Manipulation of pyrophosphate fructose 6-phosphate 1-phosphotransferase activity in sugarcane

by

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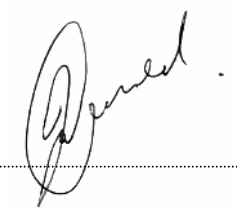
DECLARATION

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



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SUMMARY

The main aim of the work presented in this thesis was to elucidate the apparent role of pyrophosphate fructose 6-phosphate 1-phosphotransferase (PFP) in sucrose accumulation in sugarcane. PFP activity in sugarcane internodal tissue is inversely correlated to the sucrose content and positively to the water-insoluble component across varieties which differ in their capacities to accumulate sucrose. This apparent well defined and important role of PFP seems to stand in contrast to the ambiguity regarding PFP's role in the general literature as well as the results of various transgenic studies where neither the down-regulation nor the over-expression of PFP activity had a major influence on the phenotype of transgenic potato and tobacco plants. Based on this it was therefore thought that either the kinetic properties of sugarcane PFP is significantly different than that of other plant PFPs or that PFP's role in sucrose accumulating tissues is different from that in starch accumulating tissues.

In the first part of the study sugarcane PFP was therefore purified and its molecular and kinetic properties were determined. It consisted of two subunits which aggregated in dimeric, tetrameric and octameric forms depending on the presence of Fru 2,6-P_2. Both the glycolytic and gluconeogenic reactions had broad pH optima and the kinetic parameters for all the substrates were comparable to that of other plant PFPs. The conclusion was therefore that sugarcane PFP's molecular and kinetic characteristics do not differ significantly from that of other plant PFPs.

The only direct way to confirm if PFP is involved in sucrose accumulation in sugarcane is to alter its levels in the same genetic background through genetic engineering. This was therefore the second focus of this study. PFP activity was successfully down-regulated in sugarcane. The transgenic plants showed no visible phenotype under greenhouse and field conditions and sucrose concentrations in their immature internodes were significantly increased. PFP activity was inversely correlated with sucrose content in the immature

internodes of the transgenic lines. Both the immature and mature internodes of the transgenic plants had significantly higher fibre contents.

This study suggests that PFP plays a significant role in glycolytic carbon flux in immature, metabolically active sugarcane internodal tissues. The data presented here confirm that PFP can indeed have an influence on the rate of glycolysis and carbon partitioning in these tissues. It also implies that there are no differences between the functions of PFP in starch and sucrose storing tissues and it supports the hypothesis that PFP provides additional glycolytic capacity to PFK at times of high metabolic flux in biosynthetically active tissue. This work will serve as a basis to refine future genetic manipulation strategies and could make a valuable contribution to the productivity of South African sugarcane varieties.

OPSOMMING

Die hoofdoelwit van die werk wat in hierdie proefskrif beskryf word, was om die potensiële rol wat pirofosfaat fruktose 6-fosfaat 1-fosfotransferase (PFP) in sukrose akkumulering in suikerriet mag speel, te ontrafel. PFP-aktiwiteit in suikerriet-internodale-weefsel is omgekeerd eweredig aan die sukrose-inhoud en direk eweredig aan die wateronoplosbare komponent in talle variëteite wat verskillende kapasiteite het om sukrose te akkumuleer. Hierdie blykbaar duidelike en goed gedefinieerde rol van PFP staan in kontras teenoor die onsekerheid aangaande die rol vir PFP soos wat dit in die literatuur beskryf word. Resultate van verskeie transgeniese studies het ook getoon dat geen aansienlike fenotipiese veranderinge deur die afregulering of die ooruitdrukking van PFP-aktiwiteit in transgeniese aartappel- en tabakplante teweeg gebring is nie. Daar is dus op grond van hierdie inligting afgelei dat die kinetiese eienskappe van suikerriet-PFP óf aansienlik van dié van ander plant-PFPs verskil óf dat PFP se funksie in sukrose-akkumulerende weefsel verskil van dié in stysel-akkumulerende weefsel.

In die eerste deel van die studie is PFP gesuiwer en die molekulêre- en kinetiese eienskappe daarvan is bepaal. Die ensiem is uit twee subeenhede saamgestel wat in dimeriese-, tetrameriese- of oktameriese vorme kan aggregeer, afhangend van die teenwoordigheid van Fru 2,6-P_2. Beide die glikolitiese- en die glukoneogeniese reaksies het ‘n breë pH-optimum en die kinetiese parameters vir alle substrate het met ander plant-PFPs ooreengestem. Die resultate het dus bevestig dat die molekulêre- en kinetiese eienskappe van suikerriet-PFP nie aansienlik van dié van ander plant-PFPs verskil nie.

Die enigste direkte manier om die betrokkenheid van PFP by sukrose-akkumulering in suikerriet te bevestig, is om die ensiemvlakke in presies dieselfde genetiese agtergrond, dmv genetiese manipulering, te verander. Hierdie manipulering was dan die tweede fokus van die studie. PFP-aktiwiteit is suksesvol afgereguleer in suikerriet. Die transgeniese plante het nie enige sigbare fenotipe onder glashuis- of veldtoetstande getoon nie en die

sukrose-konsentrasies in die jong internodes was aansienlik verhoog. PFP-aktiwiteit was omgekeerd eweredig aan sukrose-inhoud in die jong internodes van transgeniese lyne. Die veselinhoud van beide die jong- en volwasse internodes van die transgeniese plante was aansienlik verhoog.

Resultate wat in hierdie studie verkry is, dui daarop dat PFP ‘n baie belangrike rol in glikolitiese koolstoffluks in jong, metabolies-aktiewe suikerriet internodale weefsel speel. Die data bevestig verder dat PFP wel die glikolise-tempo en koolstofverdeling in hierdie weefsels beïnvloed en dat daar geen verskille tussen die funksie van PFP in sukrose- en styselstorende weefsel is nie. Dit ondersteun dus die hipotese dat PFP bydra tot die glikolitiese kapasiteit ten tye van hoë metaboliese fluks in biosinteties-aktiewe weefsel. Hierdie werk vorm ‘n basis waarvandaan toekomstige genetiese manipuleringstrategieë verfyn kan word en kan ‘n baie belangrike bydrae tot die produktiwiteit van Suid-Afrikaanse suikerrietvariëteite lewer.

Vir Sarita, Marko en Tian – want julle gee betekenis aan alles.

"If you stood on the bottom rail of a bridge, and leant over, and watched the river slipping slowly away beneath you, you would suddenly know everything that there is to be known."

Winnie the Pooh on knowledge

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PREFACE

This dissertation is presented as a compilation of six chapters. In Chapter 1 the overarching aim and approach to the study is introduced and the aims and outcomes of each individual chapter is summarised. Similarly, Chapter 6 concludes the work with a general discussion which aims to integrate the work presented in all the other chapters and focuses on the general conclusions. Chapter 2 is a literature review in which, after critical discussion of the available literature, two probable mechanisms through which PFP can influence sucrose accumulation is presented. Each of the experimental chapters (Chapter 3-5) has a distinct aim and outcome and is introduced separately. Each chapter that will be submitted for publication is written according to the style of the particular journal as listed below; these papers will be co-authored by FC Botha.

Chapter 1	General introduction. Will not be submitted for publication. Style: Plant Physiology.
Chapter 2	Literature review: Characteristics and potential function of pyrophosphate: fructose-6-phosphate 1-phosphotransferase with special reference to the sucrose accumulation phenotype of sugarcane culm. Plant physiology and biochemistry.
Chapter 3	Purification and characterisation of pyrophosphate fructose-6-phosphate 1-phosphotransferase from sugarcane. Journal of plant physiology.
Chapter 4	Development and characterisation of transgenic systems for the manipulation of PFP activity in sugarcane. Will not be submitted for publication. Style: Plant Physiology.
Chapter 5	Down-regulation of pyrophosphate fructose 6-phosphate 1-phosphotransferase activity in sugarcane enhances sucrose accumulation in immature internodes. Transgenic research.
Chapter 6	General Discussion and Conclusions. Will not be submitted for publication. Style: Plant Physiology.

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy acetic acid
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
CaMV-35S	Cauliflower mosaic virus' 35S ribosomal subunit's promoter sequence
DEPC	diethyl pyrocarbonate
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
Fru	fructose
Fru 6-P	fructose 6-phosphate
Fru 1,6-P_2	fructose 1,6-bisphosphate
Fru 2,6-P_2	fructose 2,6-bisphosphate
FBPase	fructose-1,6-bisphosphatase (EC 3.1.3.11)
FW	fresh weight
Glc	glucose
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IGEPAL	Polyoxyethylene nonyl phenol
kb	kilo base pairs
kDa	kilo Dalton
Km	substrate concentration producing half maximal velocity
Ki	kinetic inhibition constant
MES	2(N-morpholino) ethanesulphonic acid
MS	Murashige and Skoog, i.e. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiology Plantarum 15:473-497
μM	micromolar (10^{-6}M)
mM	milimolar (10^{-3}M)
nM	nanomolar (10^{-9}M)
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFP	pyrophosphate: fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90)

PFK	ATP: fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.11)
Pi	inorganic phosphate
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PPi	inorganic pyrophosphate
PVPP	polyvinil polypyrrolidone
RNAi	RNA interference
SDS	sodium dodecyl sulphate
SPS	sucrose phosphate synthase (EC 2.4.1.14)
SuSy	sucrose synthase (EC 2.4.1.13)
UDPGlc	uridine 5'-diphosphoglucose
UDPGlc-DH	uridine 5'-diphosphoglucose dehydrogenase
VPPase	vacuolar H^+-translocating inorganic pyrophosphatase (EC 3.6.1.1)
x g	times gravitational force

CHAPTER 1

General introduction

Sugarcane is one of the most valuable agricultural crops in South Africa and cane sugar, i.e. sucrose, is the main product that is derived from it. Cane sugar generates an annual income of approximately R6 billion and contribute an estimated R2 billion to the country's foreign exchange earnings (www.sasa.org.za). Approximately 350,000 South Africans are directly or indirectly employed by the sugarcane industry and this translates into more than a million people being dependent on the industry. To stay competitive in a global commodity market prone to overproduction, the South African industry has to focus on more cost effective production systems. Increasing the sucrose concentration in commercial sugarcane varieties will be one of the most important factors contributing towards improved cost effectiveness. The emphasis should fall here on increased sucrose yield per ton cane and not only on an increase in tons sucrose per unit area. For this reason sucrose storage was identified recently as the most important research priority for the industry (Inman-Bamber *et al.* 2005).

Commercial sugarcane varieties are interspecific hybrids that are capable of storing sucrose up to 62% of their dry weight or 25% of their fresh weight (Bull and Glasziou 1963, Welbaum and Meinzer 1990). Historically, increases in sucrose yield have been accomplished through conventional breeding programs. Variety improvement through breeding is estimated to have increased sucrose yield by 1-1.5% per annum over the last half of the 20th century in Australia (Chapman 1996). However, these increases were attained mainly *via* improvements in cane yield and not in sucrose content (Jackson 2005). In addition, there are ample suggestions that sugarcane is approaching a yield plateau (Moore 2005 and reference therein). A possible reason for this might be that the natural genetic potential for sucrose production has been exhausted (Grof and Campbell 2001). Genetic engineering therefore represents an opportunity to add to this potential by the specific manipulation of endogenous genetic traits or by introducing desired genetic traits from exogenous sources.

Sucrose accumulation involves a multitude of metabolic and physical processes in the cells and tissues that are involved in sucrose synthesis, transport and storage. Cytosolic sucrose metabolism in the storage parenchyma is one of these processes that might influence sucrose accumulation, particularly in the way it governs carbon partitioning in these cells (Whittaker and Botha 1997, Bindon and Botha 2002). Pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP), in combination with ATP: fructose 6-phosphate 1-phosphotransferase (PFK) and fructose 1,6-bisphosphatase (FBPase), plays a central role in cytosolic carbon metabolism, representing the first committed catalytic step towards respiration (Figure 1). PFP catalysis the reversible conversion of fructose 6-phosphate (Fru-6-P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate (Fru-1,6-P_2) and inorganic phosphate (Pi) (Carnal and Black 1979). This is thought to be a near-equilibrium reaction and is therefore able to respond to cellular metabolism in a very flexible manner (Edwards and ap Rees 1986).

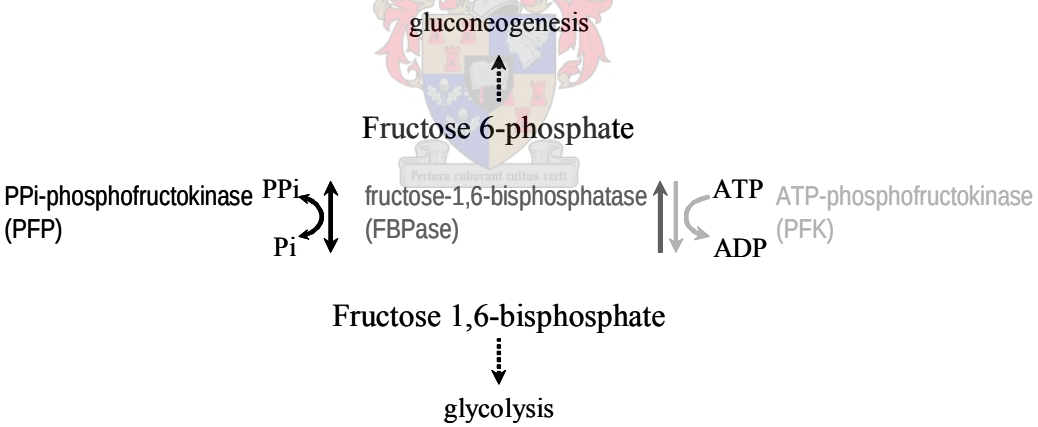

Figure 1. Interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate. While PFK and FBPase catalyses irreversible reactions in the glycolytic and gluconeogenic directions respectively, PFP catalyses a freely reversible reaction.

Results from transgenic tobacco and potato plants in which PFP activity has been reduced by much as 97% suggest that the investigated tissues either have a huge excess of PFP and/or complementary activity or that PFP does not play a crucial role in plant metabolism (Hajirezaei *et al*. 1994, Paul *et al*. 1995, Nielsen and Stitt 2001). Similarly, the over-expression of non-regulated PFP activity in tobacco plants did not cause

dramatic phenotypic or physiological effects either (Wood *et al.* 2002a, 2002b). In all these examples the levels of PFP's substrates/products were influenced as could be expected of a net glycolytic reaction but all other changes in metabolite levels were of a transient nature, disappearing as the tissues mature.

In contrast to this apparently insignificant role of PFP in these specific plants/tissues the changes in its activity (Botha and Botha 1991, Murley *et al.* 1998, Krook *et al.* 2000) and the subtle regulation of its activator, fructose 2,6-phosphate (Paz *et al.* 1985, Van Praag and Agosti 1997, Van Praag *et al.* 1997), during the various stages of normal growth and development and under changing environmental conditions suggest a more prominent role for PFP in carbohydrate metabolism. Similarly, it also seems to play an important role in sucrose accumulation in sugarcane. PFP activity is inversely correlated to the sucrose content and positively to the water-insoluble component in maturing sugarcane internodal tissues (Whittaker and Botha 1999). In addition, a significant amount of carbon is cycled between the triose-phosphate and hexose-phosphate pools, for which PFP is at least partially responsible (Whittaker 1997, Bindon and Botha 2002). The extent of this cycling is also inversely correlated to sucrose accumulation across varieties (Whittaker 1997) and maturing internodal tissue (Bindon and Botha 2002). The available data therefore suggest that PFP plays an important role in carbon partitioning in sugarcane storage tissues. Consequently, reduced PFP activity might directly decrease glycolytic carbon flow and also reduce the extent of the triose-phosphate : hexose-phosphate cycle, resulting in increased sucrose synthesis and/or accumulation.

The main aim of this study was therefore to investigate this potential role of PFP in sucrose accumulation in sugarcane. This was done by firstly characterising the sugarcane enzyme to determine whether it has significantly different properties to other plant PFPs, which could explain its apparent role in sucrose accumulation. Secondly, PFP activity was down-regulated in transgenic plants to determine whether the inverse correlation between its activity and sucrose content could be demonstrated in this direct manner.

To conclude, an overview of all the aims and outcomes of this study is presented in context of the various chapters in which they were dealt with.

Chapter 2: Characteristics and potential function of pyrophosphate: fructose-6-phosphate 1-phosphotransferase with special reference to the sucrose accumulation phenotype of sugarcane culm.

Aim: To present the background of this study in the format of a review paper that includes a critical discussion on the apparent role of PFP in sucrose accumulation in sugarcane internodal tissues.

Outcomes: An overview of the molecular, kinetic and regulatory characteristics of a representative sample of plant PFPs is presented. The potential roles of PFP in sink tissues are discussed with specific reference to the sucrose accumulation phenotype in sugarcane and two hypotheses are presented that might explain PFP's role in sucrose accumulation in sugarcane.

Chapter 3: Purification and characterisation of pyrophosphate: fructose-6-phosphate 1-phosphotransferase from sugarcane.

Aim: To determine the molecular and kinetic properties of sugarcane PFP in order to assist in elucidating its apparent role in sucrose accumulation.

Outcomes: Sugarcane PFP was purified to homogeneity and its molecular and kinetic parameters were determined for the first time. No significant differences between sugarcane and other plant PFPs were found and it was shown that the apparent correlation between sucrose content and PFP activity is probably linked to the genetically determined amount of activity present in the tissue and not to fine regulatory mechanisms.

Chapter 4: Development and characterisation of transgenic systems for the manipulation of PFP activity in sugarcane.

Aim: To establish various transformation systems that could be used to up- and down-regulate PFP activity in sugarcane.

Outcomes: Three gene sequences, i.e. the sugarcane PFP-β, *Giardia lamblia* and *Propionibacterium* PFP genes, were cloned and used for the construction of six plant expression vectors that can be used for the constitutive or phloem specific up- or down-regulation of PFP activity in sugarcane. In addition, the two heterologous PFP proteins were expressed, purified and characterised to confirm their bio-activity and potential properties under *in vivo* conditions. Finally, antisera were raised against the two purified proteins to enable the easy characterisation of transgenic plants.

Chapter 5: Down-regulation of Pyrophosphate: fructose 6-phosphate 1-phosphotransferase activity in sugarcane enhances sucrose accumulation in immature internodes.

Aim: To verify the potential role of PFP in sucrose metabolism in sugarcane and in particular its apparent direct influence on sucrose accumulation.

Outcomes: PFP activity was successfully down-regulated in several transgenic sugarcane lines using antisense and co-suppression constructs of the sugarcane PFP-β gene. Reduced PFP activity significantly increased sucrose concentrations in immature, metabolically active internodal tissues but no significant differences were apparent in mature tissues. The data presented here support the suggested role of PFP as a bypass to PFK at times of high metabolic flux in biosynthetically active tissues.

Chapter 6: General discussion and conclusions.

Aim: To integrate the observations and discussions of the experimental chapters.
Outcomes: An overarching conclusion regarding the role of PFP in sucrose accumulation in sugarcane is presented and the potential focus of future research on this topic is discussed.

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CHAPTER 2

Characteristics and potential function of pyrophosphate: fructose-6-phosphate 1-phosphotransferase with special reference to the sucrose accumulation phenotype of sugarcane culm [*]

ABSTRACT

Despite the apparent ubiquity of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) in plants no clear physiological role has emerged for it. Transgenic plants with up- or down-regulated PFP activity showed only small changes in the levels of metabolites directly associated with it but no significant, stable phenotypic changes. In sugarcane PFP activity is inversely correlated to sucrose content in maturing internodal tissues, suggesting a more prominent role for it in carbohydrate partitioning in these tissues. In this review I will therefore first give an overview of the molecular, kinetic and regulatory characteristics of plant PFPs, which could help elucidate its apparent role in sucrose metabolism and then I will discuss these potential roles with specific reference to the sucrose accumulation phenotype in sugarcane. Finally, I present two hypotheses that might explain PFP's role in sucrose accumulation in sugarcane.

INTRODUCTION

Sugar metabolism has been studied extensively in sugarcane in an attempt to elucidate the molecular basis of the sucrose storing phenotype (Batta *et al.* 1995, Moore 1995, Botha *et al.* 1996, Moore and Maretzki 1996, Lingle 1999, Moore 2005, Rae *et al.* 2005 and the references in these). In doing so, many of the enzyme reactions involved in sucrose metabolism in sugarcane sink tissues (Figure 1) have been characterised. These include sucrose phosphate synthase (SPS, Grof *et al.* 1998, Botha and Black 2000), sucrose phosphatase (Gutierrez-Miceli *et al.* 2002), sucrose synthase (SuSy, Lingle and

[*] To be submitted to Plant Physiology and Biochemistry

Irvine 1994, Lingle and Dyer 2001, Schäfer *et al.* 2004a, 2004b and 2005), the invertases (Zhu *et al.* 1997, Echeverria 1998, Vorster and Botha 1999, Rose and Botha 2000, Bosch and Botha 2004), hexo- and fructokinases (Hoepfner and Botha 2003 and 2004), ATP: fructose 6-phosphate 1-phosphotransferase (PFK, Whittaker and Botha 1999) and pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP, Whittaker and Botha 1999). Of all these enzymes only PFP activity shows a consistent inverse correlation with sucrose concentrations across commercial varieties and within a segregating F1 population (Whittaker and Botha 1999).

This apparent important role of PFP in determining carbon flux in sugarcane sink tissues is in contrast to evidence from transgenic tobacco and potato in which PFP activity was reduced by much as 97%. Although there was a marked reduction in 3-phosphoglycerate (3-PGA) and PEP and an increase in Fru 2,6-P_2 levels in these plants, there was no significant effect on fluxes or growth and morphology (Hajirezaei *et al*. 1994, Paul *et al*. 1995, Nielsen and Stitt 2001). These findings led the authors to conclude that the investigated tissues either have a huge excess of PFP and/or complementary activity or that PFP does not play a crucial role in metabolism. More recently, the over-expression of non-regulated PFP activity in tobacco plants also did not cause dramatic phenotypic or physiological effects but did result in a decrease in starch accumulation in both source and sink tissues and an increase in the total lipid content of seeds (Wood *et al.* 2002a, 2002b). In addition, the onset of lipid deposition was advanced by up to 48 hours in the developing transgenic embryos (Wood *et al.* 2002b).

