present study might be the result of different experimental conditions and plant materials between the two studies. Whittaker and Botha (1997) used field-grown sugarcane (variety NCo376) whereas fully ripened possibly stressed greenhouse-grown plants (variety NCo310) were used in the present study. In addition the rinds of the internodes were not removed before metabolite extraction in the present study whereas the internodes' rinds were removed in the study conducted by Whittaker and Botha (1997).

Despite the unexpected increased internodal Fru 2,6-P₂ levels and profile, as for leaves opposite trends for internodal Fru 2,6-P₂ and sucrose were observed when internodes 3+4 or internodes 7+8 of the plant lines were compared. Lingle and Smith (1991) also found a significant negative correlation between Fru 2,6-P₂ and sucrose within individual wild type sugarcane stems but this relationship was not evident in every trial.

Results from the field trial indicated that the tissue culture control contained lower pol (sucrose) than the control plants. This agrees with the conception that transgenic sugarcane plants produce lower sucrose content and yield probably caused by somaclonal variation, i.e. permanent genetic effects as a result of a long tissue culture procedure (Grof and Campbell, 2001). In contrast the ODe transgenic lines possessed higher pol than the wild type control plants and is therefore promising in terms of yield.

The above finding contradicts the result obtained from the glasshouse trials where the ODe transgenic lines contained lower sucrose levels than the control plants. Fru 2,6-P₂ levels were not determined for the field-grown cane. It is therefore difficult to explain but this might indicate that there is only under certain situations, e.g. fully ripened or stress conditions, a relationship between Fru 2,6-P₂ and sucrose.

To summarise, plants expressing a recombinant FBPase2 contained decreased leaf Fru 2,6-P₂ and an unexpected increase in internodal Fru 2,6-P₂ levels compared to the control plants. Opposite trends for Fru 2,6-P₂ and sucrose were evident when leaves, internodes 3+4 or internodes 7+8 of the plant lines were compared, which indicate a possible role for Fru 2,6-P₂ in sugar metabolism in these tissues of sugarcane. No consistent trends between Fru 2,6-P₂ and sucrose were evident for the ODe (recombinant 6PF2K expressing) transgenic plants.



APPENDIX A

The basic principles of chlorophyll fluorescence and the JIP-test

When a dark-adapted leaf is illuminated the photon flux is absorbed (ABS) by the antenna pigments (figure 5.18). A fraction of the excited chlorophyll's (Chl*) energy is dissipated (DI) mainly as heat and some as fluorescence emission. The remaining energy is trapped (TR) to the reaction centre (RC), converted to redox energy and utilised for photochemistry. The reduction and re-oxidation of acceptors creates an electron transport (ET) that pumps protons across the thylakoid membrane. This electrochemical gradient is utilised for adenosine 5'-triphosphate (ATP) production and ultimately for CO₂ fixation in the photosynthetic carbon reduction cycle.

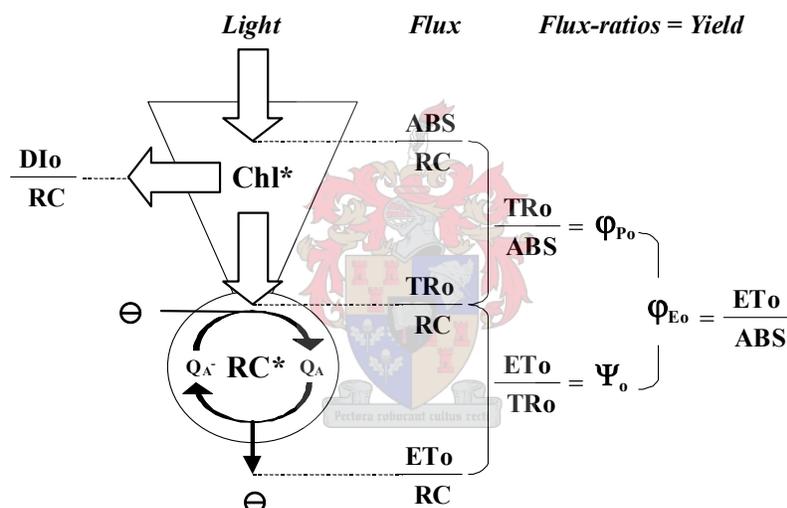


Figure 5.18. A simplified scheme for the energy cascade through photosystem II (PS II) (from Hermans *et al.* (2003)). See text for details.