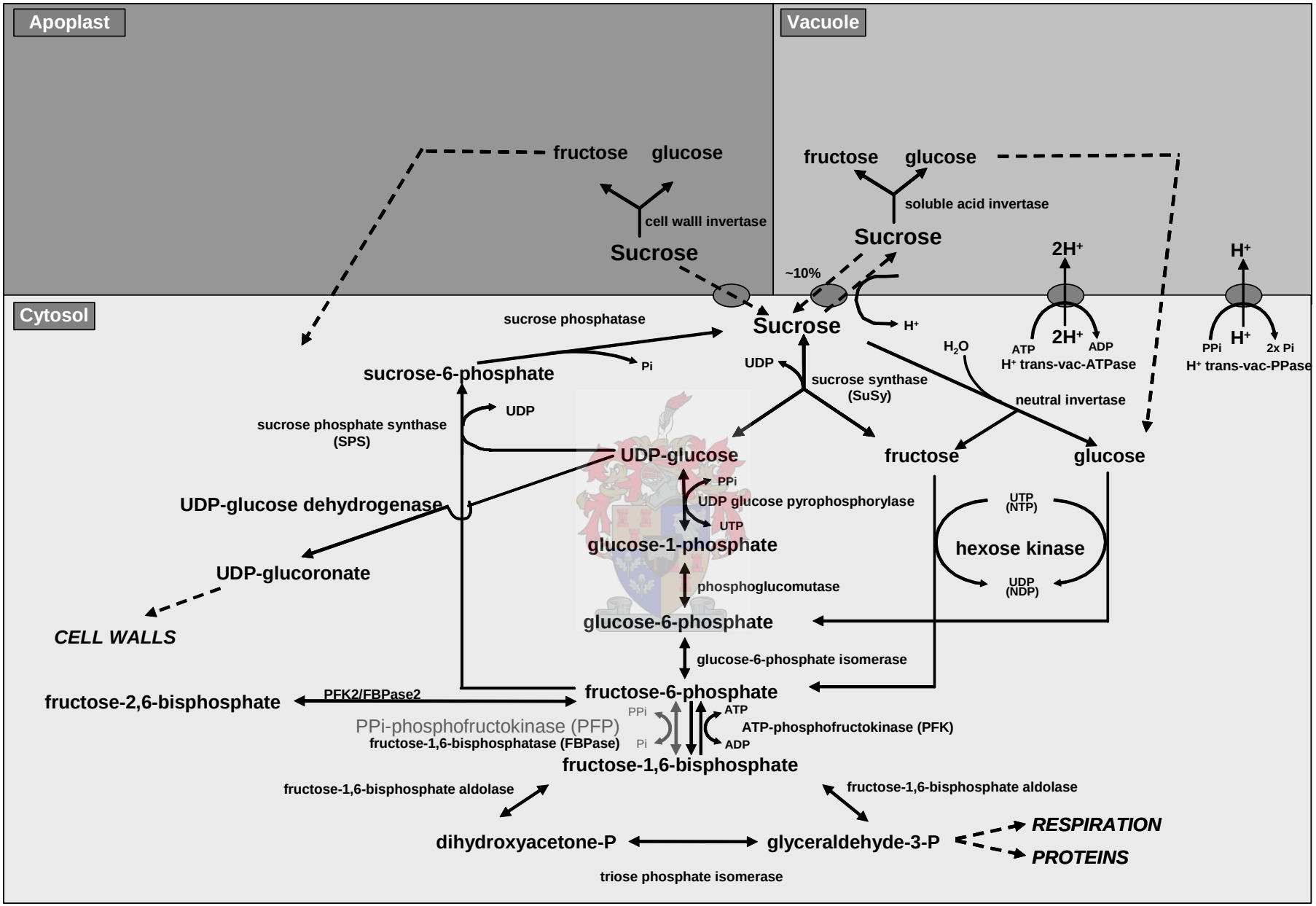

Figure 1. A summary of the most important reactions in sucrose metabolism in the sink tissues of sugarcane. Potential symplastic loading and the diffusion of sugars across membranes are not indicated in the diagram.

The apparent role of PFP in sucrose accumulation in sugarcane therefore still requires confirmation and explanation. In this review I will firstly describe the molecular, kinetic and regulatory characteristics of PFP from other plants that might contribute to our understanding of its role in metabolism and secondly discuss these potential roles. In the discussion I will specifically refer to the sucrose accumulation phenotype in sugarcane and present two hypotheses that might explain PFP's role in sucrose accumulation.

CATALYTIC ACTIVITY OF PFP

PFP (EC 2.7.1.90) catalyses the reversible conversion of fructose 6-phosphate (Fru 6-P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate (Fru 1,6-P_2) and inorganic phosphate (Pi)(Figure 1). The enzyme was first isolated from the lower eukaryote *Entamoeba histolytica* (Reeves *et al*. 1974) and later also from a limited number of prokaryotes and lower eukaryotes such as *Propionibacterium* (O'Brien *et al*. 1975), *Rhodospirillum* (Pfleiderer and Klemme 1980) and *Giardia lamblia* (Rozario *et al*. 1995). The first plant PFP was isolated from pineapple leaves by Carnal and Black (1979) and is now considered to be ubiquitous in plants (Stitt 1990).

Plant PFP is a strictly cytosolic enzyme and is one of three enzymes involved in the interconversion of Fru 6-P and Fru 1,6-P_2 in this cellular compartment. The other two being PFK (EC 2.7.1.11) and fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) (Figure 1). In contrast to PFP, PFK and FBPase catalyse irreversible reactions in the glycolytic (Fru 1,6-P_2 forming) and gluconeogenic (Fru 6-P forming) directions respectively. In addition, ATP is used as the phosphoryl donor in the PFK catalysed reaction. In most non-photosynthetic tissues the interrelationship between these three enzymes is further convoluted by the absence of detectable levels of FBPase activity (Entwistle and ap Rees 1990, Hatzfeld and Stitt 1990, Fernie *et al*. 2001), implying that PFP is responsible for all gluconeogenic carbon flux through this step in these tissues. In non-photosynthetic tissues where FBPase activity is present, the levels of activity are often inadequate to sustain gluconeogenic flux (Botha and Botha 1993a, Focks and Benning 1998), although the kinetic properties of grapefruit juice sac vesicle FBPase suggest that it might play an important gluconeogenic role (Van Praag 1997b).

PFP is thought to catalyse a near-equilibrium reaction *in vivo* (K_{eq} = 3.3, calculated for the glycolytic direction) and is therefore able to respond to cellular metabolism in a very flexible manner because it can catalyse a net flux of carbon in either the glycolytic or gluconeogenic direction (Edwards and ap Rees 1986, Weiner *et al*. 1987). Its activity is strongly modulated by metabolites such as fructose 2,6-bisphosphate (Fru 2,6-P_2), Fru 6-P, Fru 1,6-P_2, PPi and Pi (Cséke *et al*. 1982, Van Schaftingen *et al*. 1982, Kombrink *et al*. 1984, Stitt 1989, Montavon and Kruger 1992, Nielsen and Wischmann 1995, Theodorou and Plaxton 1996, Fernie *et al*. 2001) and its activity varies according to developmental stage and environmental conditions (Botha and Botha 1991a, Hajirezaei and Stitt 1991, Botha and Botha 1993b, Murley *et al*. 1998, Whittaker and Botha 1999, Krook *et al*. 2000).

MOLECULAR CHARACTERISTICS OF PFP

Most plant PFPs are multimeric enzymes, composed of two immunologically distinct peptides, namely the α- and β-subunits. The respective molecular weights of the α- and β-subunits are approximately 66 and 60 kDa (Table 1) and these subunits can be aggregated in di-, tetra- or octameric arrangements. The relative amounts of the two subunits and the specific composition of the various holoenzymes can vary depending on factors such as the developmental stage of the tissue and specific environmental conditions and can play a role in the regulation of enzyme activity (Kruger and Dennis 1987, Botha and Botha 1991a, 1991b, Theodorou *et al*. 1992, Theodorou and Plaxton 1996). In addition, it has been shown that the two subunit genes are differentially expressed in germinating castor seeds (Blakeley *et al*. 1992) and that isoforms of the enzyme exist (Yan and Tao 1984, Wong *et al*. 1990, Botha and Botha 1993b). Active PFP isoforms consisting of only the β-subunit has also been isolated and characterised (Table 1). These enzymes can either be exclusively present in the specific plant, e.g. pineapple (Trípodi and Podestá 1997), or as one of several isoforms with distinctive kinetic properties, e.g. wheat and tomato (Yan and Tao 1984, Wong *et al*. 1990).

Table 1. The molecular properties of selected plant PFPs.

Source		Molecular weight (kDa)			α:β	Reference
Plant	Tissue	Holoenzyme	α-subunit	β-subunit	ratio	
Banana	Ripe fruit	490 (octamer)	66	60	1:1	Turner and Plaxton 2003
Barley	Seedlings	500 (+20mM PPi, octamer), 240 (-PPi, tetramer)	65	60	1:1	Nielsen 1994
Carrot	Tap root	294 (tetramer)	61[a]	59[a]	1:1	Wong *et al*. 1988
Castor bean	Germinating seeds	n.a.	67	60	0.1:1 to 0.6:1	Blakely *et al.* 1992
C. lanatus	Cotyledons	n.a. (tetramer, α-β-dimer, β-dimer)	68	65	4.8:1 to 0.8:1	Botha and Botha 1991a
Mustard	Suspension cultures	520 (octamer)	66	60	1:1	Theodorou and Plaxton 1996
Pea	Cotyledons	12.7S (+Fru 2,6-P_2), 6.3S (-Fru 2,6-P_2)	n.a.[b]	n.a.	n.a.	Wu *et al*. 1984
Pineapple	Leaves	97.2 (homodimer)	-	61.5	0:1	Trípodi and Podestá 1997
Potato	Tuber	265 (tetramer), 129.6 (+20mM PPi, dimer)	65	60	1:1	Kruger and Dennis 1987
Rice	Seeds	103 (monomer)	-	-	-	Enomoto *et al*. 1992
Spinach	Leaves	242 (+Fru 2,6-P_2, tetramer), 165 (-Fru 2,6-P_2, dimer)	n.a.	n.a.	n.a.	Balogh *et al*. 1984
Tomato	Fruit	443 ("oligomer"), 68 (β-dimer) and 68 (β-monomer)	66	60	1:1 or 0:1	Wong *et al*. 1990
Wheat	Seedlings	234 (tetramer), 60 (β-dimer)	67	60	1:1 or 0:1	Yan and Tao 1984
Wheat	Endosperm	170 (dimer)	90	80	n.a.	Mahajan and Singh 1989

[a] Subunits not immunologically distinct. [b] n.a. = data not available.

The quaternary structure of PFP can also be influenced *in vitro* by the enzyme's interaction with metabolites such as PPi and Fru 2,6-P_2. Several authors reported the dissociation of the heterotetramer in the presence of PPi (Wu *et al*. 1983, Balogh *et al*. 1984, Kruger and Dennis 1987). Fru 2,6-P_2 can prevent this dissociation and also mediates the reassociation of the lower molecular weight, dimeric forms. In contrast, Nielsen (1994) could only isolate an octameric form in the presence of 20 mM PPi. This holoenzyme dissociated into tetramers in the absence of PPi and Fru 2,6-P_2 had no effect on the elution profile during gel filtration. Carrot PFP's state of aggregation on the other hand is insensitive to either the presence or absence of these metabolites (Wong *et al*. 1988). Although it has been proposed that the aggregation state of the enzyme can provide a mechanism by which Fru 2,6-P_2 activation can favour the glycolytic reaction (Wu *et al*. 1983, Wu *et al*. 1984, Black *et al*. 1985), the evidence is

inconclusive (Stitt 1990). In addition, activation does not necessarily lead to changes in the molecular mass of PFP (Bertagnolli *et al*. 1986, MacDonald and Preiss 1986, Wong *et al*. 1988).

The β-subunit has been identified as the catalytic subunit while the α-subunit is involved in the regulation of enzyme activity through Fru 2,6-P_2 (Yan and Tao 1984, Wong *et al*. 1988, 1990, Carlisle *et al*. 1990, Cheng and Tao 1990, Botha and Botha 1993b). Accordingly, although isoforms containing only β-subunits are still activated by Fru 2,6-P_2, PFP isoforms containing the α-subunit have lower K_a values, i.e. have a higher affinity for Fru 2,6-P_2 (Yan and Tao 1984, Wong *et al*. 1988, 1990). Theodorou *et al*. (1992) also found that induction of PFP activity under Pi starvation was due to the *de novo* synthesis of the α-subunit, leading to a significant enhancement in activation by Fru 2,6-P_2. Likewise, a computer model developed for grapefruit PFP supports the involvement of the α-subunit in the regulation of activity through Fru 2,6-P_2 (Van Praag 1997a). The model further suggests that Fru 2,6-P_2 can only bind when both the α- and β-subunits are present. In contrast, the homodimeric (β_2) pineapple PFP has a high affinity for Fru 2,6-P_2 compared to heteromeric PFPs (Table 2) and increase activity more than 2-fold at optimum pH values (Trípodi and Podestá 1997).

KINETIC AND REGULATORY CHARACTERISTICS OF PFP

The kinetic and regulatory properties of various plant PFPs have been studied in detail in an attempt to shed more light on the physiological relevance of the enzyme (Table 2). Unfortunately, the integration and interpretation of the available kinetic data is difficult because (i) the data were obtained under optimum conditions that do not necessarily represent *in vivo* conditions, (ii) the reaction conditions used by various researchers vary and (iii) PFP's activity is affected by various metabolites and other buffer components, including its substrates/products, which will inevitably be reflected in the data obtained. Only selected kinetic and regulatory characteristics, which have clear physiological implications, will therefore be discussed under three headings, i.e. the influence of pH on activity, substrate/product interactions and activation by Fru 2,6-P_2.

Table 2. Kinetic parameters of selected plant PFPs.

Plant (tissue)	Glycolytic			Gluconeogenic			pH optimum [b]	Reference
	K_m [a]		K_a	K_m [a]		K_a		
	Fru 6-P (μM)	PPi (μM)	Fru 2,6-P_2 (nM)	Fru 1,6-P_2 (μM)	Pi (μM)	Fru 2,6-P_2 (nM)		
Banana (ripe fruits)	32	9.7	8	25	410	n.a.[c]	f: 7.1	Turner and Plaxton 2003
Barley (seedlings)	200	8	2.8	11	480	60	n.a.	Nielsen 1994
Carrot (tap root)[d]	430	19	n.a.	200	2300	n.a.	n.a.	Wong *et al*. 1988
Castor bean (endosperm)	300	15	10-123	23	630	60-300	f:7.3-7.7, r:7.75	Kombrink *et al*. 1984
Cucumber (seeds)	180	12.9	35-100	74.9	480.4	n.a.	f:7.5-8.0, r:7.5-8.0	Botha *et al*. 1986
Grapefruit (juice sac)	159	33	6.7	61	700	n.a.	n.a.	Van Praag 1997a
Mustard (cell suspension)	50	15	15-4750	9	250	49	f:6.5-7.2, r:6.7-7.7	Theodorou and Plaxton 1996
Pineapple (leaves)	890	11	26.3-43.5	94	149	2.4-2.7	f:7.7, r:6.6-8.4	Trípodi and Podestá 1997
Tomato (fruit)[d]	380-600	20-40	4-13	40-70	610-950	n.a.	n.a.	Wong *et al*. 1990
Wheat	322	31	n.a.	139	129	n.a.	7.5	Mahajan and Singh 1989

[a] K_m values in presence of saturating Fru 2,6-P_2. [b] f = forward reaction (glycolytic) and r = reverse reaction (gluconeogenic) in the presence of Fru 2,6-P_2. [c] n.a. = data not available. [d] Fru 2,6-P_2 reduces or have little effect on affinity for Fru 6-P.

The influence of pH on activity

The pH dependence for both the forward and reverse reactions is similar and PFP usually has a relatively broad activity range with optimum activity between 6.5 and 8.0 (Yan and Tao 1984, Kombrink *et al*. 1984, Botha *et al*. 1986, Nielsen 1994, Theodorou and Plaxton 1996, Trípodi and Podestá 1997). The most important effect of pH is probably its role in the activation of PFP by Fru 2,6-P_2. Fully activated PFP can be less sensitive to changes in pH than the non-activated enzyme; i.e. the extent to which Fru 2,6-P_2 activates PFP will be greater at non-optimum pH values (Yan and Tao 1984, Kombrink *et al*.1984, Theodorou and Plaxton 1996, Trípodi and Podestá 1997). For particularly the glycolytic reaction, this can also be interpreted as a shift, or at least an extension, of the optimum pH towards more acidic pH values under fully activated conditions (Yan and Tao 1984, Kombrink *et al*.1984, Enomoto *et al*. 1992, Theodorou

and Plaxton 1996). This suggests a glycolytic role for these PFPs under conditions that will induce a decrease in cytosolic pH, e.g. anoxia (Dancer and ap Rees 1989). Exceptions to this are, for example, cucumber and wheat PFP where Fru 2,6-P_2 does not change the pH dependence and the maximum activation effect of Fru 2,6-P_2 is at the optimum pH values (Botha *et al*. 1986, Mahajan and Singh 1989).

Substrate/product interactions

PFP requires a bivalent cation and has the highest affinity for Mg^{2+} (Kombrink *et al*. 1984, Botha *et al*. 1986). Various other cations have been tested of which only Mn^{2+} and Co^{2+} can replace Mg^{2+}, but at lower efficiencies. In addition, PFP's affinity for Mg^{2+} is increased in the presence of Fru 2,6-P_2, and Mg^{2+} concentrations in excess of 1mM inhibit PFP activity in the glycolytic direction (Kombrink *et al*. 1984, Montavon and Kruger 1992). More recent studies indicated that the Mg^{2+} cation is complexed with PPi before it is used as the substrate in the glycolytic reaction (Montavon and Kruger 1992, Trípodi and Podestá 1997). Moreover, PFP uses free Fru 6-P and MgPPi in the glycolytic reaction and free Pi, free Fru 1,6-P_2 and Mg^{2+} in the gluconeogenic reaction. The use of the MgPPi complex is significant because more than 98% of the PPi will be chelated *in vivo* (Trípodi and Podestá 1997).

PFP exhibits hyperbolic kinetics for all its substrates in both the glycolytic and gluconeogenic reactions and in the presence or absence of Fru 2,6-P_2. In the glycolytic reaction each of the substrates, Fru 6-P and PPi, decreases the affinity of the other substrate with increasing concentrations, i.e. the K_m of Fru 6-P increases slightly with increasing concentrations of PPi and *vice versa* (Stitt 1989). The same holds true for Fru 1,6-P_2's effect on the K_m of Pi in the gluconeogenic reaction, but in contrast the K_m of Fru 1,6-P_2 is significantly increased by Pi (Stitt 1989). Moreover, Pi also significantly decreases PFP's affinity for Fru 6-P and PPi in the glycolytic reaction. This inhibitory behaviour of Pi for different plant PFPs has been characterised as either the mixed (Kombrink *et al*. 1984, Enomoto *et al*. 1992) or noncompetitive (Botha *et al*. 1986, Stitt 1989) type with respect to both Fru 6-P and PPi. In contrast to Fru 6-P, increasing Pi concentrations also strongly decrease PFP's affinity for Fru 2,6-P_2, which effectively

prevents the activation of PFP and results in a parallel inhibition of both the glycolytic and gluconeogenic reactions (Kombrink and Kruger 1984, Botha *et al*. 1986, Mahajan and Singh 1989, Stitt 1989, Theodorou and Plaxton 1996). Although Pi increases the K_a for Fru 2,6-P_2 in both the directions its inhibitory effect is more pronounced in the glycolytic direction.

PPi is a powerful product inhibitor of the gluconeogenic reaction and Fru 2,6-P_2 cannot relieve this effect (Stitt 1989). It is a competitive inhibitor with respect to Fru 1,6-P_2 and a non-competitive inhibitor with respect to Pi (Stitt 1989). Finally, Fru 1,6-P_2 has been shown to act as an allosteric activator of PFP (Sabularse and Anderson 1981, Nielsen 1995), but it is unlikely to be an effective activator *in vivo* because of the reduced affinity of the enzyme under physiological conditions (Theodorou and Kruger 2001). The kinetic properties of PFP therefore strongly suggest that its activity is tightly regulated *in vivo*. This regulation is mediated not only by PFP's substrates and products but also by Fru 2,6-P_2.

Activation by Fru 2,6-P_2

Fru 2,6-P_2 is a potent activator of PFP. It activates the glycolytic reaction by increasing V_{max} and the enzyme's affinity for Fru 6-P (Sabularse and Anderson 1981, Van Schaftingen *et al*. 1982, Botha *et al*. 1986, Theodorou and Plaxton 1996). The effect on the K_m of PPi is not as clear and can vary from a decrease to a slight increase (Van Schaftingen *et al*. 1982, Kombrink *et al*. 1984, Bertagnolli *et al*. 1986, Botha *et al*. 1986). The gluconeogenic reaction is also activated through an increase in V_{max} and a decrease in the K_m of Fru 1,6-P_2 (Van Schaftingen *et al*. 1982, Kombrink *et al*. 1984, Bertagnolli *et al*. 1986, Botha *et al*. 1986, Theodorou and Plaxton 1996). PFP's affinity for Pi may be slightly increased, not influenced or decreased (Kombrink *et al*. 1984, Botha *et al*. 1986, Theodorou and Plaxton 1996). Fru 2,6-P_2 can alleviate the inhibitory effect of PPi on the gluconeogenic reaction to some extent (Sabularse and Andeson 1981, Van Schaftingen *et al*. 1982). Both Fru 6-P and Fru 1,6-P_2 increase PFP's affinity for Fru 2,6-P_2 (Van Schaftingen *et al*. 1982, Kombrink *et al*. 1984) and Pi decreases its affinity (Kombrink and Kruger 1984, Botha *et al*. 1986, Mahajan and Singh 1989, Stitt

1989). Based on Fru 2,6-P_2's *in vitro* K_a (nM range, Van Schaftingen *et al*. 1982, Kombrink *et al*. 1984) and its estimated *in vivo* concentrations (μM range, Cséke *et al*. 1982, Scott and Kruger 1994) it was initially thought that PFP is always fully activated *in vivo*. Nielsen and Wischmann (1995) suggested, however, that the concentration of PFP subunits might be higher than the Fru 2,6-P_2 concentration in some tissues, resulting in non-activated PFP even when the concentration of Fru 2,6-P_2 exceeds its K_a by several orders of magnitude. More recently it was also shown that the inhibition of Fru 2,6-P_2 binding by physiological levels of Pi and phosphorylated intermediates can decrease the affinity of PFP to the extent that the enzyme is sensitive to the changes in Fru 2,6-P_2 concentrations *in vivo* (Theodorou and Kruger 2001, Turner and Plaxton 2003). This implies that the activation state of PFP at specific Fru 2,6-P_2 concentrations can vary continuously due to changes in the enzyme's affinity for this effector.

Although the same concentration of Fru 2,6-P_2 will activate the glycolytic reaction more than the gluconeogenic reaction (Kombrink *et al.* 1984, Nielsen 1994, Turner and Plaxton 2003) only a single Fru 2,6-P_2 binding site is present, which means that the glycolytic and gluconeogenic reactions are always activated symmetrically (Stitt and Vasella 1988, Stitt 1990, Nielsen 1994). Activation by Fru 2,6-P_2 is therefore not able to influence the direction of carbon flux directly, but would rather determine the rate by which equilibrium is restored. Although PFP catalyses a net glycolytic reaction and increasing Fru 2,6-P_2 levels are often associated with conditions under which the rate of glycolysis is stimulated, it is not surprising that this correlation is not absolute (Van Schaftingen and Hers 1983, ap Rees *et al*. 1985a, Stitt *et al*. 1986, Stitt 1990, Hatzfeld and Stitt 1991).

PFP'S ROLE IN METABOLISM

Theoretically PFP has only one "function"; to facilitate the establishment of an equilibrium between [Fru 6-P][PPi] and [Fru 1,6-P2][Pi]. Not withstanding, many authors suggest that despite numerous molecular and kinetic studies its *exact physiological role* is still elusive (Stitt 1989, Trípodi and Podestá 1997, Murley *et al*. 1998, Stitt 1998, Fernie *et al*. 2001). Although its ubiquity, tight regulation *in vivo* and

spatial and temporal specificity suggest that it plays a critical role in plant metabolism its apparent redundancy, as suggested by studies on transgenic plants with a reduction of up to 97% in PFP activity (Hajirezaei *et al.* 1994, Paul *et al.* 1995, Nielsen and Stitt 2001), contributes to this ambiguity. Here we would like to argue that PFP does not have a single, "definitive" physiological role but that its significance or "role" rather emanates from two of its distinct (in comparison to the other enzymes catalysing the same reactions) characteristics, i.e. (i) its ability to catalyse a reversible reaction and (ii) by serving as a link between carbohydrate and PPi metabolism. In other words, PFP has the ability to play various "roles" within the scope of these two traits, depending on the specific physiological conditions.

To illustrate, PFP has been implicated in the following roles; the regulation of cytosolic PPi concentrations (Simcox *et al*. 1979, ap Rees *et al*. 1985b, ap Rees 1988, Dancer and ap Rees 1989, Claassen *et al*. 1991), the equilibration of the hexose- and triose-phosphate pools (Dennis and Greyson 1987, Hatzfeld *et al*. 1990, Hajirezaei *et al*. 1994), the synthesis of PPi, which is required for the breakdown of sucrose through the sucrose synthase pathway (Huber and Akazawa 1986, Black *et al*. 1987, ap Rees 1988), providing a bypass to PFK at times of high metabolic flux in biosynthetically active tissue (Dennis and Greyson 1987), regulating gluconeogenic carbon flow when FBPase activity is low or absent (Botha and Botha 1993a, Focks and Benning 1998), relieving stress during periods of phosphate limitation or starvation by providing an adenylate bypass for glycolysis (Duff *et al*. 1989, Theodorou *et al.* 1992, Murley *et al*. 1998) and being the preferred glycolytic path during spells of anaerobiosis and anoxia (Mertens 1991, Kato-Noguchi 2002). In all these suggested roles the two distinct traits of PFP, as mentioned above, are relevant and will confer some advantage to the system in comparison to alternative reactions – if available at all. Specific examples include the ability to use PPi as phosphoryl donor during adenylate stress, gluconeogenic carbon flux in the absence of FBPase activity and perceptive responsiveness to metabolite levels because of the reversibility of the reaction. Based on these arguments two different hypotheses that explain the apparent role of PFP in the sucrose accumulation phenotype in sugarcane are proposed; the first is based on the direct influence the PFP-

catalysed reaction can have on carbon flux and the second on the indirect influence it can have on PPi metabolism.

Hypothesis 1: PFP plays an important role in determining carbon partitioning between the hexose-phosphate pool and total respiratory flux in sugarcane internodal tissues.

As mentioned earlier, PFP activity in internodal sugarcane tissue is inversely correlated to sucrose content across varieties with different sucrose yielding capacities (Whittaker and Botha 1999). These authors further showed that although PFP activity varied significantly between these varieties their PFK activities were very similar. In addition, it was demonstrated that the low sucrose storing varieties allocate a significantly higher proportion of carbon to their total respiratory pool, i.e. CO_2 production and anabolic biosynthesis, in similar tissue types (Whittaker and Botha 1997). It is therefore reasonable to conclude that the majority of this "additional" respiratory flux in the low sucrose storing varieties is catalysed by PFP. Reduced PFP activity under these circumstances should therefore lead to a reduction in respiratory flux, which could increase the availability of the precursors for sucrose synthesis. The potential impact of reduced PFP activity on carbon flux in sugarcane should also be seen in the light of the very high ratio of PFP:PFK activity in low sucrose storing sugarcane varieties compared to high yielding varieties. This ratio is for example 2.1:1 for US6656-15, a low sucrose yielding variety, and 0.9:1 for N24, a high sucrose yielding variety (Whittaker and Botha 1999).

Testing this hypothesis against the available transgenic data is complicated by the variety of tissues involved, i.e. photosynthetic vs. non-photosynthetic, sink vs. source and different levels of metabolic activity. Although the silencing studies suggest that these specific tissues are (eventually) able to compensate for the large decrease in PFP activity by the allosteric activation of the remaining PFP and/or by the activation of PFK (Hajirezaei *et al*. 1994, Paul *et al*. 1995, Nielsen and Stitt 2001), more transient aspects of metabolism are influenced significantly. For example, although the final sucrose concentration in mature transgenic tubers is unchanged, the flux into sucrose in

the growing (metabolically active) tubers is 13 times higher when compared to the wild type (Hajirezaei *et al*. 1994). It most probably indicates that PFP's contribution to glycolytic carbon flux is crucial at times of high metabolic flux in biosynthetically active tissues, with a high demand for glycolytic precursors. This is also supported by data from lipid rich seeds. PFP is implicated in the inability of *wrinkled1* mutant *Arabidopsis* seeds to accumulate triacylglycerol – seeds that also accumulate up to five-times more sucrose than the wild type seeds (Focks and Benning 1998). In addition, the total lipid content of tobacco seeds constitutively over expressing *G. lamblia* PFP increased significantly and the onset of lipid deposition was advanced by up to 48 hours in the developing transgenic embryos (Wood *et al.* 2002a, 2002b).

Finally, sugarcane varieties also cycle a significant amount of carbon between the triose-phosphate and hexose-phosphate pools, for which PFP is at least partially responsible (Whittaker 1997, Bindon and Botha 2002). The extent of this cycling is also inversely correlated to sucrose accumulation across varieties (Whittaker 1997) and maturing internodal tissue (Bindon and Botha 2002). Similarly, in sucrose storing carrot suspension cells, high respiratory activity stimulates triose-phosphate:hexose-phosphate cycling, but reduces the cells' ability to accumulate sucrose (Krook *et al.* 2000). Reduced PFP activity should therefore not only directly decrease glycolytic carbon flow but also reduce the extent of this seemingly wasteful cycle.

Hypothesis 2: PFP influences sucrose metabolism indirectly through its impact on PPi levels. (a) Its ability to synthesise PPi could contribute to sucrose mobilisation via SuSy. (b) Its inability to utilise PPi could favour the activity of the H^+-translocating vacuolar pyrophosphatase (VPPase) and in doing so, improve the secondary translocation of sucrose into the vacuole.

PPi is primarily located in the cytosol at concentrations between 200 and 300 μM, which are very accurately maintained (Weiner *et al.* 1987, Takeshige and Tazawa 1989). Moreover, PPi levels in the cytosol are remarkably insensitive to abiotic stresses such as anoxia or Pi starvation or following the addition of respiratory poisons, which

elicit a significant reduction in cellular ATP pools (Plaxton 1996, Stitt 1998). Cellular ATP levels on the other hand changes dramatically under these conditions. In addition, over expressing a soluble alkaline pyrophosphatase from *E. coli* in transgenic tobacco and potato plants resulted in plants containing significantly less PPi, which showed a dramatic phenotype with altered levels of metabolites in primary metabolism and major changes in their carbohydrate levels, sink-source relations, development and growth rate (Jellito *et al.* 1992, Sonnewald 1992). PPi therefore seems to play an essential role in plant metabolism, growth and development.

A potential role for PFP in sucrose mobilisation through the SuSy and subsequent UDP-glucose pyrophosphorylase (UGPase, EC 2.7.7.9) catalysed reactions has been proposed by various authors (Huber and Akazawa 1986, Black *et al*. 1987, ap Rees 1988). In fact, the main difference in the PFP-catalysed reaction between sucrose and starch storing tissues was suggested to be the direction of the net flux, i.e. PPi generation or consumption respectively, to allow the mobilisation of sucrose in sucrose storing tissues (Hajirezaei and Stitt 1991). However, this contrasts with the findings of Wong *et al*. (1988, 1990) in carrot root and tomato fruits, also sucrose storing tissues, which suggests that the kinetic characteristics of PFP might be adapted to favour the gluconeogenic reaction to supply the necessary precursors for sucrose synthesis.

In sugarcane SuSy activity is associated with the elongation of the internodes (Lingle and Smith 1991) and in general decreases with maturation in internodal tissue (Lingle and Smith 1991, Zhu *et al.* 1997, Lingle 1999, Schäfer *et al.* 2004a) and suspension cells (Wendler *et al.* 1990, Goldner *et al.* 1991). Variation in the ratio between the breakdown and synthetic activity of SuSy prevents the direct correlation of activity and sucrose utilisation (Goldner *et al.* 1991, Schäfer *et al.* 2004a) and although sucrose accumulation seems to correspond with a decrease in SuSy activity in suspension cells (Wendler *et al.* 1990, Goldner *et al.* 1991) this does not correspond to similar changes in PFP activity and the PPi concentrations (Wendler *et al.* 1990). Additional support that the PFP reaction and the mobilisation of sucrose *via* SuSy are not directly linked comes from transgenic potato plants that over express a soluble pyrophosphatase. Although

sucrose cleavage was inhibited due to PPi deficiency it did not alter the activity of PFP in these plants (Mustroph *et al.* 2005).

Although the mobilisation of sucrose via SuSy, using the PPi generated by PFP cannot be excluded, there is no evidence suggesting that this play an important role in the accumulation of sucrose in sugarcane. Moreover, even in the case of the relatively straight forward correlation between cell wall synthesis and the mobilisation of the required carbon *via* SuSy activity (Lingle and Smith 1991, Amor *et al.* 1995), PPi should not play a major role because the UGPase reaction is not directly involved. In conclusion, although the inverse correlation between PFP activity and sucrose concentrations (Whittaker and Botha 1999) apparently fits with the potential of high PFP activities to lower sucrose concentrations (mobilise sucrose) it is not supported by the available PFP and PPi data.

Regarding the second part of the hypothesis: Although the sugar concentrations are probably similar in the apoplast, cytoplasm and vacuole (Welbaum and Meinzer 1990, Preisser *et al.* 1992) the vacuolar compartment represents more than 90% of the intracellular space in mature sugarcane parenchyma cells and is therefore the most significant sub-cellular compartment where sucrose is stored (Komor 1994). It also represents a relatively stable compartment for stored sucrose from which very little is remobilised (Bindon and Botha 2001). Increasing the flux of sucrose into this compartment therefore has the potential to increase the amount of stored sucrose.

Despite numerous attempts to characterise a potential H^+-sucrose antiport system in the sugarcane tonoplast similar to that of sugar beet (Briskin *et al.* 1985, Getz and Klein 1995) success has not yet been achieved. Although ATP stimulates sucrose transport across the tonoplast of sugarcane cells, the mechanism for this is not clear because an H^+-sucrose antiport system could not be unequivocally demonstrated (Williams *et al.* 1990, Getz *et al.* 1991). Similarly, although both ATP and PPi can stimulate H^+ translocation across the tonoplast, these experimental systems could not yield any evidence for proton-coupled sucrose translocation either (Williams *et al.* 1990, Preisser

and Komor 1991). These conflicting results are at least in part due to experimental difficulties in preparing pure and intact tonoplast preparations from sugarcane cells and the existence of an H^+-sucrose antiport system could therefore not be excluded (see Moore 1995 for a review). The rest of the discussion will therefore be based on the assumption that there is indeed an H^+-sucrose antiporter in the tonoplast of sugarcane parenchyma cells that facilitates the active transport of sucrose into the vacuole.

Both vacuolar pyrophosphatase (VPPase, EC 3.6.1.1) and H^+-translocating vacuolar ATPase (VATPase; EC 3.6.1.3) catalyse the electrogenic translocation of protons from the cytosol to the vacuolar lumen to generate an inside-acidic pH and a cytosol-negative electrical potential difference, which can be used to drive the secondary transport of various solutes, including ions, amino acids and sugars, into the vacuole (Sze 1985; Hedrich and Schroeder 1989; Hedrich *et al*. 1989). VPPase could therefore theoretically utilise the phosphoanhydride energy bond in PPi to pump H^+ into the vacuole and thereby activate the secondary transport of sucrose into the vacuole.

In the absence of a soluble inorganic pyrophosphatase, PPi levels in the cytosol can only be regulated by a combination of the activities of the three PPi utilising enzymes present, i.e. UGPase, VPPase and PFP. If these three enzymes work collectively to regulate PPi concentrations it is reasonable to argue that if one of these activities is low / reduced, one or both of the other two activities will have to increase to reach and maintain the desired PPi levels - an apparently crucial metabolic parameter as discussed above. Moreover, because high PPi concentrations will inhibit many biosynthetic reactions the removal of the excess PPi from the system is crucial to maintain normal growth and development. This should be especially true when there is a greater need for the down-regulation of PPi concentrations, e.g. at times of high biosynthetic activity in young, metabolically active tissues where PPi is a by-product of many biosynthetic reactions. Inherently low PFP activity could therefore translate into increased VPPase activity, which should lead to the more efficient energisation of the tonoplast, which in turn could improve the secondary transport of sucrose into the vacuole. A direct link

between PPi, PFP activity and proton transport across maize tonoplasts was demonstrated by Dos Santos *et al.* (2003).

To conclude, although PFP activity is developmentally regulated, the maximum activity and the ratio between PFP and PFK activity are influenced more by genotype than by these developmental changes and fine regulation (Whittaker and Botha 1999, Krook *et al.* 2000). In a sugarcane genotype with inherently low PFP activity a bigger burden could therefore rest on VPPase to regulate PPi concentrations and in doing so indirectly increase the efficiency of all H^+-antiport systems, including a possible H^+-sucrose antiport system. This hypothesis clearly relies on the presence of a H^+-sucrose antiport system and should be further investigated.

CONCLUSION

If the negative correlation between PFP expression and sucrose levels in sugarcane is real, two probable mechanisms through which PFP could impact on sucrose content can be offered based on the current literature. The first is based on a reduction in total respiratory flux, resulting in an increased allocation of carbon to sucrose synthesis and storage. The second is based on the interconnection between cytosolic carbon and PPi metabolism where the inability of PFP to regulate PPi concentrations could lead to increased VPPase activity, resulting in the energisation of the tonoplast and improved translocation of sucrose into the vacuole. However, before these can be evaluated it is important to establish if there is indeed a direct correlation between PFP activity and sucrose content. The only direct way of testing this is to alter PFP levels in the same genetic background through genetic engineering. The primary aim of this study is therefore to confirm the potential role of PFP in sucrose accumulation in sugarcane, which could serve as basis for further investigations and genetic manipulation strategies.

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CHAPTER 3

Purification and characterisation of pyrophosphate: fructose-6-phosphate 1-phosphotransferase from sugarcane*

ABSTRACT

Pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PFP) activity in sugarcane internodal tissue is inversely correlated to sucrose content. To help elucidate this apparent role in sucrose accumulation we determined sugarcane PFP's molecular and kinetic properties. Sugarcane PFP was purified 285-fold to a final specific activity of 4.23 μmol $min^{-1}mg^{-1}$ protein. It contained two polypeptides of 63.2 and 58.0 kDa respectively, at near equal amounts that cross-reacted with potato PFP-α and –β antiserum. In gel filtration analyses the native enzyme eluted in three peaks of 129, 245 and 511 kDa, corresponding to dimeric, tetrameric and octameric forms respectively. Fructose 2,6-bisphosphate (Fru 2,6-P_2) influenced the aggregation state of the enzyme. Both the glycolytic (forward) and gluconeogenic (reverse) reactions had relative broad pH optima between pH 6.7 and 8.0 and Fru 2,6-P_2 shifted the pH optimum for the forward reaction to a more acidic pH (6.7-7.0). The Fru 2,6-P_2 saturation curves were sigmoidal with approximate K_a values of 69 and 82 nM for the forward and reverse reactions respectively. The enzyme showed hyperbolic saturation curves for all its substrates with K_m values comparable to that of other plant PFPs, i.e. 150, 37, 39 and 460 μM for fructose 6-phosphate, inorganic pyrophosphate, fructose 1,6-bisphosphate and inorganic phosphate respectively. This study showed that sugarcane PFP's molecular and kinetic characteristics do not differ significantly from that of other plant PFPs.

KEY WORDS

PFP, Pyrophosphate: fructose-6-phosphate 1-phosphotransferase, sucrose, sugarcane

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INTRODUCTION

Pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP; EC 2.7.1.90) is found exclusively in the cytosol and is one of three enzymes catalysing the interconversion of fructose 6-phosphate (Fru 6-P) and fructose 1,6-bisphosphate (Fru 1,6-P_2), a key reaction in primary carbohydrate metabolism (see Plaxton 1996 for a review). High PFP activity has been associated with strong sink strength, i.e. sucrose cleavage (ap Rees *et al.* 1985, Black *et al.* 1987, Xu *et al.* 1989, Hajirezaei and Stitt 1991), increased glycolytic flux (Dennis and Greyson 1987, Hatzfeld *et al.* 1989, Mertens *et al.* 1991), gluconeogenic flux (Botha and Botha 1991a, Botha and Botha 1993) and regulation of cytosolic PPi concentrations (Simcox *et al*. 1979, ap Rees *et al*. 1985, Dancer and ap Rees 1989, Claassen *et al*. 1991) but no clear physiological function has yet emerged (Stitt 1989, Trípodi and Podestá 1997, Murley *et al.* 1998).

In most plants PFP is present as a heterotetramer consisting of two catalytic (PFP-β) and two regulatory (PFP-α) subunits, approximately 66 and 60 kDa respectively (Yan and Tao 1984, Kruger and Dennis 1987, Wong *et al.* 1990, Botha and Botha 1991a, Nielsen 1994). Depending on various cellular conditions the relative amount and ratio between the α- and β-subunits may also vary, influencing the regulatory properties of the enzyme (Kruger and Dennis 1987, Botha and Botha 1991a, 1991b, Theodorou *et al.* 1992, Theodorou and Plaxton 1994). The reaction catalysed by PFP is thought to be close to equilibrium *in vivo* (Edwards and ap Rees 1986), its activity is strongly activated by Fru 2,6-P_2 (Van Schaftingen *et al*. 1982, Kombrink *et al*. 1984) and Fru 1,6-P_2 (Sabularse and Anderson 1981, Nielsen 1995) and inhibited by Pi (Kombrink and Kruger 1984, Botha *et al.* 1986, Stitt 1989) and PPi (Stitt 1989). In addition, its activity can be modulated by pH (Yan and Tao 1984, Dancer and ap Rees 1989, Trípodi and Podestá 1997) and its molecular composition (Kruger and Dennis 1987, Botha and Botha 1991a, 1991b), suggesting it to be an adaptive enzyme, which should respond sensitively to developmental and environmental cues (Edwards and ap Rees 1986) as is also suggested by the environmental sensitivity of its activator Fru 2,6-P_2 (Van Praag *et al.* 1997).

Despite all these characteristics, no significant changes in the visible phenotype or metabolic fluxes and only small changes in metabolite concentrations are observed in transgenic potato and tobacco plants in which PFP activity is reduced up to 97% (Hajirezaei *et al.* 1994, Paul *et al.* 1995, Nielsen and Stitt 2001). This is true even when these plants are exposed to limiting phosphate and nitrogen conditions and cold stress. Similarly, transgenic tobacco plants over expressing a non-regulated PFP from *Giardia lamblia* also do not show dramatic phenotypic or physiological effects (Wood *et al.* 2002a, 2002b). The alteration induced only a small decrease in starch accumulation in both source and sink tissues and an increase in the total lipid content of the seeds. In addition, the onset of lipid deposition was advanced by up to 48 hours in the developing transgenic embryos (Wood *et al.* 2002b).

In sugarcane PFP activity in internodal tissue is inversely correlated to sucrose content across commercial varieties and a segregating F1 population (Whittaker and Botha 1999). Differences in activity are reflected by corresponding changes in the relative amount of the PFP-β subunit, pointing to coarse regulation. These results are consistent with the general consensus that PFP catalyses a net glycolytic flux (see Stitt 1990 for review) and also with the view that PFP synthesises PPi which is needed for the mobilisation of sucrose through sucrose synthase (SuSy, EC 2.4.1.13; Huber and Akazawa 1986, Black *et al*. 1987, ap Rees 1988). It contrasts, however, with the findings of Wong *et al*. (1988, 1990) who suggested that the kinetic characteristics of PFP in sucrose storing tissues, e.g. carrot root and tomato fruits, might be adapted to favour the gluconeogenic reaction, thereby maintaining substrate levels for sucrose synthesis. In addition, findings in *in vitro* grown potato tubers suggest a sugar-inducible effect on coarse control of PFP (Appeldoorn *et al*. 1999).

The contradiction between the strong inverse correlation of PFP activity and sucrose content in sugarcane and its apparent redundancy as suggested by transgenic studies could be due to possible unique properties of the enzyme in this species. The aim of the current work was therefore to investigate the expression, molecular and kinetic characteristics of sugarcane PFP to determine if it has any features that are in accord

with its apparent role in the sucrose storage phenotype. Here we report that most of sugarcane PFP's properties are similar to that of other plant PFPs although some notable differences do occur. The possible role of these differences in sucrose accumulation is also discussed.

MATERIALS AND METHODS

Chemicals

All chemicals, auxiliary enzymes, cofactors, substrates and kits were of molecular biology grade and were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, Missouri, USA), Roche Diagnostics (Mannheim, Germany) or Promega (Madison, Wisconsin, USA). Antiserum for potato PFP-α and -β were obtained from Dr NJ Kruger (Oxford, England) and has previously been described (Kruger and Dennis 1987). The anti-rabbit IgG-alkaline phosphatase conjugate was obtained from Roche.