The chlorophyll emission or fluorescence transient of chlorophyll a was first observed by Kautsky and Hirsch (1931). It consists of a rise from initial fluorescence (F_0) to maximum fluorescence (F_p) that lasts for about 1 s before it declines. At normal conditions at room temperature the chlorophyll a fluorescence rise is almost entirely attributed to PSII and reflects the closure / reduction of the primary quinone acceptor (Q_A to Q_{A^-}) (Krause and Weiss, 1991). The three energy release processes of Chl^* (heat, fluorescence and photochemistry) are competitive. Therefore although chlorophyll a fluorescence is typically only 2.5 – 5% of the absorbed energy, information on the performance of the photosynthetic apparatus can be obtained (Krause and Weiss, 1991; Govindjee, 1995).

When recorded with a high time-resolution fluorometer (e.g. the Plant Efficient Analyzer (PEA), Hansatech, King's Lynn, Norfolk, UK) and plotted on a logarithmic time scale the fluorescence rise is polyphasic with two of the intermediate steps at 2×10^{-3} s (J) and at $20 - 30 \times 10^{-3}$ s (I) (Strasser and Govindjee, 1992). The JIP-test (Strasser and Strasser, 1995; Strasser and Tsimilli-Michael, 2001), after the basic steps of the transient, uses parameters derived from the energy fluxes through PSII to link the energetic behaviour of the photosynthetic apparatus and the fluorescence signal. The JIP-test parameters are defined in table 5.3.

Table 5.3. The JIP-test parameters for the analysis of the fluorescence transient of chlorophyll a.

The specific fluxes expressed per reaction centre (RC)		
Absorption	ABS/RC	$= (M_0/V_J) / (1-F_0/F_M)$
Trapping at time zero	TRo/RC	$= M_0/V_J = (ABS/RC) \phi_{P_0}$
Dissipation at time zero	Dlo/RC	$= (ABS/RC) - (TRo/ABS)$
Electron transport at time zero	ETo/RC	$= (TRo/RC) \Psi_0$
The phenomenological fluxes expressed per cross section (CS) of the leaf tissue		
Absorption	ABS/CS	measured by absorption techniques or approximated by F_0 or F_M
Trapping at time zero	TRo/CS	$= (TRo/ABS) / (ABS/CS)$
Dissipation at time zero	Dlo/CS	$= (ABS/CS) - (TRo/CS)$
Electron transport at time zero	ETo/CS	$= (ETo/RC) (RC/CS)$
Density of RCs per CS	RC/CS	$= (ABS/CS) (RC/ABS)$
The yields (fluxes ratios)		
Maximum quantum yield of primary photochemistry	ϕ_{P_0}	$= TRo/ABS = (F_M - F_0) / F_M$ $= 1 - (F_0/F_M)$
Maximum quantum yield of non photochemical de-excitation	ϕ_{D_0}	$= Dlo/ABS = 1 - \phi_{P_0} = F_0/F_M$
Probability that a trapped exciton moves an electron further than Q_A^-	Ψ_0	$= ETo/TRo = 1 - V_J$
Probability that an absorbed photon moves an electron further than Q_A^-	ϕ_{E_0}	$= \phi_{P_0} \Psi_0 = (TRo/ABS) (ETo/TRo)$ $= ETo/ABS = (1-F_0/F_M) (1-V_J)$
Vitality indexes		
Density of RCs per chlorophyll	RC/ABS DF(RC)	$= (RC/TRo) (TRo/ABS)$ $= (V_J/M_0) (F_V/F_M)$ $= \text{Log}(RC/ABS)$
Conformation term for primary photochemistry	$(\phi_{P_0}/(1 - \phi_{P_0}))$ DF$_{\phi}$	$= TRo/Dlo = k_P/k_N = F_V/F_0$ $= \text{Log}(\phi_{P_0}/(1 - \phi_{P_0}))$
Conformation term for the thermal reactions (non light depending reactions) beyond Q_A^-	$(\Psi_0/(1 - \Psi_0))$ DF$_{\Psi}$	$= ETo/(dQ_A^-/dt_0)$ $= \text{Log}(\Psi_0/(1 - \Psi_0))$
Performance Index	PI(ABS)	$= [RC/ABS][\phi_{P_0}/(1 - \phi_{P_0})][\Psi_0/(1 - \Psi_0)]$
Photosynthetic driving force on a cross-section basis	DF(CS)	$= \text{Log} [PI_{ABS}] + \text{Log}(\phi_{P_0}/(1 - \phi_{P_0}))$ $= \text{Log}(\Psi_0/(1 - \Psi_0))$ $= DF_{RC} + DF_{\phi} + DF_{\Psi}$