Plant material and sample preparation

Plant tissues

Mature, non-flowering, field grown (Stellenbosch, South Africa) sugarcane plants, *Saccharum* spp. hybrid varieties NCo310 and N19, were randomly selected and harvested. The leaf with the uppermost visible dewlap, the node it was attached to and internode immediately above it was defined as leaf-, node- and internode one respectively, according to the system of Kuijper (Van Dillewijn, 1952). Internodes selected for analysis were excised from the stalk and ground to a fine powder in liquid nitrogen after the rind was carefully removed. Leaf roll samples were ground in a similar manner and consisted of the first 50 mm of tissue just above the apical meristem, only including leaves younger than leaf -1 (minus one).

Callus

Callus growth was induced in the dark from leaf roll explants on MS nutrient media containing 3 mg l^{-1} 2,4-D (Taylor *et al.* 1992, Snyman *et al*. 1996). Type 2 yellowish, mucilaginous callus and type 3 white embryogenic callus, consisting of densely

cytoplasmic cells (Taylor *et al*. 1992), were harvested after four to five weeks and ground in liquid nitrogen. All prepared tissue samples were stored in sealed plastic containers at –80^{o}C until used. A combination of type 2 and type 3 calli was harvested and used for the large-scale purification of PFP.

Enzyme extraction and purification

Crude extracts

Proteins were extracted from approximately 500 mg of the ground tissues by stirring it for 15 min on ice in 2.5 volumes of freshly prepared extraction buffer (100 mM Tris (pH 7.2), 5 mM $MgCl_2$, 150 mM KCl, 5 mM EDTA, 5% (m/v) PEG 6,000, 0.05% (v/v) IGEPAL, 2% (m/v) PVPP, 10 mM DTT and 1x Complete™ protease inhibitor cocktail (Roche)). Cell debris was precipitated by centrifugation (15 min at 10,000 g) to render a clear supernatant in which enzyme activity was determined.

PFP Purification

All the extraction and purification steps were performed on ice or at 4^{o}C. Thirty grams of ground tissue was extracted with 2.5 volumes of extraction buffer (as for crude extracts) by stirring it for 20 min. The homogenate was clarified through centrifugation (15 min at 10,000 g) and subsequent filtration through nylon mesh. PEG 6,000 was slowly added to the filtrate to a final concentration of 15%, stirred for 15 min and incubated statically for a further 15 min. The fractionated proteins were precipitated as above and resuspended in 10 ml phosphocellulose buffer (100 mM PIPES (pH 6.6), 2 mM $MgCl_2$, 3 mM EDTA, 10% (v/v) glycerol and 5 mM DTT). The equivalent of 0.5 g dry weight, washed and pre-equilibrated phosphocellulose (washed with 10 volumes (m/v) 1 M KOH (pH>10) for 10 min, rinsed with water until the pH was neutral, washed with 10 volumes (m/v) 10% HCl (pH<3) for 10 min, rinsed again with water as above and pre-equilibrated three times with five bed volumes of phosphocellulose buffer for 15 min each time) was added to the solution and stirred for 30 min. This slurry was applied to a 30 mm column and washed until the A_{280} decreased to the baseline. Bound proteins were eluted with a 5, 10, 20 mM Na_2PPi (in phosphocellulose buffer) step gradient and collected in 5 ml fractions. Fractions containing more than 20% of the total activity were pooled and one volume of ion exchange buffer (20 mM

HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol and 5 mM DTT) was added to this. The protein was adsorbed at 1 ml min^{-1} onto an anion-exchange column (5 ml HiTrap™ Q-Sepharose, Amersham Biosciences, Uppsala, Sweden) and washed with ion exchange buffer until the A_{280} decreased to the baseline. PFP was eluted with 60 ml of a linear 0 to 500 mM KCl gradient in ion exchange buffer; collecting 2 ml fractions. Fractions containing more than 25% of the total activity were pooled, frozen in liquid N_2 and stored at -80^{o}C.

SDS-PAGE and protein blotting

One microgram of purified protein and 20 µg of crude protein extracts were resolved on a 10% (m/v) SDS-PAGE according to the modified method of Moorhead and Plaxton (1991) as described by Whittaker (1997). For the silver staining of gels Amersham Biosciences' PlusOne™ silver staining kit for proteins (Cat. Nr. 17-1150-01) was used according to the manufacturer's instructions. Spot densitometry was done using the AlphaEaseFC software (version 4.0.1) of Alpha Innotech Corp. (San Leandro, CA, USA). Protein blots were done according to the methods described by Whittaker and Botha (1999). In brief, after the peptides were transferred to nitrocellulose membranes the blots were blocked overnight in 4% (m/v) BSA and then incubated in a 1:500 dilution of the specific antiserum. Thereafter the blots were washed and incubated with a 1:2,000 dilution of a goat anti-rabbit IgG-alkaline phosphatase conjugate (Roche), washed again and developed through the enzymatic cleavage of 5-bromo-4-chloro-3-indolyl-phosphate, using nitroblue tetrazolium as a stain enhancer (Blake et al. 1984).

Gel filtration chromatography

Gel filtration chromatography was done at room temperature on a Superose® 6 HR 10/30 column (Amersham Biosciences) using a ÄKTA*prime* FPLC-system (Amersham Biosciences). Thirty milli-units of PFP was applied to the column and fractionated in gel filtration buffer (100 mM Tris (pH 7.2), 5 mM $MgCl_2$, 150 mM KCl, 5 mM EDTA, 10 mM DTT and ½x Complete™ protease inhibitor cocktail (Roche)) at a flow rate of 0.3 ml min^{-1}. One hundred microliter fractions were collected and assayed for PFP

activity. PFP's native molecular weight was determined by linear regression analysis using ferritin (440 kDa), aldolase (158 kDa), BSA (67 kDa) and ovalbumin (43 kDa) as molecular weight markers. To determine the influence of Fru 2,6-P_2 on the aggregation state of the enzyme it was incubated for 1h in the presence of 25 μM Fru 2,6-P_2 and the sample was fractionated in the presence of 2 μM Fru 2,6-P_2 (in the above buffer).

PFP assays and kinetic analysis

PFP activity was measured using the conditions as described by Whittaker and Botha (1999). All enzyme assays were carried out in a final volume of 250 μl at 30°C. Activity was measured by quantifying the oxidation of NADH (forward reaction) or the reduction of $NADP^+$ (reverse reaction) at 340nm using a Power $Wave_X$ microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA). Kinetic constants were determined by varying the different substrate concentrations and the analysis of the data by non-linear regression analyses (two-parameter rectangular hyperbola) using the Sigma Plot 2000 (V6.1), Enzyme Kinetics Module (V1.0) software package (SPSS, Chicago, Illinois, USA).

RESULTS

Purification and molecular characterization

PFP activity in different sugarcane tissues

PFP activity varies significantly between various sugarcane tissue types; activity was therefore measured in crude extracts of several tissue types from two commercial sugarcane varieties, NCo310 and N19, to determine the best source for PFP protein. For the two tissue types originating directly from plants, PFP activity (U g^{-1} fresh weight) was 12 to 18 times higher in the leaf roll than in internode seven. The specific activity (U mg^{-1} protein) of the leaf roll extract was also 2.5 to 3.5 times higher than that of the internode seven extract (Fig. 1). Compared to the leaf roll tissue, type 2 and 3 callus yielded up to five times more PFP activity per gram fresh weight, at a four times higher specific activity (Fig. 1). There was no significant difference in yield between the two callus types, but in general N19 callus yielded PFP at twice the specific activity of

NCo310. Compared to the plant tissues, callus also had the additional advantage of very low levels of phenolic contamination, which increased the overall efficiency of the extraction.

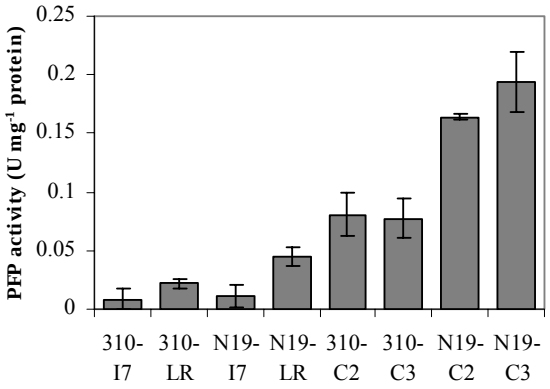

Fig. 1 PFP activity in various sugarcane tissue types as determined in crude extracts. Protein extracts were prepared from the two sugarcane varieties, NCo310 (310) and N19, and the tissue types sampled were internode seven (I7), leaf roll (LR), type 2 (C2) and type 3 (C3) callus.

Subsequently PFP was partially purified (PEG-fractionated) from both internode seven and callus tissue of variety N19 to determine if the enzymes present in these tissues had similar kinetic properties. Based on the very big differences in the reported kinetic parameters for plant PFPs (refer to Table 2 in Chapter 2), it is evident from the data presented in Table 1 that the kinetic parameters for the forward reaction of sugarcane PFP extracted from internodal and callus tissue respectively are comparable.

Table 1. Kinetic parameters for the forward reaction of partially purified sugarcane PFP from internodal and callus tissue.

Parameter	Internode 7		Callus	
	0μM Fru 2,6-P_2	50μM Fru 2,6-P_2	0μM Fru 2,6-P_2	50μM Fru 2,6-P_2
K_m (Fru 6-P) (mM)	0.690 ± 0.042	0.207 ± 0.019	0.594 ± 0.020	0.120 ± 0.013
K_m (PPi) (mM)	0.056 ± 0.004	0.035 ± 0.003	0.061 ± 0.005	0.043 ± 0.004
V_{max} (U mg^{-1} protein)	0.007 ± 0.001	0.054 ± 0.006	0.359 ± 0.054	1.045 ± 0.071

Purification

Based on these findings it was decided to purify sugarcane PFP from N19 type 2 and 3 calli. The results for a typical purification are presented in Table 2. The enzyme was purified 285-fold to a final specific activity of 4.28 and 3.18 U mg^{-1} protein for the forward and reverse reactions respectively, with an overall recovery of 14%. The inclusion of a protease inhibitor cocktail (Complete™, Roche) was essential; without these inhibitors up to 65% of the activity was lost during the first purification step. PFP activity eluted as a single peak during both the phosphocellulose and Q-Sepharose chromatography steps. The final preparation, which was used for all kinetic analyses, was essentially free (less than 1% of PFP activity) of contaminating aldolase, hexosephosphate isomerase, phosphatase and pyrophosphatase activity.

Table 2. Purification of PFP from sugarcane callus.

Step	Activity [a] (U)	Yield (%)	Protein (mg)	Specific activity (U mg^{-1})	Purification (fold)
Crude extract	9.00	100.0	335.92	0.015	1.0
5-15% PEG	7.38	82.0	175.52	0.046	3.1
Phosphocellulose [b]	2.13	23.7	5.63	0.361	24.1
Anion exchange [b]	1.25	13.9	1.61	4.281	285.4

[a] Measured in the forward direction at pH 7.25. [b] Pooled fractions.

Molecular composition

SDS-PAGE analyses using both silver- and Coomassie staining revealed two dominant polypeptides at 63.2 ± 3.8 and 58.0 ± 2.3 kDa respectively, corresponding to the two subunits of PFP found in most other plant species (Fig. 2). Judged by these analyses the two peptides were present in near equal amounts. Moreover, the average ratio between the 63.2 and 58.0 kDa peptides was calculated to be 1 : 1.2, using spot densitometry.

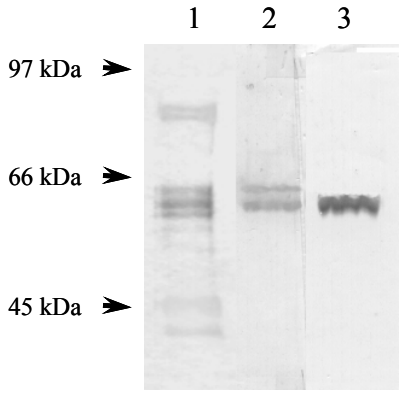

Fig. 2 SDS-PAGE and immunoblot analysis of purified sugarcane PFP. (1) Silver stained gel with 1μg of the purified protein and immunoblots of the same samples using potato PFP-α (2) and PFP-β (3) antisera respectively.

Protein blot analyses were done using antisera generated against potato PFP-α and -β subunits respectively (Kruger and Dennis 1987). Although the putative β-subunit cross-reacted specifically with the potato PFP-β antisera the PFP-α antisera cross-reacted to both the peptides (Fig 2). The PFP-α antisera was also less immunoreactive than the PFP-β antisera.

Molecular weight

The native size of the PFP enzyme was determined using gel filtration chromatography. PFP activity eluted in three peaks with apparent molecular masses of 524 ± 18, 239 ± 8 and 132 ± 4 kDa respectively (Fig. 3). In the absence of Fru 2,6-P_2 the 132 kDa peak dominated (96% of total activity) but in the presence of Fru 2,6-P_2 more than 65% of the total activity was eluted in the 524 kDa peak. The 239 kDa peak represented approximately 3% of the total activity in the absence of Fru 2,6-P_2 and only 1% in the presence of Fru 2,6-P_2 .

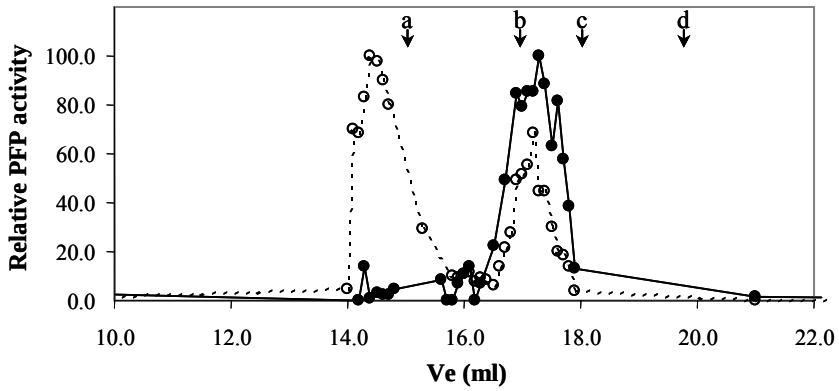

Fig. 3 Gel filtration chromatography of purified sugarcane PFP in the absence (●) and presence (○) of Fru 2,6-P_2. Arrows indicate the elution volumes of ferritin (a), aldolase (b), albumin (c) and ovalbumin (d) respectively.

Kinetic and regulatory characterisation

pH dependence

The pH dependence of the activity was similar for the forward and reverse reactions. Both reactions had relatively broad activity ranges, retaining more than 80% of their maximum activity between pH 6.75 and 8.0 (Fig. 4). The pH optima for both reactions were between 7.25 and 7.5. In the absence of Fru 2,6-P_2 the pH optimum for the forward reaction shifted slightly to a more acidic pH (6.75-7.0). The pH activity profile of the reverse reaction in the absence of Fru 2,6-P_2 had a very broad activity range (6.0-9.0). Compared to a HEPES-acetate based buffer, a Tris-HCl based buffer did not inhibit PFP activity as was reported for potato, mung bean and Shamouti orange PFP (Agosti *et al.* 1992, Van Praag *et al.* 2000). All subsequent kinetic studies for both the forward and reverse reactions were performed at pH 7.25.

Substrate saturation kinetics and Fru 2,6-P_2 activation

In general, the enzyme showed hyperbolic saturation curves for all the substrates. The kinetic parameters of the enzyme were determined using non-linear regression analyses. Apparent K_m and V_{max} values obtained for the various substrates of PFP are summarised in Table 3. The enzyme's affinity for Fru 6-P, PPi and Fru 1,6-P_2 was increased 27-, 2.5- and 16-fold respectively by the addition of 50 μM Fru 2,6-P_2.

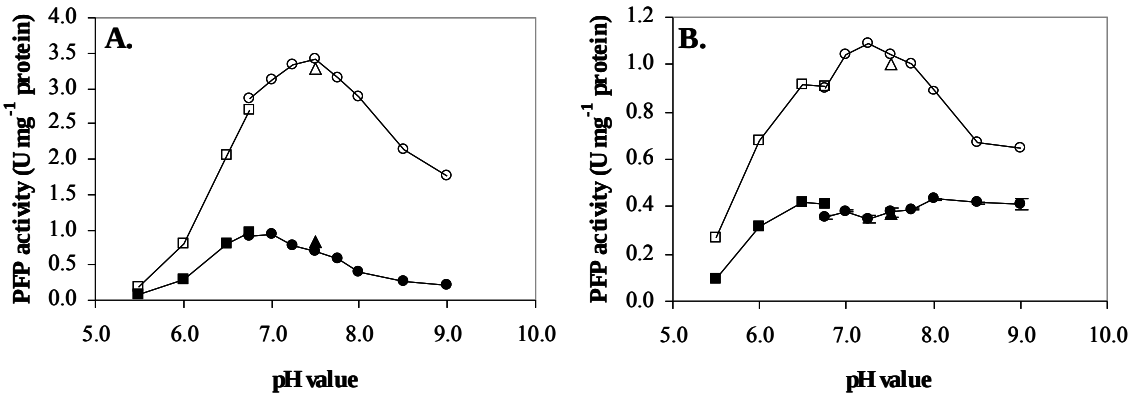

Fig. 4 pH dependence of sugarcane PFP activity. PFP activity in the presence (open symbols) and absence (closed symbols) of Fru 2,6-P_2 in the forward (A) and reverse direction (B). A MES based buffer (□ and ■) was used at the low pH values and a Tris based buffer (○ and ●) at high pH values. The potential inhibitory effect of Tris was tested with a HEPES based buffer (△ and ▲).

In contrast, Fru 2,6-P_2 decreased the enzyme's affinity for Pi almost 2-fold (Table 3). In the presence of 50 μM Fru 2,6-P_2 and saturating substrate concentrations V_{max} was stimulated 2-fold in the forward direction and 1.5-fold in the reverse direction (Table 3). At subsaturating concentrations of Fru 6-P (5 mM) and Fru 1,6-P_2 (0.5 mM) V_{max} increased 5- and 3-fold for the forward and reverse reactions respectively, in the presence of 50 μM Fru 2,6-P_2 (Fig. 2). The Fru 2,6-P_2 saturation curves were sigmoidal with approximate K_a values of 69.3 ± 10.1 and 82.2 ± 14.7 nM for the forward and reverse reactions respectively at the above mentioned substrate concentrations.

Table 3. Kinetic and regulatory properties of sugarcane PFP at saturating substrate concentrations.

Parameter	Fru 2,6-P_2 concentration	
	0μM	50μM
Forward reaction		
K_m^{app} (Fru 6-P) (mM)	4.03 ± 0.29	0.15 ± 0.02
K_m^{app} (PPi) (mM)	0.090 ± 0.006	0.037 ± 0.004
V_{max} (U mg^{-1} protein)	2.13 ± 0.06	4.28 ± 0.10
Reverse reaction		
K_m^{app} (Fru 1,6-P_2) (mM)	0.62 ± 0.10	0.039 ± 0.005
K_m^{app} (Pi) (mM)	0.27 ± 0.04	0.46 ± 0.06
V_{max} (U mg^{-1} protein)	2.26 ± 0.14	3.26 ± 0.08

DISCUSSION

PFP activity in different sugarcane tissues

Initial investigations identified type 2 and 3 callus as an ideal source for sugarcane PFP. Callus did not only yield up to 75 times more PFP per gram fresh weight than internodal tissue, but also had a 20 times higher specific activity and very little contaminating phenolics. Comparative molecular and kinetic studies on partially purified PFP extracted from internodal and callus tissues confirmed that the enzymes had similar properties. In combination with earlier findings that only a single allele of the PFP-β gene is expressed in various sugarcane tissues (Huckett *et al.* 2001) it is reasonable to argue that PFP isolated from callus will have similar molecular and kinetic properties than the PFP from internodal tissues.

The specific activity of PFP in the crude extracts from internode seven tissue was comparable to that obtained in previous studies (Lingle and Smith 1991, Whittaker and Botha 1999). Similarly, the specific activity in the NCo310 callus was the same as that obtained by Wendler and co-workers (1990) in sugarcane suspension cultures, although N19 callus yielded PFP at approximately twice that specific activity. In contrast to the difference in specific activity between the two varieties, the variation between the two different callus types was not significant. This suggests that PFP activity is genetically, rather than developmentally determined in sugarcane callus cultures, similar to that reported for different lines and cell types in carrot suspension cultures (Krook *et al.* 2000). Finally, the specific activities of PFP in the various sugarcane tissues reported here, falls comfortably within the range published for other plant tissues, including sugar storing tissues such as carrot tap root (0.012 U mg^{-1} protein, Wong *et al.* 1988), tomato fruits (0.15 U mg^{-1} protein, Wong *et al.* 1990) and ripe banana fruits (0.012 U mg^{-1} protein, Turner and Plaxton 2003).

Purification and molecular properties

Sugarcane PFP was purified 285-fold to a final specific activity of 4.23 and 3.18 U mg^{-1} protein for the forward and reverse reactions respectively. Fru 2,6-P_2 had a profound effect on the apparent molecular weight of the native enzyme. In the absence of Fru 2,6-

P_2 96% of the total activity eluted in a 132 kDa peak corresponding to the dimeric form of the enzyme. Under these conditions the apparent tetrameric and octameric forms of the enzyme represented only 3% and 1% of the total activity respectively. The addition of Fru 2,6-P_2 induced aggregation, which resulted in the direct conversion of the dimeric form into the octameric form. Similar influences have been described for pea (Wu *et al.* 1984), spinach (Balogh *et al.* 1984) and carrot (Wong *et al.* 1988) PFP, but in these cases the aggregations were always sequential, i.e. from the dimeric to the tetrameric (Balogh *et al.* 1984, Wu *et al.* 1984) or from the tetrameric to the octameric state (Wong *et al.* 1988). Based on this data the kinetic parameters for the enzyme reported above, in the absence or presence of Fru 2,6-P_2, should therefore be for the dimeric and octameric forms respectively. Moreover, PFP should be predominantly in the octameric form *in vivo*, based on the calculated concentrations of Fru 2,6-P_2 in sugarcane (Lingle and Smith 1991, Whittaker and Botha 1999).

SDS-PAGE analyses revealed that the purified enzyme consisted of two polypeptides at near stoichiometric amounts, corresponding to an α- (63 kDa) and β-subunit (58 kDa). This data matched the sequence deduced peptide sizes, which became available more recently, i.e. 67 and 61 kDa for the α- and β-subunits respectively (unpublished data, refer to Telles *et al.* 2001). The putative β-subunit interacted specifically with potato PFP-β antisera, confirming the antigenic similarity between these two peptides. Alignment of their amino acid sequences lent further support to their functional complementarity indicating 81% homology based on amino acid identity. In contrast the potato PFP-α antiserum apparently cross-reacted with both peptides. Any cross-reactivity will be surprising because both the PFP-α and -β antisera are highly specific for their respective target peptides in potato (Kruger and Dennis 1987). Moreover, there is only a 41% homology between the deduced amino acid sequences of the sugarcane PFP-α and -β proteins and an even lower homology (38%) between potato PFP-α and sugarcane PFP-β. A more feasible explanation might therefore be that the PFP-α peptide was subjected to post-translational or *in vitro* modification that resulted in a second peptide similar in size to the PFP-β protein. The apparent lower immunoreactivity of the PFP-α antisera could be explained by the lower homology

between the sugarcane and potato proteins; 65% compared to the 81% for PFP-β. Alternatively it might be an artefact based on the dispersal of the signal if the PFP-α peptide is indeed subjected proteolytic activity.

Kinetic and regulatory properties

Both reactions had relatively broad pH optima between pH 6.75 and 8.00 and the reverse reaction was particularly insensitive to pH in the absence of Fru 2,6-P_2. In addition, Fru 2,6-P_2 shifted the pH optimum of the forward reaction to a slightly more basic pH, i.e. from 6.75 to 7.5, and the biggest degree of activation was also measured at the higher pH values (7.25 – 7.5). A pH response to Fru 2,6-P_2 activation, although exactly the opposite, has only been reported for a few PFPs. In rice seedlings (Enomoto *et al.* 1992), Pi stressed black mustard suspension cells (Theodorou and Plaxton 1996) and pineapple leaves (Trípodi and Podestá 1997) Fru 2,6-P_2 shifts the enzyme's pH optimum to a more acidic pH and the degree of activation increases in this direction. All these authors argued that this activation pattern suggests a glycolytic role for PFP under conditions that result in a decrease in cytosolic pH, e.g. anoxia and night acidification in CAM plants. The significance of this slight change in optimum pH is not clear, especially in light of the fact that maximum activation, for both the forward and reverse reactions, takes place at the optimum pH for the activated reactions.

The Fru 2,6-P_2 saturation curves for the forward and reverse reactions were sigmoidal with approximate K_a values of 0.069 and 0.082 μM respectively. Although the reported Fru 2,6-P_2 levels in sugarcane (~3μM; Lingle and Smith 1991, Whittaker and Botha 1999) is much higher than the K_a values reported here the *in vivo* activation of PFP will depend much on the prevailing concentrations of Pi and other phosphorylated intermediates (Kombrink and Kruger 1984, Theodorou and Kruger 2001). Fru 2,6-P_2 induced a relatively small increase in the V_{max} (2x) in the forward direction and a similar increase (1.5x) in the reverse direction. The fact that sugarcane PFP's V_{max} is influenced very similarly by Fru 2,6-P_2 in both directions is dissimilar to most other plant PFPs for which the forward reaction's V_{max} is in general increased much more (>5x) than the reverse (Kombrink *et al.* 1984, Wong *et al.* 1988, Enomoto *et al.* 1992,

Nielsen 1994, Theodorou and Plaxton 1996, Trípodi and Podestá 1997 and Turner and Plaxton 2003) or where the reverse reaction is not affected by Fru 2,6-P_2 at all (Wong *et al.* 1990, Van Praag *et al.* 2000).

The enzyme showed hyperbolic saturation curves for all the substrates with K_m values comparable to that of other plant PFPs. In contrast to the findings in other sugar storing tissues, i.e. carrot (Wong *et al.* 1988) and tomato PFP (Wong *et al.* 1990), Fru 2,6-P_2 significantly increased (27x) the enzymes' affinity for Fru 6-P. The suggestion of these authors that PFPs in sugar storing tissues might favour gluconeogenic flux because of their low affinity for Fru 6-P especially under conditions that favour high Fru 2,6-P_2 levels, therefore does not hold true for sugarcane PFP. Based on the reported levels for the relevant metabolites in sugarcane internode seven tissue, Fru 6-P, 85µM; PPi, 189µM; Fru 1,6-P_2, 37µM; and Pi, 5.1mM (Whittaker and Botha 1997), PFP could operate close to its V_{max} *in vivo*. In conjunction with the calculated substrate ratios that suggest that the PFP-catalysed reaction is close to equilibrium (Whittaker and Botha 1997) and the similarity in the activation patterns of both the forward and reverse reactions reported above, it suggests a very responsive system that could react in a flexible manner to changes in metabolite levels.

Implications to the sucrose accumulation phenotype

Sugarcane PFP's molecular and kinetic properties do not differ significantly from that of other plant PFPs in a way that clearly suggests a direct role for it in the accumulation of sucrose, e.g. reduced glycolytic or increased gluconeogenic carbon flux. The information presented here therefore supports the study by Whittaker and Botha (1999) in that the apparent link between PFP activity and sucrose accumulation is based on the total amount of catalytic activity present in the tissue and not the fine regulation thereof.

It is important to stress that the above mentioned study correlates PFP activity with sucrose content specifically in internode seven. Although the sucrose content of older internodes is significantly higher (Welbaum and Meinzer 1990, Botha *et al.* 1996)

internode seven has one of the highest sucrose accumulation rates (Whittaker and Botha 1997, 1999). The relevance of this lies in the fact that the influence of PFP in sucrose concentration might be of a transient nature, i.e. might be obscured over time. Internode seven represents tissues with high metabolic activity and carbohydrate flux (Whittaker and Botha 1997, Bindon and Botha 2002). A perturbation in the metabolic network could therefore lead to an altered flux, e.g. into sucrose accumulation, which could temporarily lead to altered concentrations, but which would be restored over time to basal levels. This argument is supported by the transient influence of reduced PFP activity on sucrose concentrations in transgenic potato tubers. Although the final concentration is unchanged the flux into sucrose in the growing (metabolically active) tubers is 13-times higher in comparison to the wild type (Hajirezaei *et al*. 1994). The argument is used to support the hypothesis that PFP influence flux rather than the ability of the tissue to store sucrose. It is therefore probably more accurate to refer to PFP's influence on the sucrose accumulation rate in these tissues instead of their sucrose content.

Lower PFP activity could improve sucrose accumulation directly by (i) increasing the concentration of precursors for sucrose synthesis, i.e. reduced glycolytic flux (Wong *et al.* 1988, Focks and Benning 1998, Krook *et al.* 2000) or indirectly by (ii) limiting the production of PPi which could be used for the mobilisation and utilisation of sucrose through SuSy (ap Rees *et al.* 1985, Black *et al.* 1987, Huber and Akazawa 1986, Xu *et al.* 1989, Hajirezaei and Stitt 1991). However, the role of PFP in sucrose accumulation in sugarcane can only be irrefutably demonstrated in transgenic plants with altered PFP activity, which is currently under investigation in our laboratories.

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CHAPTER 4

Development and characterisation of transgenic systems for the manipulation of PFP activity in sugarcane*

ABSTRACT

To manipulate PFP activity in sugarcane the endogenous PFP-β coding sequence and two monogenic, non-plant PFP genes, those of *G. lamblia* and *P. freudenreichii*, were cloned and characterised. The 1668 bp sugarcane PFP-β cDNA sequence showed high homology to other plant PFP-β sequences and was subsequently used to construct antisense and co-suppression expression vectors for the down-regulation of PFP activity. The activity and kinetic parameters of the non-plant PFPs were determined using purified proteins produced in bacterial expression systems. Based on these parameters it was concluded that the non-plant PFPs should function at least at $½V_{max}$ in transgenic sugarcane plants. Polyclonal antibodies raised against these two enzymes showed no cross reactivity with sugarcane PFP. The two non-plant genes were used to construct plant expression vectors for the up-regulation of PFP activity, either constitutively or specifically in the phloem.

KEY WORDS

PFP, Pyrophosphate: fructose-6-phosphate 1-phosphotransferase, bacterial expression, expression vector, transgenic sugarcane, sucrose metabolism

INTRODUCTION

Pyrophosphate fructose 6-phosphate 1-phosphotransferase (PFP; EC 2.7.1.90) is most probably present in all plants (Stitt 1990) and in a limited number of prokaryotes and lower eukaryotes such as *Propionibacterium shermanii* (O'Brien *et al.* 1975),

* Parts of this work have been published as follows: 1) Patent PA128025/ZA, A high level, stable, constitutive promoter element for plants. 2) Groenewald *et al*. 2000. Plant Cell Rep 19: 1098-1101.

Giardia lamblia (Mertens 1990), *Entamoeba histolytica* (Reeves *et al.* 1974), *Rhodospirillum* (Pfleiderer and Klemme 1980) and *Euglena gracilis* (Miyatake *et al.* 1984). The enzyme catalyses the reversible conversion of fructose 6-phosphate (Fru 6-P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate (Fru 1,6-P_2) and inorganic phosphate (Pi) (Reeves *et al.*1974). This is thought to be a near-equilibrium reaction *in vivo* and would therefore be able to respond to cellular metabolism in a very flexible manner (Edwards and ap Rees 1986).

Although the exact physiological role of PFP in plants is still unknown, its kinetic properties have been studied in some detail and it is evident that it plays an important role in sucrose cycling, breakdown and subsequent respiratory carbon flow (Sabularse and Andersen 1981, Kombrink *et al.* 1984, Bertagnolli *et al.* 1986, Botha and Small 1987, Stitt 1990). PFP activity in sugarcane internodal tissues is inversely correlated to sucrose content across commercial varieties and a segregating F1 population (Whittaker and Botha 1999). These differences in activity are also reflected by corresponding changes in the relative amount of the PFP-β subunit, implying coarse regulation. Potentially, the levels of both PPi and Fru 6-P could control the rate of glycolysis, i.e. respiratory flux (Plaxton 1996 and references therein) and, in addition, PFP has been implicated in the determination of sink strength (Edwards and ap Rees 1986, Botha *et al*. 1992, Black *et al*. 1995). Manipulation of PFP activity in sugarcane could therefore have an impact on carbon distribution between stored sucrose and respiration.

Plant PFPs are usually heterotetramers of approximately 250 kDa, composed of two α- and two β-subunits with molecular weights of approximately 66 and 60 kDa respectively (Kruger and Dennis 1987). The β-subunits are the catalytic subunits while the α-subunits are involved in the regulation of enzyme activity through fructose 2,6-bisphosphate (Fru 2,6-P_2) (Yan and Tao 1984, Carlisle *et al.* 1990, Cheng and Tao 1990, Van Praag 1997). In contrast, the PFPs from the prokaryotes and lower eukaryotes are homodimers, which are insensitive to Fru 2,6-P_2 activation (O'Brien *et al.* 1975, Mertens 1990).

PFP activity has been increased indirectly in transgenic plants by over expressing Fru 2,6-P_2 (Kruger and Scott 1994, Scott and Kruger 1995), or directly through the expression of a heterologous enzyme (Wood *et al.* 2002a and b). The digenic nature of plant PFP and the fact that its activity is finely regulated *in vivo* complicates the direct up-regulation of activity in transgenic plants. However, both these difficulties can be overcome by the use of homodimeric PFP enzymes such as those isolated from prokaryotes and lower eukaryotes. The successful expression of any of these PFP genes in transgenic plants should be reflected in an increase in the *in vivo* PFP activity, because only a single gene product is necessary to produce a functional enzyme and the resulting enzyme is not sensitive to the endogenous regulatory mechanisms in plants.

Down-regulation of PFP activity, on the other hand, can be done by down regulating the expression of any one of the two endogenous genes encoding each of the two subunits of plant PFP. Using homology-dependent gene silencing technology, gene expression can be specifically down-regulated by transforming the plant with either an antisense or untranslatable form of the target gene (Fagard and Vaucheret 2000 and references therein).

Here we report the establishment of various transformation systems that will allow the up- and down-regulation of PFP activity in sugarcane. The goals for this work did not only include the cloning of the relevant gene sequences and the construction of appropriate plant expression vectors but also the expression, purification and characterisation of the heterologous PFP proteins to confirm their bio-activity and potential properties under *in vivo* conditions. Finally, antisera were raised against the two purified proteins to enable the easy characterisation of transgenic plants.

MATERIALS AND METHODS

Chemicals and enzymes

All chemicals, enzymes, including their reaction buffers, cofactors and kits were of molecular biology grade and were obtained from the sources mentioned in Chapter 3.

Recombinant DNA technology

Cloning and expression vectors

All PCR derived fragments were cloned directly into the pGEM®-T Easy cloning vector (Promega) and the pGEX-4T1 bacterial expression system (Pharmacia, Milwaukee, USA; GENBANK U13853) was used for the expression and purification of recombinant proteins. For constitutive gene expression in transgenic plants the plant expression vector pUBI 510 (ECACC deposit reference number: 00042603, Groenewald *et al.* 2000) was used. Gene expression in this vector is driven by the CaMV 35S (Odell *et al.* 1985) and the maize polyubiquitin (*UBI-1*, Christensen *et al.* 1992) promoters in tandem and, in addition, also contains the first intron of *UBI-1*, which acts as an enhancer. Vectors for the phloem specific expression of transgenes were constructed by using the *rolC* promoter (Chilton *et al*. 1982, Groenewald 1999) from the plasmid pBIN*rolC* (provided by Dr. J. Schell, Max Planck Institute, Köln, Germany). Standard cloning techniques were used during the construction of these vectors and characterisation of the final constructs was done by restriction analyses and sequencing (Sambrook *et al.* 1989).

RT-PCR

RT-PCR was done using the Titan™ RT-PCR system (Roche) according to the manufacturer's specifications.

DNA sequencing

Automated DNA sequencing was performed with an Applied Biosystem ABI Prism 373 Genetic Analyser using an ABI BigDye™ terminator cycle sequencing ready reaction kit according to the manufacturer's recommendations (Perkin-Elmer, Boston, Massachusetts, USA).

Library construction and screening

mRNA was isolated from the leaf roll of sugarcane commercial variety N19 (Bugos *et al.*1995) and cDNA was synthesised using the Universal RiboClone® cDNA Synthesis System (Promega) according to the manufacturer's instructions. A cDNA library was constructed using Stratagene's (La Jolla, California, USA) Lambda ZAP II system

according to the manufacturer's instructions. A library with a titre of 3 x 10^9 plaque forming units per millilitre (pfu ml^{-1}) was obtained. Library screening was done according to standard methods as described earlier (Groenewald 1999).

P. freudenreichii cultivation and isolation of gDNA

P. freudenreichii was grown for five days at 37°C under anaerobic conditions in a medium containing 0.5% (m/v) yeast extract, 0.2% (m/v) Peptone P, 36.7 mM KH_2PO_4, 1.0% (v/v) lactic acid, 0.1% (v/v) Tween 80 and 0.001% (m/v) Hemin. To isolate gDNA the cells were harvested through centrifugation, resuspended in lysis buffer (50 mM Tris (pH 8.0), 1 mM EDTA, 0.2% (m/v) lysozyme) and incubated at 37°C for 30 min. Thereafter SDS and proteinase K was added to final concentrations of 1% (m/v) and 50 μg ml^{-1} respectively and the suspension was incubated at 50°C for 4 h. The suspension was extracted twice with an equal volume of chloroform:isoamyl alcohol (Chl:IAA, 24:1) and the aqueous phase was transferred to a small glass beaker and overlaid with 2.5 volumes of –20°C, 96% (v/v) ethanol. High molecular weight gDNA was spooled out with a glass hook and resuspended in 1 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The gDNA was treated with RNase (0.2 mg ml^{-1}) for 30 min at 37°C and the Chl:IAA (24:1) extraction was repeated. Finally, the gDNA was quantified spectrophotometrically.

Expression, purification and characterisation of recombinant proteins

Bacterial expression system and purification of proteins

To verify the bioactivity and kinetic properties of the homodimeric PFP gene products and to purify the proteins for the generation of polyclonal antibodies the two genes were cloned into the pGEX-4T1 bacterial expression system (Pharmacia). This system expresses the recombinant protein as a GST-fusion to allow the easy purification of the product. Both the production, preparation of crude extracts and affinity purification of the recombinant proteins were done according to the instructions of the manufacturer. *G. lamblia* PFP cultures were grown at 37°C while the *P. freudenreichii* cultures had to be grown at 22°C to ensure the production of a soluble product. The GST-fusion proteins were purified using Glutathion Sepharose 4B affinity chromatography as

recommended by the manufacturer. Proteins used for the kinetic characterisation of the enzymes were eluted after Thrombin digestion to remove the GST-peptide and the GST-fusion proteins used to raise antibodies were eluted using reduced glutathion.

Polyclonal antiserum

Rabbits were immunised by injecting them with eight fractions of 100 µg purified protein according to the method described by Bellstedt *et al.* (1987). Whole blood, from which the antiserum was obtained, was collected after 38 days.

Enzyme assays and kinetics

PFP activity was measured using the conditions as described by Whittaker (1997) with the exception that Fru 2,6-P_2 was omitted from the reaction mixture. All enzyme assays were carried out in a final volume of 250 µl at 30^{o}C. Activity was measured by quantifying the oxidation of NADH (forward reaction) or the reduction of $NADP^{+}$ (reverse reaction) at 340 nm using a Power $Wave_X$ microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA). Kinetic constants were determined by varying the different substrate concentrations and the analysis of the data by non-linear regression analyses (two parameter rectangular hyperbola) using the Sigma Plot 2000 (V6.1), Enzyme Kinetics Module (V1.0) software package (SPSS, Chicago, Illinois, USA).

SDS PAGE and protein blot analyses

SDS PAGE and protein blot analyses were done as described in Chapter 3.

RESULTS AND DISCUSSION

Selection of non-plant PFPs

The primary structure of a transgene can determine the efficiency with which it is expressed in transgenic plants (Perlak *et al.* 1991, Sutton *et al.* 1992). Potential non-plant PFPs, to be used for the up-regulation of PFP activity in transgenic sugarcane, were therefore evaluated in terms of the availability of their gene sequences and differences in their nucleotide and amino acid sequences, GC-content and codon usage (Table 1). Based on these criteria two genes were identified. *G. lamblia* PFP consists of

two 59.8 kDa monomers, which are encoded by a 1635 bp gene (Rozario *et al.* 1995), while the 43.3 kDa monomer of *P. freudenreichii* PFP is encoded by a 1215 bp gene (Ladror *et al.* 1991). The most important properties of these two genes are summarised and compared to plant PFPs in Table 1.

Table 1. Comparison of plant and non-plant PFPs.

Origin	Protein	Gene	Exons	%GC	GENBANK	Activator	Reference
Castor bean	250 kDa heterotetramer	α- 1.9 kb β- 1.7 kb	α- 19 β- 16	α- 44.4% β- 44.5%	α- Z32849 β- Z32850	Fru 2,6-P_2	Todd *et al.* 1995
Potato	250 kDa heterotetramer	α- 1.9 kb β- 1.5 kb	α- n.a. β- n.a.	α- 43.4% β- 44.6%	α- M55190 β- M55191	Fru 2,6-P_2	Kruger and Dennis 1987
G. lamblia	120 kDa homodimer	1.6 kb	None	50.3 %	U12337	None	Rozario *et al.* 1995
P. freudenreichii	90 kDa homodimer	1.2 kb	None	66.7 %	M67447	None	Ladror *et al.* 1991

n.a. = not available

Isolation and characterisation of the PFP gene sequences

Sugarcane PFP-β gene

To ensure optimal antisense and co-suppression mediated gene silencing in transgenic sugarcane the endogenous PFP-β gene sequence was isolated. A set of degenerate primers was designed (PFP-B4 and PFP-B2, Table 2), based on the consensus sequence of the castor bean and potato PFP-β gene sequences available in the international database; GENBANK accession numbers Z32850 and M55191 respectively (www.ncbi.nlm.nih.gov). These primers were used to amplify a 248 bp cDNA fragment, spanning exons eight; nine and ten, from sugarcane leaf roll RNA using the RT-PCR technique. Subsequent cloning and characterisation of the fragment confirmed its identity as being the PFP-β gene.

Table 2. Primers used to amplify a 248 bp fragment of sugarcane PFP-β.

Primer	Binding site	Sequence
PFP-B4	bp 802 – 821 (forward)	5′- CAC ATT ACI TTI GIA TGC GC –3′
PFP-B2	bp 1049 – 1029 (reverse)	5′- TCA TCI ACA ACA TCA TGI GCC –3′

The amplified PFP-β cDNA fragment was used as a probe to screen a sugarcane leaf roll cDNA library for putative PFP-β clones. Isolated clones were sequenced to verify the inserts' identity and integrity. One such clone, PFP#5, contained a 1135 bp fragment, which represents 53% (875/1668 bp) of the PFP-β coding sequence, spanning exons 8 to 16 (Fig. 1).

ATGGCGGCGC CGAGCGGACC ATCACCTGGG ACTGGGAGGT TGGCGTCGGT TTACAGCGAG
GTGCAGACGA GCCGCCTCCA TCACGCGATC CGGCTCCCCT CCGTCCTCTG CTCCCAATTC
TCCCTCGTCG ATGGACCTCC CAGCTCAGCC ACGGGGAACC CGGATGAGAT CGCGAAGCTG
TTCCCTAACT TGTTTGGGCA GCCGTCGGCG ACATTGGTGC CGGCCAAAGA GGCGGTGGAG
GGGAAGGCGC TGAAGGTCGG GGTGGTGCTC TCTGGTGGAC AAGCACCCGG TGGGCACAAT
GTGATCTGCG GTATCTTCGA TTTCTTGCAG AAACACGCAA AGGGAAGCAC AATGTATGGA
TTCAAAGGAG GCCCAGCAGG GGTGATGAAG TGCAAGTACG TCAAACTCAA TACCGATTTC
GTCTATCCCT ACAGAAACCA GGGTGGTTTT GATATGATCT GTAGTGGAAG GGATAAGATT
GAAACACCAG AGCAGTTTAA GCAAGCCGAA GATACAGCCA ACAAACTTGA GTTGGACGGA
CTTGTTGTTA TTGGACGGGA CGATTCAAAT ACTCATGCTT GCCTCTTTGC TGAATACTTC
AGGAGTAAAA ATTTGAAAAC CCGTGTCATT GGCTGCCCAA AGACCATTGA TGGTGATCTC
AAATGCAAAG AGGTTCCAAC CAGTTTTGGA TTTGACACTG CATGCAAGAT CTATTCAGAA
ATGATTGGAA ATGTCATGAT TGATGCCCGA TCAACTGGAA AATATTATCA CTTTGTACGG
CTTATGGGGC GTGCTGCTTC TCACATTACA TTGGGATGCG CTTTGCAAAC ACACCCCAAT
GCTGCACTCA TTGGGGAAGA GGTTGCTGCA AAGAAGCAAA CCCTTAAGAA CGTCACAAAC
TACATTACTG ATATCATCTG CGAGCGTGCA GATCTTGGTT ACAACTATGG TGTTATCCTT
ATACCAGAAG GCCTGATTGA TTTCATCCCA GAGGTGCAGA ATATCATTGC TGAATTGAAT
GAAATTTTGG CACATGATGT TGTTGATGAG GCAGGGGCCT GGAAAAGCAA GCTTCAGCCT
GAATCAAAGG AGCTGTTTGA GTTTTTGCCC AAAACTATTC AGGAGCAACT TATGCTTGAA
AGGGGCCCCC ATGGCAATGT TCAGGTTGCA AAAATTGAAA CCGAGAAAAT GCTTATTAGC
ATGGTGGAAA CTGAACTGGA GAAGAGAAAA GCAGAGGGGA GATACTCTGC ACATTTCAGA
GGGCAAGCTC ATTTCTTTGG GTACGAAGGA AGATGTGGCC TTCCTACCAA TTTTGATTCT
AACTATTGCT ATGCATTAGG CTATGGGGCT GGTGCCCTTC TCCAAAGTGG GAAGACAGGA
CTTATTTCAT CGGTTGGCAA CCTTGCGGCT CCAGTAGAAG AATGGACTGT TGGTGGAACA
GCATTGACAT CACTGATGGA TGTGGAGAGG AGGCATGGCA AGTTCAAGCC AGTGATCGAG
AAGGCTATGG TGGAACTTGA TGCTGCACCT TTCAAGAAAT ATGCATCAAT GCGGGATGAG
TGGGCCACCA AGAACAGATA CATCAGCCCT GGCCCCATCC AGTTCAGTGG CCCTGGAAGT
GATGACTCGA ACCACACTTT GATGCTGGAA CTCGGTGCTG AGTTA**TAG**AG ATGCGTCCTT
TGCTTATTTT TGTTTCTTAC AGTTTTGGGA GTGGAGACTG GACACTGGGT CTCCTGGAGC
AGCCTGCAGT CTCCATATTG TGAATTGTTT AATAAGAGGT TCGATGTGAG TTTTCTGCGT
AGCGGACTGG ATGTAGCAAA TAAGAACTGG TTTTAGCATT TTTTGTATGA TTTACGCACC
AACTGACTTG TCTTGTAACC CTGATTCTGT TCCACTGGTT GCAATCTCGT GAGAATGAAC
AAGTTGATAT GAGGCTAAAT CGGAATTCCT GCAGCCCGGG GGATCCACTA GTTCTAGAGC
GGCCGCCACC GCGGTGGAGC TCCAGCTTTT TTCCCTTTAG TCAGGGTAAT TCGAACTGCG
A

Fig. 1 Coding sequence and 3' UTR of the sugarcane PFP-β gene. The initiation and termination codons are indicated in bold and the sequence of the cDNA clone, PFP#5, is underlined.