CHAPTER 6

General discussion

There were three main objectives in this study. Firstly, to determine whether the recombinant rat 6-phosphofructo 2-kinase (6PF2K, EC 2.7.1.105) and fructose 2,6-bisphosphatase (FBPase2, EC 3.1.3.11) enzymes, which catalyse the synthesis and degradation of fructose 2,6-bisphosphate (Fru 2,6-P₂) respectively, showed any catalytic activity as fusion proteins. Secondly, to alter the levels of Fru 2,6-P₂ in sugarcane by expressing these genes. Thirdly, to investigate whether sugar metabolism in photosynthetic (leaves) and non-photosynthetic tissues (internodes) were subsequently influenced.

Activity tests performed on the bacterially expressed glutathione-S-transferase (GST) fusion 6PF2K and FBPase2 proteins showed that they are catalytic active (chapter 3). The expression of similar recombinant rat enzymes (driven by the 35S promoter from the cauliflower mosaic virus) has altered the levels of Fru 2,6-P₂ in tobacco (Scott *et al.*, 1995; Scott *et al.*, 2000) and *Kalanchoe daigremontiana* (Truesdale *et al.*, 1999).

The polyclonal antibody raised against the GST fusion recombinant FBPase2 was not very sensitive in detecting that protein in crude protein extracts of the transgenic sugarcane plants. The recombinant FBPase2 was only detected with enhanced chemiluminescence (ECL) Western blotting that is at least 10-times more sensitive than colorimetric methods. It is possible that many or most of the antibodies were raised against GST that resulted in a low titre against the recombinant FBPase2. It might have been better to remove the GST tag before antibodies were raised against the recombinant proteins. Another reason for poor detection of the recombinant FBPase2 could simply be that only low recombinant protein levels were present *in vivo*.

Fru 2,6-P₂ levels have been reported for sugarcane internodes (Whittaker *et al.*, 1997) but until the present study no data were available for sugarcane leaves. Fru 2,6-P₂ is often difficult to extract from plant tissues because it is extremely susceptible to hydrolysis by non-specific phosphatases. Furthermore the *in vivo* Fru 2,6-P₂ concentration is very low: 1.7 x 10⁴ and 2.0 x 10⁷-times lower than sucrose levels in sugarcane leaves and internodes respectively (leaves and internodes 7+8 of the control sugarcane plants (variety NCo310), chapter 5).

The optimised chloroform/methanol extraction procedure gave reproducible Fru 2,6-P₂ recoveries of 93% from sugarcane leaves and 85% from internodal tissues (chapter 4). Internodes contain 36-times lower Fru 2,6-P₂ levels than leaves (variety NCo310, chapter 5; assuming that 1 gram fresh weight (FW) leaves contain 0.5 mg chlorophyll), which might

contribute to the lower recovery from internodes compared to leaves. In addition the optimised methods produced linear standard curves for Fru 2,6-P₂ against PFP activity, used in the PFP-coupled assay to calculate Fru 2,6-P₂ levels, for both leaves and internodal extracts. Linear curves are a prerequisite for accurate Fru 2,6-P₂ measurements (Trevanion, 2000).

Transgenic sugarcane plants expressing a recombinant FBPase2 or 6PF2K (ODe and OCe transgenic lines respectively) were generated (chapter 5). The ODe transgenic lines contained decreased leaf Fru 2,6-P₂ levels and higher leaf sucrose and reducing sugar levels than the control plants. Clones with the highest leaf Fru 2,6-P₂ levels contained the lowest sucrose levels suggesting that Fru 2,6-P₂ is involved in sugar metabolism in sugarcane leaves. This agrees with the observation that the most dramatic changes in sugarcane Fru 2,6-P₂ levels were observed with the transition from dark to light (decline in Fru 2,6-P₂ levels) and from light to dark (rise in Fru 2,6-P₂ levels) (chapter 4). This is consistent with the described function of Fru 2,6-P₂ in leaves of other plants where the drop in Fru 2,6-P₂ levels upon illumination abolishes the inhibition of fructose 1,6-bisphosphatase (FBPase1, EC 3.1.3.11) that allows sucrose to accumulate during photosynthesis (Stitt, 1990a). In the dark the rise in Fru 2,6-P₂ levels inhibits FBPase1 and subsequently sucrose accumulation.