The rest of the insert consisted of a 260 bp 3' untranslated sequence. Comparing the sequence to the international database (BLASTN software; www.ncbi.nlm.nih.gov/cgi-bin/BLAST) confirmed its identity as being PFP-β - it was 74.9% and 75.0%

homologous to the castor bean and potato PFP-β sequences respectively. Moreover, this sequence was also 96.9% homologous to a recently published maize sequence (GENBANK AY104192). This gene fragment was therefore used in the construction of antisense and co-suppression plant expression vectors, aimed at down-regulating the expression of the sugarcane PFP-β gene. Subsequently, the complete sugarcane PFP-β coding sequence, as presented in Fig. 1, was cloned and characterised.

Non-plant PFP genes

The *G. lamblia* PFP gene, originally characterised by Rozario *et al*. (1995, GENBANK U12337), was obtained from Dr D Dennis (Performance Plants, Queens University, Kingston, Canada). To isolate the *P. freudenreichii* PFP gene specific primers were designed to amplify the complete coding sequence from gDNA. The primer sequences were based on the published *P. freudenreichii* PFP gene sequence of Ladror *et al*. (1991, GENBANK M67447) and *BamH* I restriction sites were introduced into the primers to facilitate the subsequent cloning of the gene (Table 3). As expected, a 1.2 kb fragment was amplified from *P. freudenreichii* gDNA, which was cloned into the pGEM®-T Easy cloning vector and sequenced. Comparison with the international database confirmed the cloned sequence to be the PFP gene of *P. freudenreichii* (BLASTN software; www.ncbi.nlm.nih.gov/cgi-bin/BLAST).

Table 3. Primer sequences used to amplify the *P. freudenreichii* PFP gene from gDNA. The initiation and termination codons are indicated in bold and the introduced *BamH* I sites are underlined.

Primer	Binding site	Sequence
Prop-PFP-F	bp -11 – 15 (forward)	5′-GG CCC GGA TCC **ATG** GTG AAA AAG GTC–3′
Prop-PFP-R	bp 1240 – 1213 (reverse)	5′-TC GCC GGA TCC CTA **TTA** CGC GGC GGC G–3′

Expression, purification and kinetic characterisation of non-plant PFPs

Because the non-plant PFP genes were earmarked for the over expression of PFP activity in transgenic sugarcane, it was imperative to verify the bioactivity of the two gene products and to confirm their potential effectiveness in transgenic sugarcane plants, i.e. determine their kinetic properties under sugarcane's physiological

conditions. Both genes were therefore cloned into the pGEX-4T1 bacterial expression system, which would express the enzymes as GST-fusion proteins to facilitate the purification of the recombinant enzymes.

Although both systems expressed active enzymes, *P. freudenreichii* PFP was only detectable when the cultures were grown at low temperatures, e.g. 22°C (Fig. 2). Subsequent protein blot analyses indicated that this was not due to incorrect folding of the peptides but rather the complete absence of soluble protein (results not shown). Preparative cultures were prepared based on these findings and the recombinant enzymes were purified using affinity chromatography and subsequent thrombin cleavage to remove the GST-fusion. *G. lamblia* and *P. freudenreichii* PFP were respectively purified to final specific activities of 3.82 and 2.53 U mg^{-1} protein.

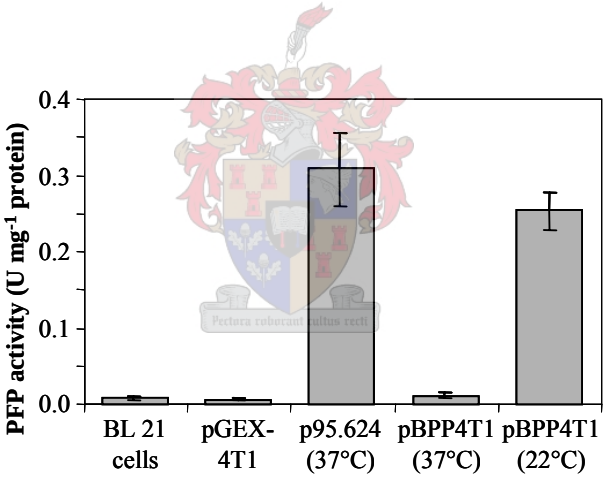

Fig. 2 Activity of recombinant *G. lamblia* and *P. freudenreichii* PFP in crude extracts from bacterial expression systems. BL 21 cells represent the untransformed *E.coli* strain used, pGEX-4T1 is the cloning vector without an insert, p95.624 is the vector containing the *G. lamblia* PFP gene and pBPP-4T1 contains the *P. freudenreichii* PFP gene.

Both enzymes had relatively broad pH optima, between 6.5 and 7.8, for both the forward and reverse reactions (Fig. 3) and displayed typical Michaelis-Menten kinetics for all four substrates. The kinetic parameters of the enzymes were determined with non-linear regression analyses as described in the materials and methods section. The kinetic parameters determined for the two enzymes are summarised and compared to the concentrations of these substrates in sugarcane in Table 4. In addition, both enzymes were also shown to be completely active in the absence of Fru 2,6-P_2 (Fig. 4).

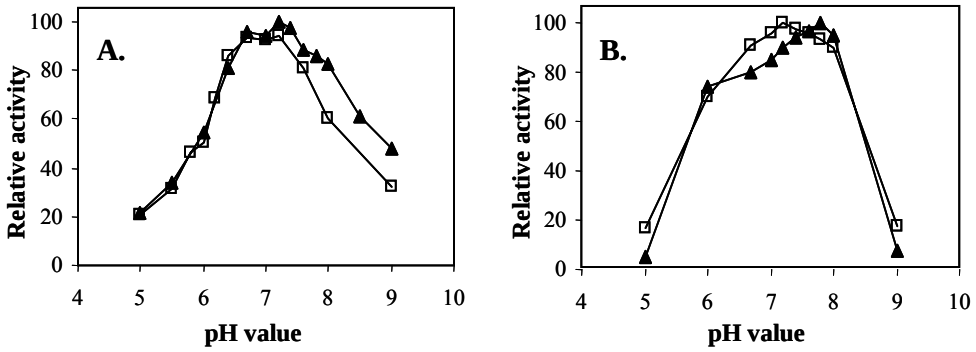

Fig. 3 pH dependence of *G. lamblia(*▲) and *P. freudenreichii* (□) PFP activity for the forward (A) and reverse (B) reactions respectively.

Table 4. Kinetic properties of *G. lamblia* and *P. freudenreichii* PFP compared to the metabolite concentrations in sugarcane culm (Whittaker and Botha 1999).

Metabolite	K_m (µM)		Concentration in sugarcane culm (µM)
	G. lamblia	*P. freudenreichii*	
Fru 6-P	79.60	196.70	85-178
PPi	37.57	41.40	166-290
Fru 1,6-P_2	26.98	37.83	32-46
Pi	1331.89	1491.83	3400-6100
pH optimum	6.5 - 7.8	6.5 – 7.8	6.7 – 8.0

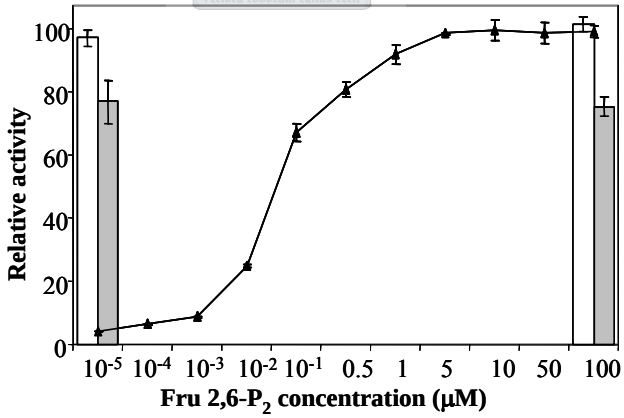

Fig. 4 Influence of Fru 2,6-P_2 on the activity of *G. lamblia* (open bars) and *P. freudenreichii* (filled bars) PFP, compared to that of a plant (potato, ▲) PFP. V_{max} for *G. lamblia*, *P. freudenreichii* and potato PFP was 3.54, 2.63 and 0.61 µmol min^{-1} mg^{-1} protein respectively.

Based on the K_m values for the four different substrates and their concentrations in sugarcane, both enzymes should function at least at $^1/_2V_{max}$ in transgenic sugarcane

plants. In addition, the confirmed insensitivity of these enzymes to Fru 2,6-P_2 suggest that they would be insensitive to the usual *in vivo* regulatory mechanisms of PFP in sugarcane. The successful expression of the proteins in transgenic plants should therefore be translated into increased PFP activity.

After the kinetic characterisation of the enzymes additional protein was purified for the production of polyclonal antibodies to facilitate the future characterisation of putative transgenic clones. In this instance the whole GST-fusion protein was eluted from the affinity column using reduced glutathion. Rabbits were immunised against the purified proteins and 38-day antiserum was tested at a 1:500 dilution to verify the specificity of the raised antibodies (Fig. 5). The polyclonal antibodies showed no cross reactivity to sugarcane PFP, which makes it ideal for the characterisation of transgenic plants. This lack of cross reactivity is not surprising considering the low homology between these peptides on amino acid level, i.e. the highest percentage homology (identity) between any of the four peptides is the 47% between *G. lamblia* PFP and the sugarcane PFP-β protein.

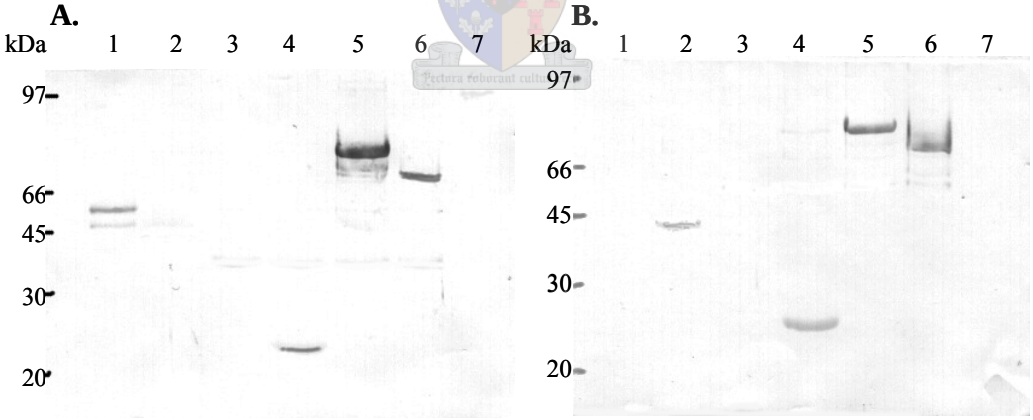

Fig. 5 Protein blot analysis using thirty-eight day serum raised against the GST fusions of *G. lamblia* (A) and *P. freudenreichii* PFP (B). The following protein extracts were loaded in the respective lanes: 1) purified *G. lamblia* PFP, 2) purified *P. freudenreichii* PFP, 3) untransformed *E. coli*, 4) pGEX-4T1 transformed cells, 5) crude extract of the GST-*G. lamblia* PFP fusion protein, 6) crude extract of the GST-*P. freudenreichii* PFP fusion protein, 7) crude extract of sugarcane internode seven protein (variety NCo 310).

Construction of plant expression vectors

Six plant expression vectors were constructed for the genetic manipulation of PFP activity in sugarcane. These included vectors for the up- and down-regulation of PFP activity, either constitutively or specifically in the phloem. A tandem promoter construct consisting of the CaMV-35S (Odell *et al.* 1985) and maize *UBI-1* (Christensen *et al.* 1992) promoters, previously shown to drive high level expression in sugarcane tissues (Groenewald *et al.* 2000), was used as constitutive promoter and the *rolC* promoter (Chilton *et al*. 1982, Groenewald 1999) was used to confer phloem specific expression. The general properties of the vectors are summarised in Table 5. All the expression vectors were designed for direct transformation protocols, i.e. particle bombardment, and had to be co-transformed with a vector containing a selectable marker, e.g. *NPT II*. Plasmid maps of two of these expression vectors are presented in Fig. 6 as examples.

Table 5. Properties of the plant expression vectors constructed to manipulate PFP expression in transgenic sugarcane.

Vector	Promoter	Gene	Regulation	Specificity
pEGP 510	35S-*UBI-1*	*G. lamblia*	Up	Constitutive
pEPP 510	35S-*UBI-1*	*P. freudenreichii*	Up	Constitutive
pEGP 800	*rolC*	*G. lamblia*	Up	Phloem
pASPc 510	35S-*UBI-1*	Sugarcane PFP-β	Down	Constitutive
pUSPc 510	35S-*UBI-1*	Sugarcane PFP-β	Down	Constitutive
pASPc 800	*rolC*	Sugarcane PFP-β	Down	Phloem

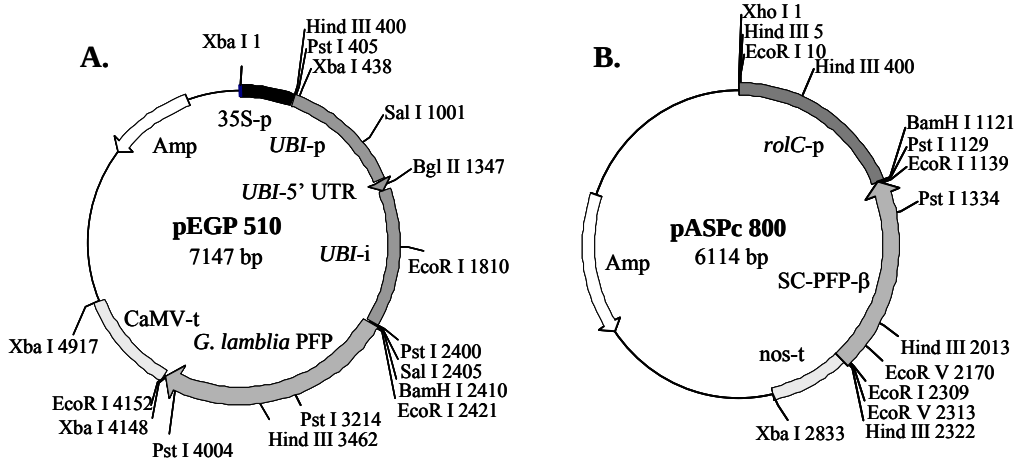

Fig. 6 Schematic maps of two of the plant expression vectors constructed for the genetic manipulation of PFP activity in sugarcane.

CONCLUSION

The main aim of the work described here was to establish relevant transformation systems for the up- and down-regulation of PFP activity in sugarcane; six plant expression vectors were constructed which should do so, either constitutively or phloem specifically. The sugarcane PFP-β gene was cloned and characterised for the first time and two monogenic, non-plant PFP genes were also cloned and characterised. In addition the bio-activity of these heterologous PFP proteins, to be used for the up-regulation of activity, was confirmed and their potential properties in sugarcane under *in vivo* conditions were determined. Finally, antisera were raised against the two purified heterologous PFP proteins, which should facilitate the characterisation of transgenic plants.

Although the over expression of PFP activity in sugarcane will be investigated to complement this study the rest of the work described here will focus only on the down-regulation of PFP activity in transgenic plants to assess the apparent inverse correlation described earlier.

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CHAPTER 5

Down-regulation of pyrophosphate fructose 6-phosphate 1-phosphotransferase activity in sugarcane enhances sucrose accumulation in immature internodes*

ABSTRACT

Pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity was successfully down-regulated in sugarcane using constitutively expressed antisense and untranslatable forms of the sugarcane PFP-β gene. In young internodal tissue activity was reduced by to 70% while no residual activity could be detected in mature tissues. The transgenic plants showed no visible phenotype or significant differences in growth and development under greenhouse and field conditions. Sucrose concentrations were significantly increased in the immature internodes of the transgenic plants but not in the mature internodes. This contributed to an increase in the purity of the immature tissues, resembling an early ripening phenotype. PFP activity was inversely correlated with sucrose content in the transgenic lines. Both the immature and mature internodes of the transgenic plants had significantly higher fibre contents. These findings suggest that PFP influences the ability of biosynthetically active sugarcane cells to accumulate sucrose but that the equilibrium of the glycolytic intermediates, including the stored sucrose, is restored when ATP-dependent phosphofructokinase and the residual PFP activity is sufficient to sustain the required glycolytic flux as the tissue matures. Moreover, it suggests a role for PFP in glycolytic carbon flow, which could be rate limiting under conditions of high metabolic activity.

KEY WORDS

PFP, Pyrophosphate: fructose-6-phosphate 1-phosphotransferase, carbon metabolism, sucrose, transgenic sugarcane, gene silencing

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INTRODUCTION

Pyrophosphate fructose 6-phosphate 1-phosphotransferase (PFP; EC 2.7.1.90) catalyses the reversible conversion of fructose 6-phosphate (Fru 6-P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate (Fru 1,6-P_2) and inorganic phosphate (Pi); one of the principle reactions in glycolysis (Reeves *et al.* 1974, Carnal and Black 1979). In sugarcane, PFP activity in internodal tissues is inversely correlated with sucrose content and positively related to total respiration, across commercial varieties and within a segregating F1 population (Whittaker and Botha 1999). In addition, a significant amount of carbon is cycled between the triose-phosphate and hexose phosphate pools, for which PFP is at least partially responsible (Bindon and Botha 2002). The extent of this cycling decreases concomitantly with sucrose accumulation in mature internodal tissue (Bindon and Botha 2002). Similarly, in sucrose storing carrot cell suspensions a high respiratory activity stimulates the flow of hexose phosphates towards the respiratory pathway and the recycling (back-flow) of the triose-phosphates, but reduces the cells' ability to accumulate sucrose (Krook *et al.* 2000). PFP activity is directly linked to these carbon-partitioning patterns and, as in sugarcane, the ratio between PFP and fructose 6-phosphate 1-phosphotransferase (PFK; EC 2.7.1.11) activity is influenced more by genotype than by development (Whittaker and Botha 1999, Krook *et al.* 2000). In sugarcane, differences in PFP activity also correspond to changes in the amount of the PFP-β subunit, pointing to coarse regulation (Whittaker and Botha 1999).

Fructose 2,6-bisphosphate (Fru 2,6-P_2) is a potent activator of PFP activity (Sabularse and Anderson 1981, Van Schaftingen *et al.* 1982). In general it increases the V_{max} of both the forward (glycolytic) and reverse (gluconeogenic) reactions and the enzyme's affinity for the substrates (decreases K_m) (Sabularse and Andeson 1981, Van Schaftingen *et al.* 1982, Botha *et al.* 1986, Theodorou and Plaxton 1996). In contrast, the enzymes purified from carrot taproots and ripe tomato fruits have a decreased affinity for Fru 6-P (increased K_m) in the presence of Fru 2,6-P_2 (Wong *et al.* 1988, 1990). This led the authors to speculate that these enzymes might be adapted to favour the gluconeogenic reaction in sucrose storing tissues. We have, however, recently shown that this does not hold true for sugarcane PFP, where saturating Fru 2,6-P_2

concentrations decreased the K_m of Fru 6-P more than 27 times (Chapter 3). In addition, the inverse correlation between sucrose concentrations and PFP activity in sugarcane (Whittaker and Botha 1999) is consistent with the general consensus that PFP catalyses a net glycolytic flux (Stitt 1990 and references therein).

Studies on transgenic plants, in which PFP activity was down-regulated by as much as 99%, gave contradictory evidence for the role of PFP. Transgenic potato (Hajirezaei *et al*. 1994) and tobacco (Paul *et al*. 1995, Nielsen and Stitt 2001) plants show no changes in visible phenotype and in general the metabolite concentrations and fluxes are not significantly impacted upon. Although these studies provide evidence that PFP catalyses a net glycolytic flux, they also suggest that it does not contribute to the control of this flux (Hajirezaei *et al*. 1994, Nielsen and Stitt 2001). In addition, PFP was shown to be responsible for the recycling of the triose-phosphates in non-photosynthetic tissues (Hajirezaei *et al*. 1994). These studies also show that plant tissues are able to compensate for the large decrease in PFP activity by the allosteric activation of the remaining PFP and by the activation of PFK (Hajirezaei *et al*. 1994, Paul *et al*. 1995, Nielsen and Stitt 2001). Overall, one of the most significant observations that can be made from these studies is the apparent different response of the various tissues to decreased PFP activity. Moreover, Fru 2,6-P_2 concentrations are significantly increased in metabolically active tissues, e.g. growing (sink) and sprouting (source) potato tubers (Hajirezaei *et al*. 1994) and growing (sink) tobacco leaves, particularly at the base (Nielsen and Stitt 2001), in contrast to mature tubers and leaves. In addition, although the final sucrose concentration in mature transgenic tubers is unchanged, the flux into sucrose in the growing (sink) tubers is 13 times higher when compared to that of the wild type (Hajirezaei *et al*. 1994).

Recently an unregulated form of PFP from *Giardia lamblia* was also expressed in transgenic tobacco plants, which led to a 20-fold increase in PFP activity (Wood *et al.* 2002a, 2002b). The total biomass of these transgenic lines is lower and carbon partitioning is altered in both the source and sink tissues. Although total sucrose concentrations are unaffected in the leaves (source), starch concentrations are

significantly lower (Wood *et al.* 2002a). Young seeds (sink) have reduced starch levels but higher lipid levels, which results in similar total seed carbohydrate concentrations when compared to the wild type seeds (Wood *et al.* 2002a). In addition to the increase in seed lipids the specific composition of fatty acids also changed (Wood *et al.* 2002b). Transgenic seeds also display a temporal enhancement in the growth and development of the young embryo (Wood *et al.* 2002a).

As suggested for other plant PFPs (Edwards and ap Rees 1986, Weiner *et al.* 1987, Sung *et al.* 1988) the calculated mass-action ration for PFP in sugarcane indicates that the *in vivo* reaction is close to equilibrium (Whittaker and Botha, 1997). It therefore appears that sugarcane PFP might be directly involved in the regulation of carbon-flow between sucrose synthesis and eventual accumulation on the one hand and the supply of carbon to respiration and other biosynthetic pathways on the other. To verify this potential role of PFP in sucrose metabolism in sugarcane and in particular its apparent role in sucrose accumulation, PFP activity was down-regulated in transgenic plants.

In this paper we report on the effect of the reduction of PFP activity on the sugar yields of the transgenic sugarcane lines. More than 50% of the lines analysed had a significant increase in sucrose concentrations in their immature internodes, both in greenhouse and field grown plants. Although most of the transgenic lines' mature internodes also gave higher average sucrose yields these were not significant. Field grown transgenic plants also had significantly higher fibre content. The inverse correlation between PFP activity and sucrose content also held true for the transgenic lines.

MATERIALS AND METHODS

Chemicals

The general chemicals that were used are described in Chapter 3. Antiserum for potato PFP-β was obtained from Dr NJ Kruger (Oxford, England) and has previously been described (Kruger and Dennis 1987). Antisera for *G. lamblia* and *P. freudenreichii* PFP were raised against the recombinant proteins expressed in a bacterial expression system

as described earlier (Chapter 4). The anti-rabbit IgG-alkaline phosphatase conjugate was obtained from Roche.

Plant material and sample preparation

Non-flowering sugarcane stalks were randomly selected and harvested. Wild type *Saccharum* spp. hybrid variety NCo310 plants were used as controls and transgenic lines were also generated using this variety. Both greenhouse and field grown plants of various ages were used as described for each experiment. The leaf with the youngest visible dewlap, the node it was attached to and internode just above it was defined as the number one (1) leaf, node and internode respectively. For the greenhouse grown plants the selected internodes were excised from the stalk and ground to a fine powder in liquid nitrogen after the rind was carefully removed. Prepared samples were kept frozen and stored at –80^{o}C.

Expression vectors, transformation, selection and regeneration

A partial sequence of the sugarcane PFP-β gene was used to construct both an antisense and a co-suppression expression vector for the down-regulation of PFP activity in transgenic sugarcane. In both the constructs the gene sequences were under the control of a constitutive, tandem CaMV-35S : maize *UBI-1* promoter. Commercial sugarcane variety NCo310 was used to induce callus growth in the dark from leaf roll explants on MS nutrient media containing 3 mg l^{-1} 2,4-D (Taylor *et al.* 1992, Snyman *et al*. 1996). Type 3 white embryogenic calli, consisting of dense cytoplasmic cells (Taylor *et al*. 1992), were used as targets for biolistic transformation. Co-transformation was done with the respective silencing constructs and a similar expression vector containing the *npt-II* selectable marker. Selection was done on geneticin-containing media and putative transgenic plants from independent transformation events were regenerated and hardened off based on standard protocols (Snyman *et al*. 1996).

RNA extraction and northern blot analysis

RNA was extracted according to the method of Bugos *et al.* (1995). Purified RNA was suspended in diethyl pyrocarbonate (DEPC) treated water and quantified spectrophotometrically. Fifteen µg of total RNA per sample was loaded on a 1.2% (m/v) Tris-Borate/EDTA (TBE) prepared agarose gel and developed at 100 V until the bromo phenol blue dye front migrated eight cm. The gel was trimmed and then equilibrated in 10x SSC-buffer (1.5M NaCl, 0.15M $Na_3C_6H_5O_7$ (pH 6.8)) for 20 min while the positively charged Nylon membrane was wetted in water before it was equilibrated in 10x SSC-buffer for 10 min. The RNA was transferred overnight to the Nylon membrane by downward capillary blotting using 10x SSC-buffer at room temperature. After transfer the RNA was UV cross-linked to the membrane on both sides for 1.5 min at 1200 mJ cm^{-2}. The transgene sequence was used as probe and was radioactively labelled using 25 µCi [$\propto$-^{32}P] dCTP and a random labelling kit (Amersham). Membranes were prehybridised in 15 ml RAPIDhyb™ hybridization buffer at 65°C for 30 min. The probe was boiled for 5 min, added to the hybridisation bottle and incubated overnight at 65°C. The hybridised blots were washed twice for 15 min each at 50°C and 55°C in wash solution 1 (1 x SSC, 0.1% (m/v) SDS) and twice for 15 min each at 60°C and 65°C in wash solution 2 (0.5 x SSC, 0.1% (m/v) SDS). The blots were exposed to a supersensitive Cyclone Phosphor screen (Packard) for 16 to 18 hours. Hybridisation was visualised using the Cyclone™ Storage Phosphor System (Packard Instrument Co., Inc., Meriden, USA).

Protein extraction

Crude protein extracts were prepared from approximately 500 mg of the ground tissues by stirring it for 15 min on ice in 2.5 volumes of freshly prepared extraction buffer (100 mM Tris (pH 7.2), 5 mM $MgCl_2$, 150 mM KCl, 5 mM EDTA, 5% (m/v) PEG 6000, 0.05% (v/v) IGEPAL, 2% (m/v) PVPP, 10 mM DTT and 1x Complete™ protease inhibitor cocktail (Roche)). Cell debris was precipitated by centrifugation (15 min at 10000 g) to render a clear supernatant in which enzyme activity was determined.

PFP assays

PFP activity was measured using the conditions as described by Whittaker (1997). All enzyme assays were carried out in a final volume of 250 µl at 30°C. Activity was measured by quantifying the oxidation of NADH (forward reaction) or the reduction of $NADP^+$ (reverse reaction) at 340nm using a Power $Wave_X$ microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA).

Sugar extraction and quantification

Soluble sugars were extracted in 70% (v/v) ethanol and 30% HM-buffer (100 mM HEPES [pH 7.8], 20 mM MgCl2), (1/10, m/v) with incubation at 65ºC overnight. The samples were then centrifuged at 10,000 x g, for 10 minutes at room temperature. The supernatant was dried under vacuum and resuspended in 1 ml 10% (v/v) isopropanol. For sucrose determinations all samples were diluted 10 times. Sucrose, glucose and fructose concentrations were determined spectrophotometrically using the enzymatic method described by Bergmeyer and Bernt (1974). Purity was expressed as the percentage ratio between sucrose and the total sugar pool including glucose, fructose and sucrose.

Field trial

Nine transgenic lines and a wild type control line were transplanted, using setts, to a field at the South African Sugarcane Research Institute (SASRI), KwaZulu-Natal, South Africa. Each line was grown in a single 8 m row, representing eight individual stools approximately 1 m from each other. Row spacing was 1.5 m. The plant crop was harvested after approximately 15 months in the winter (July). Three independent samples of 1.5 to 2 kg each were prepared for each line by the random harvesting of whole stalks. Excess leaf material and the tops, i.e. the 3-5 youngest internodes of the cane, were removed as is customary during commercial harvesting. Sample preparation and analysis was done in a research mill using standard, industry-scale procedures (Anonymous 1977). Data collected included the total soluble sugar yield (Brix % DW and Brix % cane, i.e. FW), sucrose yield (Pol % cane), purity (%) and fibre content

(Fibre % cane) as is standard for the sugar industry. After the first harvest the sugarcane was allowed to ratoon and was again harvested after 16 months during early summer (November). Although the harvesting was done as before, on this occasion the stalks were divided approximately in half to yield a top and a bottom half that were analysed separately. At this age the cane consisted of approximately 34 harvestable internodes. The top half therefore contained immature, maturing as well as mature internodes (approximately internodes 4 to 22) and the bottom half only mature internodes (approximately internodes 23 to 39).

RESULTS AND DISCUSSION

Molecular characterisation of transgenic plants

The presence of the transgenes in the regenerated sugarcane plants was confirmed by PCR analyses during the tissue culture stage. Confirmed transgenic lines, from which a clear transgene fragment was amplified, were hardened off and grown under greenhouse conditions. No obvious phenotypic differences were apparent between the wild type control plants and the transgenic lines. Transgene expression was confirmed by northern blot analyses in approximately 10 month old cane (Figure 1). Because only 53% of the sugarcane PFP-β gene was used to construct the expression vectors it was easy to differentiate between the endogenous messenger (2.3 kb) and the transgene messengers (1.2 kb). The northern blot analyses did not only confirm the expression of the transgenes in the different transgenic lines but also within the different tissues that were analysed, i.e. leaf roll, immature and maturing internodes. In addition, a reduction of the relative amount of the endogenous transcript was evident in the transgenic tissues. Only lines in which the expression of the transgene was confirmed were used in subsequent analyses. Lines transformed with an untranslatable form of the PFP-β were enumerated with a clone number alone, e.g. 501-508, and lines transformed with an antisense construct were enumerated with a Q and a clone number, e.g. Q3.

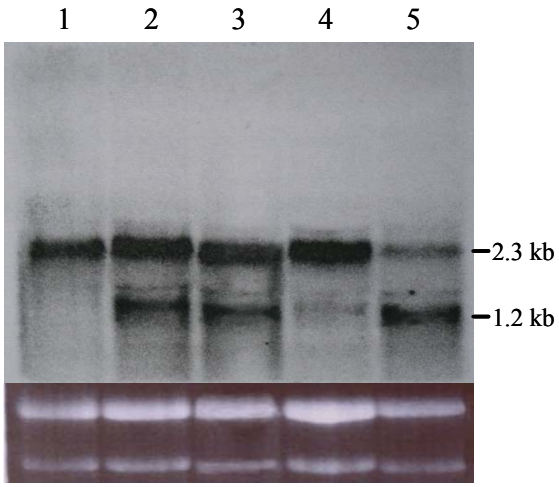

Figure 1. Northern blot analysis confirming the expression of the PFP-β transgene. Lanes (1) NCo310 (wild type control) leaf roll sample, (2) line 503 leaf roll sample, (3-5) line 502 leaf roll, internodes 3 and 5 samples respectively. The endogenous transcript is 2.3 kb while the untranslatable transgenic transcript is only 1.2 kb. The bottom gel represents the ethidium bromide stained ribosomal subunits that were used to verify equal loading.

Reduction in PFP activity

Expression of the antisense or untranslatable forms of the PFP-β gene resulted in a significant reduction in PFP activity in internodal tissues (Figure 2). The extent to which PFP activity was reduced in the internodal tissues of the transgenic plants was depended on the developmental stage / maturity of the internode. In very young, immature internodes, i.e. internodes 2-4 that had high PFP activity in the wild type, the activity was reduced by up to 70%. In mature internodes, i.e. internodes ≥10 that have low PFP activity in the wild type, activity was reduced to undetectable levels (Figure 3). This pattern of reduced activity, i.e. more in mature than immature tissues, is similar to that reported for tobacco sink (immature tissue) and source (mature tissue) leaves which were engineered in the same way (Paul *et al.* 1995).

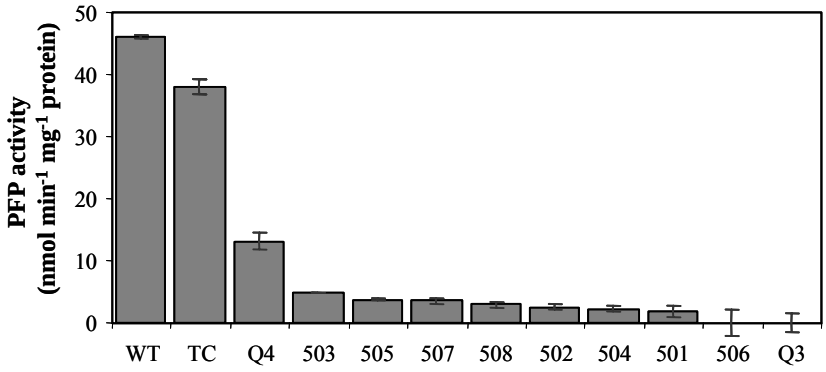

Figure 2. PFP activity in maturing internodal tissue, i.e. pooled internode 9 and 10 tissue of the wild type (WT), an untransformed tissue culture control (TC) and the various transgenic genotypes. Three replicate samples were prepared for each line.

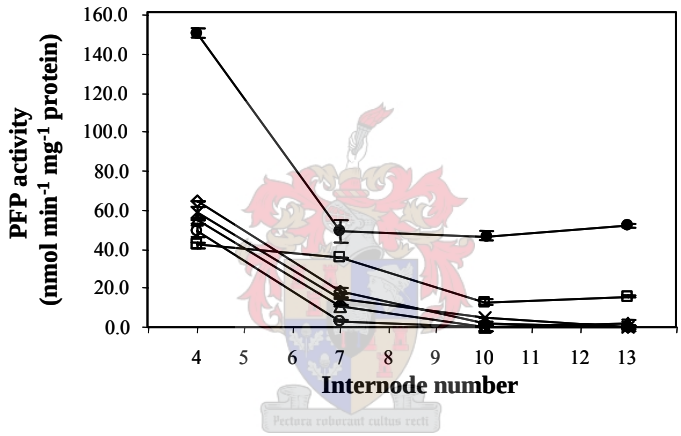

Figure 3. PFP activity in different internodal tissues of wild type (-●-) and five representative transgenic genotypes: 501 (-◇-), 503 (-x-), 506 (-○-), Q3 (-△-) and Q4 (-□-). The values reported are the means of three separate samples.

The reduction in PFP activity was accompanied by a similar decrease in the amount of PFP-β protein as determined by protein blotting (data not shown). This concomitant reduction in the amount of PFP-β protein and extractable PFP activity is consistent with the work in potato (Hajirezaei *et al.* 1994) and tobacco (Paul *et al.* 1995) in which antisense and co-suppression was also used to induce gene silencing. In combination, this data suggest that the efficiency of antisense and co-suppression technology is limited in tissues that express the endogenous gene at high levels. More recent advances in RNA silencing (RNAi) technology (see Watson *et al.* 2005 for a review) could therefore aid in down-regulating PFP activity more efficiently in young, metabolically active tissues.

Influence of reduced PFP activity on sugar yields in greenhouse grown plants

To investigate the potential role of PFP in sucrose accumulation, sugars were extracted from internodal tissue of greenhouse grown control, i.e. wild type NCo310, an untransformed tissue culture control and transgenic plants and were quantified. Tissues from internodes 3-5 were combined to represent immature internodes and that of internode 12-14 to represent mature tissue. Sugar concentrations were variable when expressed on a fresh weight basis and in comparison to the wild type the immature tissue of the untransformed, tissue culture control had significantly higher hexose and sucrose concentrations, which confounded the interpretation of these data (Figure 4).

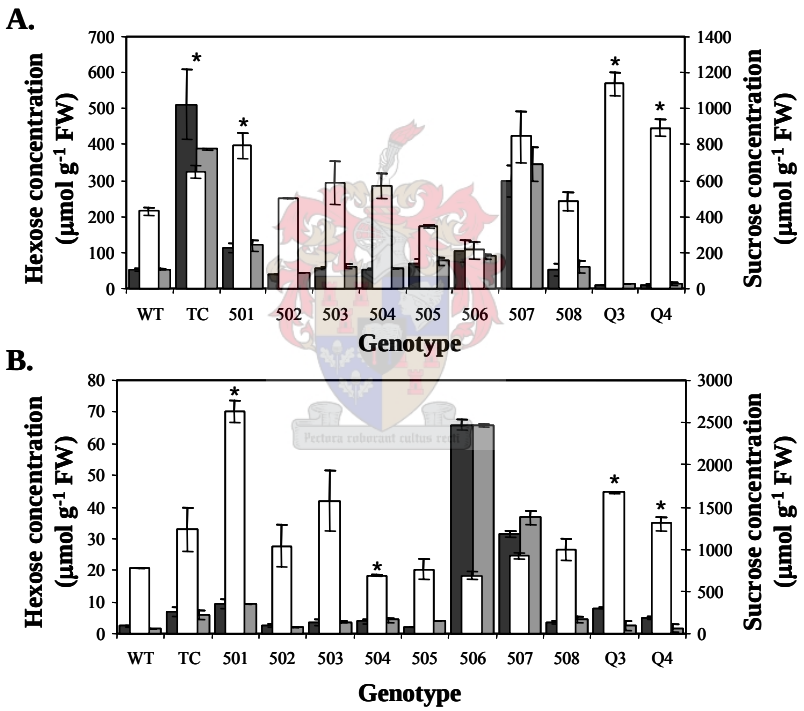

Figure 4. Sugar concentrations in internode 3-5 (A) and 12-14 (B) tissue expressed as a function of fresh weight. Glucose (-■-), fructose (-■-) and sucrose (-□-) concentrations were determined in triplicate samples; * indicates sucrose concentrations significantly ($p < 0.05$) different from that of the wild type.

The transgenic lines 506 and 507 had higher hexose concentrations in both the immature and mature tissues, resulting in a significant ($p < 0.05$) reduction in purity, i.e. sucrose concentration expressed as a percentage of the total sugar concentration, in both the tissue types of these lines. In contrast, Q3 and Q4 had significantly lower hexose concentrations in their immature tissues. Three transgenic lines, i.e. 501, Q3 and Q4,

had significantly higher sucrose concentrations in both their immature and mature tissues (Figure 4), which translated into a significant increase in purity in the immature tissues for the latter two lines. The variability in the fresh weight data makes the comparison and interpretation of these results impossible. The most important factor contributing to this variability is probably the watering regime used in the greenhouse during the time of harvest. Expressing the data on a dry weight basis might therefore allow a more standardised comparison.

In doing so, significant changes were evident in both the hexose and sucrose concentrations in the immature tissues of most of the transgenic lines, while very few significant differences were apparent in the mature tissues (Figure 5). These induced changes were, however, not consistent, e.g. while most lines had unchanged or increased hexose concentrations the two antisense lines, Q3 and Q4, had significantly lower concentrations than the control lines. Similarly, lines 506 and 507 were the only lines showing an increase in hexose concentrations in mature tissue. The variability could be ascribed to factors as widely different as clonal/transformation effects (undefined) to the physiological state of the cane at harvest (e.g. induced ripening could lead to low hexose concentrations and *vise versa*) and compartmentalisation effects (e.g. a large percentage of the sugars are localised in the vacuole and could therefore be influenced by other enzyme activities not localised in the same compartment as PFP).

Although the glucose vs. fructose levels were similar in all the lines and tissue types, the ratios between the hexose and sucrose concentrations were highly variable, especially in the immature tissues (Figure 5A). Lines with the lowest sucrose concentrations had the highest hexose concentrations and *vice versa*. Fifty percent of the transgenic lines had a significantly higher sucrose concentration than the control in the immature tissues, which translated into a significant ($p < 0.05$) increase in purity in these lines. In contrast, the only significant change in the sucrose concentrations of the mature tissues was a decrease in that of line Q3 (Figure 5B). In addition, two lines, i.e. 506 and 507, also showed a significant increase in hexose concentrations and consequently, only these two lines displayed a significant decrease in purity. The only changes that were

consistent between the fresh and dry weight data were the increase in sucrose concentrations in the immature tissues of lines Q3 and Q4 and the high hexose concentrations in lines 506 and 507.

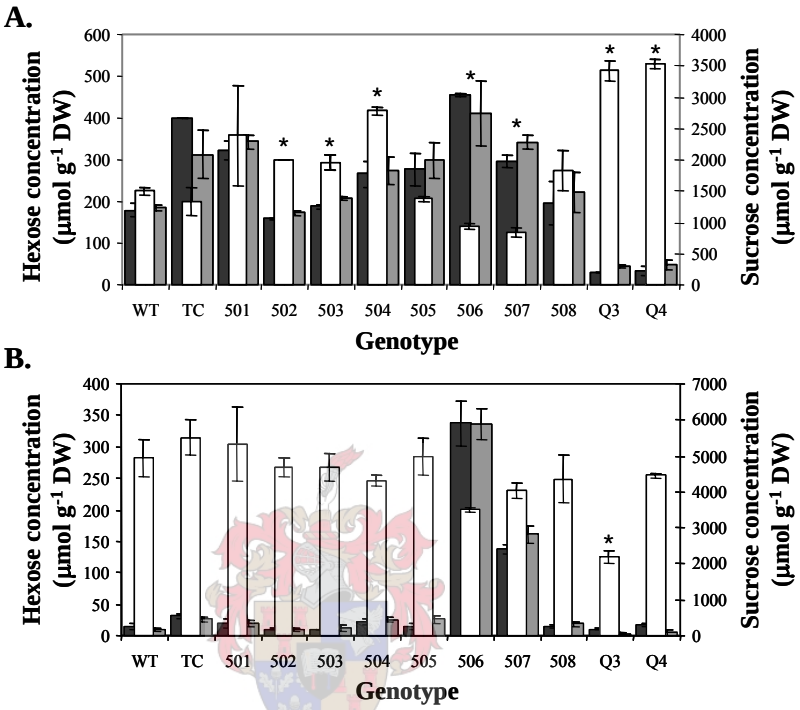

Figure 5. Sugar concentrations in internode 3-5 (A) and 12-14 (B) tissue expressed as a function of dry weight. Glucose (-■-), fructose (-■-) and sucrose (-□-) concentrations were determined in triplicate samples; * indicates sucrose concentrations significantly ($p < 0.05$) different from that of the wild type.

Correlation between PFP activity and sucrose yields

PFP activity and sucrose concentrations in immature and mature tissues from a representative number of transgenic lines were correlated. Significant ($p < 0.05$) inverse correlations were evident from the results when sucrose concentrations were expressed on a fresh weight and protein basis (Figure 6A and C). This inverse correlation between PFP activity and sucrose yields in the transgenic clones is consistent with that reported in commercial varieties and a segregating F1 population (Whittaker and Botha 1999), further supporting the apparent role for PFP in the sucrose accumulation phenotype in sugarcane.