In contrast to leaves the expression of a recombinant FBPase2 did not reduce the levels of Fru 2,6-P₂ in ripened internodes. In fact the ODe transgenic lines contained higher internodal Fru 2,6-P₂ levels than the control plants. The increased Fru 2,6-P₂ might be stress induced (discussed in chapter 5). Although genetic transformation allows specific genes to be manipulated, the expression of other genes could be influenced to compensate for the change that was introduced (Stitt, 1995). Another explanation for the increased Fru 2,6-P₂ levels in the transgenic lines might therefore be over compensation. The transformation of transgenic plants with a second transgene (double transformants) will probably become standard practice for studying plant metabolism.

In spite of the unexpected increase in internodal Fru 2,6-P₂ as for leaves opposite linear trends for Fru 2,6-P₂ and sucrose were evident for internodes 3+4 or 7+8 when the different plant lines were compared. Although the function of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90), which is activated by Fru 2,6-P₂, in plants is unclear, there is a negative correlation between PFP activity and stem sucrose content across different sugarcane varieties (Whittaker and Botha, 1999). Sucrose accumulation in sugarcane coincides with the redirection of carbon from insoluble matter, amino acids, phosphorylated intermediates, and respiration into sucrose (Moore, 1995; Whittaker and Botha, 1997; Whittaker and Botha, 1999). The decreased allocation of carbon to respiration corresponds with a decreased flux from hexoses (Whittaker and Botha, 1997) that might be attributed to the decline

in PFP activity that is observed with internode maturation (Whittaker and Botha, 1999). In addition transgenic sugarcane plants in which PFP activity was reduced by 13 – 40% contained a 20 – 50% increase in sucrose content in maturing internodes (Groenewald and Botha, 2001).

The lower sucrose levels in the HTC ODe transgenic lines than the control plants in the present study could therefore be attributed to higher PFP activity caused by increased Fru 2,6-P₂ levels. Future analysis of the ODe transgenic plants have to include PFP activity tests, hexose phosphates and triose phosphates measurements to confirm this hypothesis. Transgenic sugarcane plants with reduced PFP activity contained increased hexose phosphates and decreased triose phosphate levels (M van der Merwe and FC Botha, unpublished results⁹).

Despite lower FBpase2 transgene expression in the high transgene copy (HTC) plants than in the low transgene copy (LTC) plants, the metabolic results and chlorophyll fluorescence for the HTC plants varied more from the control. The reason for this is not clear but the following has to be kept in mind. Firstly, although a high level of transgene expression is generally required to overcome the plant's endogenous regulatory systems to have an effect on metabolism (Sonnewald *et al.*, 1994), the allosteric effectors that modulate the activities of the plant 6PF2K and FBpase2 enzymes do not influence the heterologous enzymes used in this study (Kruger and Scott, 1995). Secondly, lower transcription / protein levels do not necessarily mean that the HTC plants possessed lower FBpase2 activity. For example Banzai *et al.* (2003) reported increased Fru 2,6-P₂ levels and 6PF2K activity in NaCl- and mannitol-osmotically stressed and in dehydrated *Bruguiera gymnorhiza* plants. Increased transcript levels were detected only in the osmotically stressed plants. Thus Fru 2,6-P₂ data need to be supplemented with 6PF2K and FBpase2 activity tests in future work.

A limitation of our study was the limited number of transgenic lines available for analysis. In addition the variation between the glasshouse-grown plants of the same clone was also large. More plants of the same clone need to be analysed to verify the trends that were observed.

The transgenic plants grown at SASRI under normal agricultural conditions contained increased polarisation (sucrose) compared to the control plants. This result is only preliminary and has to be confirmed with relation to the expression of the recombinant FBpase2 and Fru 2,6-P₂ levels. These plants showed a 6% increase in polarisation, which could have a tremendous economic impact. A 1% increase in saleable sugar will increase South Africa's foreign exchange earnings with R2 million.

⁹ FC Botha, South African Sugar Research Institute, Mount Edgecombe, South Africa

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