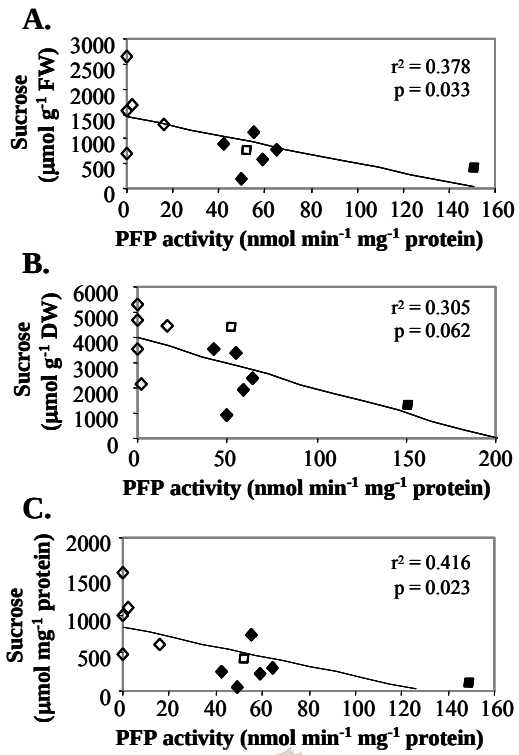

Figure 6. The relationship between sucrose yields and PFP activity in transgenic and control sugarcane lines. Solid and hollow symbols represent internode 3-5 and 12-14 tissues respectively and the wild type is represented by a square.

Influence of reduced PFP activity on sugar yields in field grown plants

To further investigate the influence of reduced PFP activity on sucrose yields, sets of the greenhouse grown plants were planted in the field and analysed as described in the materials and methods section. When the plant crop was harvested after approximately 15 months and the stalks were analysed as a whole no significant differences were evident (data not shown). The plants were therefore allowed to ratoon and were again harvested after 16 months. Based on the apparent differences between immature and mature tissues of the greenhouse grown plants, the stalk samples were divided into a top and a bottom half and analysed separately.

All the transgenic lines except 507 showed a significant decrease in total sugar yield in both the top and bottom halves of the stalks (Figure 7A). This was true when total sugar yield was expressed on a dry weight as well as a fresh weight basis. Moreover, the relative differences between the various tissues and lines were exactly the same when expressed either on a dry weight or a fresh weight basis. In other words, in contrast to

the greenhouse data, there were no differences between the fresh and dry weight data of the field trial. This could possibly be attributed to the fact that all the plants were well established at the time of the first ratoon and that they were all exposed to exactly the same growing conditions.

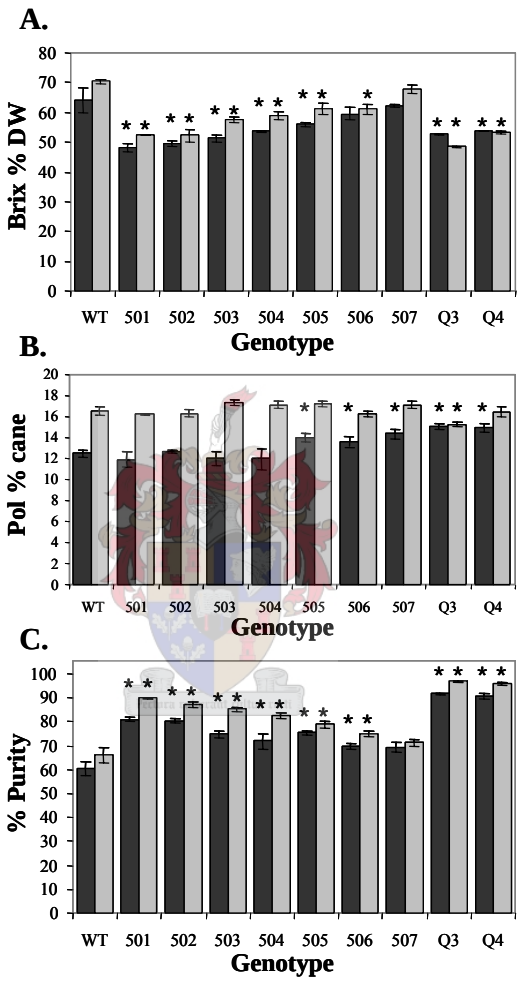

Figure 7. Sugar data for field grown wild type (WT) and transgenic sugarcane lines with reduced PFP activity. The harvested cane was divided into the top (-■-) and bottom (-□-) halves and analysed for (A) Brix % DW, (B) Pol % cane and (C) % purity. The data was generated from triplicate samples; * indicates measurements significantly ($p < 0.05$) different from that of the respective tissues of the wild type.

In contrast to the total sugar yield, sucrose yields expressed on a fresh weight basis (Pol % cane) significantly increased only in the top halves of five of the transgenic lines (Figure 7B). Although the bottom half of several lines, i.e. 503, 504, 505 and 507, had up to 5% higher average sucrose yields than the wild type, this was not significant. In

fact, the only significant difference in sucrose yields in the bottom half of the stalks was a slight decrease in yield for line Q3 (Figure 7B). Although all except one transgenic line had a higher average sucrose yield than the wild type when calculated for the whole stalk, these increases were not significant (data not shown). The increase in sucrose yields in immature / maturing internodes was therefore consistent between the greenhouse and field grown material. Although the increase seemed less pronounced in the field grown tissue; a maximum of 20% vs. 130% in the greenhouse, this was expected because the field grown material represented the average of more mature tissue, i.e. approximately internode 4-22, while the greenhouse immature samples only represented the very immature internodes 3-5. In addition, as described in the materials and methods section, the greenhouse samples represented only core tissue, predominantly storage parenchyma cells, while the field samples also included the rind and nodal tissue which do not store sucrose at high concentrations.

Similar to the total sugar data the purity of all the transgenic lines, bar 507, for both tissue types changed significantly, but in contrast to the total sugar content the purity increased (Figure 7C). Moreover, in all cases the decrease in total sugar yield correlated with a similar increase in purity. In combination with the constant or slight increase in sucrose concentrations it therefore suggests that hexose concentrations were significantly reduced in most transgenic lines. A reduction in hexose concentrations can be attributed to a reduction in the total sucrose breakdown activity or an increased flux out of the hexose pool or a combination thereof. Furthermore, if these changes can be ascribed to the reduction in PFP activity it either implies that it is the cytosolic hexose concentrations that are influenced or that the cytosolic and vacuolar hexose pools are in a dynamic equilibrium. The consistency of the data between the immature and mature tissues also indirectly supports this conclusion because the increased contribution of the vacuolar constituents should influence the changes non-symmetrically as the tissues mature.

Reduction in PFP activity leads to an increase in the hexose phosphate pool in sink tissues (Hajirezaei *et al.* 1994, Paul *et al.* 1995), which could lead to a decrease in the

rate of sucrose breakdown (Geigenberger *et al.* 1994, Hajirezaei *et al.* 1994). Under these conditions sucrose synthesis by sucrose phosphate synthase (SPS) will also be stimulated because of the increased levels of glucose 6-phosphate (Glc 6-P), a strong allosteric activator of SPS (Reimholz *et al.* 1994), and SPS's substrates (Dancer *et al.* 1990, Hajirezaei *et al.* 1994). Hajirezaei *et al.* (1994) also showed that a decrease in PFP activity leads to an increased flux of glucose into sucrose; although the hexose concentrations in these plants are highly variable in all the tissue types that were analysed. The validity of these arguments for the sugarcane plants with reduced PFP activity can only be verified by determining the actual flux into sucrose.

Fibre content was also significantly increased in both the tissue types in most of the transgenic lines (Figure 8). The fibre data mirrored the purity data, which also means that the differences, relative to the wild type, correlated inversely to those observed for the total sugars (Brix %, Figure 7A). In other words, a large decrease in total sugar content relative to the wild type, correlated with a large increase in the fibre content and a small decrease in sugar with a small increase in fibre. Again, this could be explained by an increase in the hexose phosphate pool, which could lead to an increase in the cell wall precursors *via* UDP-glucose. UDP-glucose and the hexose phosphate pool are connected by a series of highly active, reversible reactions, which should ensure equilibrium between these metabolites (ap Rees 1988, Tobias *et al.* 1992). Because the millroom data are expressed as percentages the possibility that the said reduction in total sugar yield is due to a corresponding increase in fibre content cannot be excluded and should be further investigated.

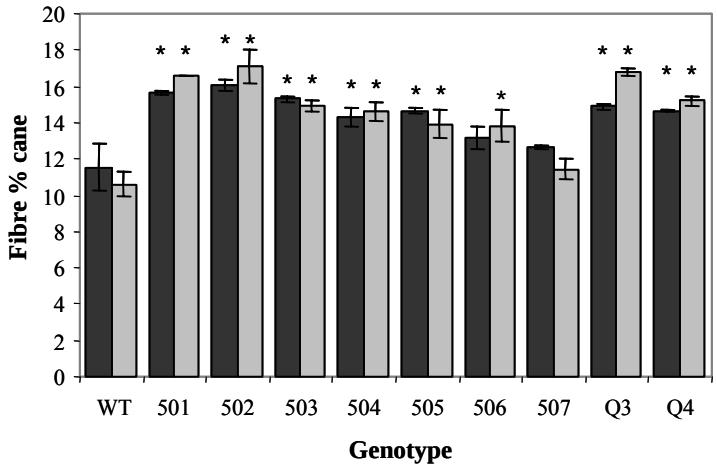

Figure 8. Fibre content of field grown wild type (WT) and transgenic lines with reduced PFP activity. The harvested cane was divided into the top (-■-) and bottom (-■-) halves, analysed for fibre content and expressed on a fresh weight basis (% cane). The data was generated from triplicate samples; * indicates measurements significantly ($p < 0.05$) different from that of the respective tissues of the wild type.

Cumulatively the field data confirms that reduced PFP activity influences sugar metabolism in sugarcane internodal tissues. Similar to the greenhouse grown plants, the effect on sucrose accumulation was the greatest in the more immature, metabolically active tissues even though PFP activity was only down-regulated to 30% of that of the wild type in these tissues. This apparent difference with which the different tissues respond to decreased PFP activity is similar to that reported for growing (sink) and sprouting (source) potato tubers (Hajirezaei *et al*. 1994) and growing (sink) tobacco leaves (Nielsen and Stitt 2001) in contrast to mature tubers and leaves. The determining parameter therefore seems not to be the absolute change in PFP activity in a particular tissue but rather the relative contribution PFP makes to the glycolytic flux in that tissue.

In immature internodal tissues the reduction in PFP activity was sufficient to impede on the high requirement for respiratory flux, probably resulting in the accumulation of the hexose phosphates as observed in similar studies (Hajirezaei *et al*. 1994, Paul *et al.* 1995). Elevated hexose phosphate levels in turn could result in increased sucrose synthesis and storage as well as elevated cell wall synthesis as discussed above. As the internodes mature and the demand for respiratory flux decreases (Bindon and Botha 2002) the residual PFP activity and the alternative reaction catalysed by PFK is sufficient and the system returns to equilibrium, ending with sugar levels comparable to that of the unimpeded system. It is important to note that although sucrose, a part of

dynamic carbon metabolism, returned to equilibrium in the older internodes the amount of fibre in the mature internodes were still significantly higher in most transgenic lines – probably because most of the carbon is fixed during the developmental stage most impeded upon by the reduced levels of PFP. The data generated with the transgenic sugarcane lines, similar to the studies in potato and tobacco (Hajirezaei *et al*. 1994, Paul *et al.* 1995), supports the suggested role of PFP as a bypass to PFK at times of high metabolic flux in biosynthetically active tissue as suggested by Dennis and Greyson (1987).

To confirm these speculative statements the net glycolytic fluxes and detailed metabolic analyses should be performed on these transgenic lines. In addition, determining the extent of the triose-hexose phosphate cycle, especially in mature internodes, could shed some light on the presence of FBPase in non-photosynthetic tissues. Finally, the more efficient down-regulation of PFP activity, particularly in immature tissues, should be attempted using RNAi technology. Al these aspects are currently under investigation in our laboratory.

CONCLUSION

The down-regulation of PFP activity in sugarcane confirmed that it is inversely correlated to the sucrose accumulation phenotype, but more specifically only in metabolically active, immature tissues. Moreover, it suggests that PFP plays a significant role in glycolytic carbon flux in immature, metabolically active sugarcane internodal tissues. The data presented here confirm that PFP can indeed have an influence on the rate of glycolysis and carbon partitioning in these tissues

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CHAPTER 6

General discussion and conclusions

The main aim of the work presented in this thesis was to elucidate the apparent role of pyrophosphate fructose 6-phosphate 1-phosphotransferase (PFP) in sucrose accumulation in sugarcane. PFP activity in sugarcane internodal tissue is inversely correlated to the sucrose content and positively to the water-insoluble component across varieties which differ in their capacities to accumulate sucrose (Whittaker and Botha 1999). This apparent well defined and important role of PFP seems to stand in contrast to the ambiguity regarding PFP's role in the general literature as well as the results of various transgenic studies where neither the down-regulation (Hajirezaei *et al*. 1994, Paul *et al*. 1995, Nielsen and Stitt 2001) nor the over-expression (Wood *et al*. 2002a, 2002b) of PFP activity had a major influence on the phenotype of the transgenic plants. Based on this it was therefore thought that either the kinetic properties of sugarcane PFP are significantly different than that of other plant PFPs or that PFP's role in sucrose accumulating tissues is different from that in starch accumulating tissues – clearly an issue that needed resolution.

If there is indeed a negative correlation between PFP expression and sucrose levels in sugarcane, two different probable explanations for this can be offered based on current literature. Firstly, it could be a direct consequence of a reduction in glycolytic carbon flow through the PFP-catalysed reaction and the subsequent alteration in carbon partitioning. Moreover, if the PFP-catalysed reaction is rate limiting in sucrose accumulating tissues, decreased activity should result in an increase in the hexose-phosphate pool, which could lead to increased sucrose synthesis and subsequent storage. This model is consistent with the general consensus that PFP catalyses a net glycolytic flux (see Stitt 1990 for review), which is also true for sugarcane (Whittaker and Botha 1999). Secondly, the effect might be linked to the presence of a H^+-sucrose antiport system in the tonoplast, which could be more effectively energised if the activity of a H^+-translocating vacuolar pyrophosphatase (VPPase) is enhanced. This could happen in

an environment where low PFP activity translates into the reduced utilisation of PPi, but because PPi concentrations need to be finely regulated (Weiner *et al.* 1987, Takeshige and Tazawa 1989) at least some of this burden could be transferred to VPPase, resulting in the enhanced energisation of the tonoplast. This could be of particular importance in metabolically active tissues where many biosynthetic reactions produce PPi as a by-product and their continuation is dependent on the effective removal of the PPi.

Reduced glycolytic carbon flow through the PFP-catalysed reaction could be the result of reduced total activity or adapted kinetic characteristics, which could for example favour the gluconeogenic reaction and in doing so, increase the concentrations of the precursors for sucrose synthesis as suggested by Wong *et al*. (1988, 1990) for other sugar storing tissues. In addition, albeit indirect, support for the apparent importance of the gluconeogenic reaction in sucrose storing tissues is also provided by the unique characteristics of grapefruit FBPase. Grapefruit juice sac FBPase has a much higher (up to 20x) affinity for Fru 1,6-P_2 than other plant FBPases, which might be an adaptation for sucrose biosynthesis in sink tissues (Van Praag 1997). However, we have shown in Chapter 3 that sugarcane PFP's molecular and kinetic properties do not differ significantly from that of other plant PFPs in a way that clearly suggests a direct role for it in the accumulation of sucrose. If the apparent link between PFP activity and sucrose accumulation is based on flux through this reaction it is therefore probably based on the total amount of catalytic activity present in the tissue rather than the fine regulation thereof. This conclusion is also consistent with the coarse regulation of activity in these tissues as illustrated by Whittaker and Botha (1999) as well as the suggestion that the total amount of PFP activity in sugarcane is influenced more by genotype than developmental and/or fine regulatory factors (Chapter 3); analogous to sucrose storing carrot suspension cells (Krook *et al.* 2000). If this is indeed the case it suggests great promise for the transgenic down-regulation of PFP activity in sugarcane.

PFP activity was successfully down-regulated in transgenic sugarcane (Chapter 5). The degree to which PFP activity was reduced varied between different tissue types, depending on the basal endogenous activity and overall metabolic activity in the

particular tissue. These findings are similar to that reported for tobacco sink (immature tissue) and source (mature tissue) leaves which were engineered in the same way (Paul *et al.* 1995). In general sugar metabolism was impeded upon most in young, metabolically active internodal tissues resulting in an "early ripening" phenotype, i.e. increased sucrose concentrations and higher purity in immature internodes. No consistent differences were apparent in the sugar concentrations of the mature internodes. This was true even though, if expressed as percentage reduction in activity, PFP activity was down-regulated to a lesser extent in the immature than in the mature internodes.

The influence of PFP on the metabolism of these tissues therefore seems to depend on the total catalytic activity (glycolytic flux) required and PFP's relative contribution to this flux rather than an exclusive function for PFP in sucrose accumulation. Another way of interpreting this is that the influence of reduced PFP activity is of a transient nature, i.e. as the effected tissues mature equilibrium is restored, even in the apparent absence of PFP activity (Figure 3, Chapter 5). As the transgenic cells mature and the required glycolytic flux decreases to levels which can be sustained by PFK and the residual PFP, the equilibrium of the glycolytic intermediates, including the stored sucrose, is restored. PFP therefore does not influence the overall ability of the sugarcane cells to accumulate sucrose but can influence the rate at which it happens in tissues with a high glycolytic flux. This explains why sucrose yields were significantly increased in immature internodes but not in mature internodes.

Commercial sugarcane plants are up to three meters tall, consisting of approximately 40 internodes, and are grown over 12 to 24 months. The proportion of immature tissue to mature tissue in the stalk at the time of harvest is small (less than 5% immature tissue). The dynamic metabolite pools in the bulk of the transgenic tissues therefore have ample opportunity to return to equilibrium, resulting in total sucrose yields similar to that of the wild type. Notwithstanding, as has been illustrated by the use of commercial ripeners this "early ripening" phenotype could contribute to the overall productivity of the crop if the right varieties are targeted. In addition, in the light of the very

discouraging yield data of a field trial with transgenic sugarcane in Australia, where 98% of the lines had a reduced sucrose content (Vickers *et al.* 2005), the data presented here are indeed promising.

All the above mentioned arguments agree with the original data that suggested a role for PFP in sucrose accumulation (Whittaker and Botha 1999). Moreover, the fact that these data were also obtained from metabolically active tissues (internode seven) with a high sucrose accumulation rate fits well with the findings of this study. Considering the above mentioned ability of the sucrose pool to return to equilibrium over time, it implies that PFP does not play a role in determining the maximum sucrose load in mature tissues.

As discussed in Chapter 5, a reduction in the glycolytic carbon flow due to low PFP activity should lead to increased levels of hexose phosphate pool, including UDP-glucose and consequently increased cell wall/cellulose synthesis (Fig. 1, Chapter 1) (ap Rees 1988, Tobias *et al.* 1992). In the transgenic plants the most active stage of cell wall synthesis therefore coincides with the stage most impeded upon by the reduced levels of PFP, resulting in tissues with significantly higher fibre than the wild type (Fig. 7, Chapter 5). In contrast to the glycolytic intermediates the carbon fixed during cell wall synthesis, which will form part of the water-insoluble component, can not be remobilised and any quantitative changes should therefore remain in the mature internodes, resulting in an increase in fibre content.

It is interesting to note that the water-insoluble component is positively correlated to PFP activity across commercial varieties (Whittaker and Botha 1999), whereas reduced PFP activity in the transgenic plants had a similar effect (Fig. 7, Chapter 5). The reason for this apparent discrepancy probably lies in the diverse composition of the water-insoluble component. One plausible explanation is that in tissues with high PFP activity it contributes to an increased total respiratory flux which could translate into a water-insoluble component with increased protein content (Bindon and Botha 2002). In the transgenic plants, on the other hand, reduced PFP activity results in an increase in the

water-insoluble component by increasing cellulose synthesis as explained above. PFP could also influence the insoluble component through its effect on fatty acid metabolism. PFP is for example implicated in the inability of *wrinkled1* mutant *Arabidopsis* seeds to accumulate triacylglycerol (Focks and Benning 1998) and also influences lipid content and the onset of lipid deposition in transgenic tobacco seeds (Wood *et al.* 2002a, 2002b).

The most obvious priority for future work is the comprehensive metabolic analysis of the transgenic plants. Potential changes in the levels of metabolites directly associated with PFP activity should be determined to confirm for example the reduction in glycolytic flux. Other aspects that need to be investigated include: (i) Determining the extent to which the triose-phosphate : hexose-phosphate cycle was influenced. (ii) Quantifying the possible change in total respiration. (iii) Qualifying potential changes in the water-insoluble component. Research priorities aimed at commercial application could include the more efficient reduction of PFP activity in the immature tissues using RNAi and the down-regulation of both PFP and UDPGlc-DH activity in transgenic plants.

With respect to the proposed second hypothesis in Chapter 2 the potential influence of reduced PFP activity on PPi metabolism should be investigated, with special emphasis on possible changes in VPPase activity in the transgenic plants. This line of investigation should be a priority because of the important insight gained in this study into the dynamic nature of the sucrose pool, i.e. its ability to return to equilibrium over time. Enhanced sucrose transport out of the metabolically active compartment into the vacuole might therefore represent a viable system to increase the maximum sucrose content in sugarcane tissues. This could also be interpreted as a terminal reaction for sucrose accumulation, which in general is a good target for manipulating the flux through a particular metabolic network (see Kinney 1998 for a review).

In conclusion, this study confirmed the negative correlation between PFP activity and sucrose content of immature sugarcane internodal tissue. Moreover, it suggests that PFP

plays a significant role in glycolytic carbon flux in immature, metabolically active sugarcane internodal tissues. The data presented here confirm that PFP can indeed have an influence on the rate of glycolysis and carbon partitioning in these tissues. Although this seems to be in disagreement with the general conclusions of the first transgenic studies that were published (Hajirezaei *et al.* 1994, Paul *et al.* 1995) the data on immature tissues in these papers and the subsequent work (Nielsen and Stitt 2001, Wood *et al.* 2002a) support this conclusion. It therefore also implies that there are no differences between the functions of PFP in starch and sucrose storing tissues and it supports the hypothesis that PFP provides additional capacity to PFK at times of high metabolic flux in biosynthetically active tissue (Dennis and Greyson 1987).

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