PYROPHOSPHATE DEPENDENT PHOSPHOFRUCTOKINASE (PFP) ACTIVITY AND OTHER ASPECTS OF SUCROSE METABOLISM IN SUGARCANE INTERNODAL TISSUES

by

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PREFACE

The experimental work described in this thesis was supervised by Professor FC Botha and conducted in the Biotechnology Department at the South African Sugar Association Experiment Station (SASEX), and the Department of Biology, University of Natal.

The results presented are original and have not been submitted in any form to another University. Where use was made of the work of others, it is duly acknowledged in the text.

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ABSTRACT

The biochemical basis for the regulation of sucrose accumulation is not fully understood. The present study was thus aimed at investigating aspects of 'coarse' (enzyme activity) and 'fine' (metabolite) control of glycolytic enzyme activity in relation to carbon partitioning in the developing stalk (internodes 3 to 10), and between varieties with significant differences in sucrose content. Particular emphasis was placed on studying pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90), since this enzyme has been implicated in sucrose metabolism in other plant species.

Within the developing stalk, internodal maturation was associated with a redirection carbon from the insoluble matter and total respiration (CO_2 production and biosynthesis) to sucrose storage. Between varieties, with significant variation in sucrose content, there was an inverse relationship between hexose monophosphate partitioning into respiration and sucrose. The reduction in carbon flux to respiration was not associated with a decline in the extractable specific activity of PK, PFK and PFP. There was also no alteration in the regulation of PK, PFK and FBPase by change in the mass action ratios. Hexose monophosphate concentration declined approximately two to three-fold from internodes 3 to 9 and Fru-6-P concentration was within the lower Km or $S_{0.5}$ range (Fru-6-P) of PFP and PFK, respectively (as reported from the literature). Within the developing stalk, substrate limitation might have contributed to the decline in carbon partitioning to respiration.

In sugarcane, the levels of PFP activity were controlled in part by PFP protein expression. Sugarcane PFP polypeptide(s) are resolved as a single protein with a molecular mass of approximately 72 kD. PFP catalysed a reaction close to equilibrium in all internodes investigated, and the concentration of Fru-2,6-P_2 was shown to be in excess of the requirement to stimulate PFP activity. Carbon flux from the triose-P to hexose monophosphate pool was apparent in sugarcane, suggesting that PFP activity

was functional *in vivo.* The developmental profile of specific PFP activity was not positively correlated to the increasing rate of sucrose accumulation in the top ten internodes of the developing stalk. Between different sugarcane varieties, specific PFP activity was shown to be inversely correlated to sucrose content.

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CHAPTER 6

ASPECTS OF THE REGULATION OF SUCROSE METABOLISM IN

<u>LIST OF ABBREVIATIONS</u>

A	:	absorbance
Acetyl-CoA	:	acetyl-coenzyme A
ADP	:	adenosine 5'-diphosphate
AMP	:	adenosine 5'-monophosphate
APMSF	:	(4-amidinophenyl)-methanesulfonyl fluoride
APS	:	ammonium persulphate
ATP	:	adenosine 5'-triphosphate
Bq	:	Becquerel
BSA	:	bovine serum albumin
CAM	:	crassulacean acid metabolism
D	:	Dalton
1,3-DPGA	:	1,3-diphosphoglycerate
DTT	:	1,4-dithiothreitol
E-64	:	N-[N-(L-3-trans-carboxirane-2-carbonyl)-L-Leucyl]-agmatine
ECL	:	enhanced chemiluminescence
EDTA	:	ethylenediaminetetraacetic acid
FBPase	:	fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase), EC 3.1.3.11
$FBPase_c$	:	cytosolic FBPase
Fru	:	D-fructose
$Fru-1,6-P_2$	:	D-fructose-1,6-bisphosphate
$Fru-2,6-P_2$	:	D-fructose-2,6-bisphosphate
Fru-6-P	:	D-fructose-6-phosphate
Glc	:	D-glucose
Glc-1-P	:	D-glucose-1-phosphate
Glc-6-P	:	D-glucose-6-phosphate
glycerol-3-P	:	glycerol-3-phosphate
Hepes	:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HK	:	hexokinase (ATP: D-hexose-6-phosphotransferase), EC 2.7.1.1
HPI	:	hexose phosphate isomerase (D-hexose-6-phosphate-ketol isomerase), EC 5.3.1.9
HPLC	:	high performance liquid chromatography
IgG	:	immunoglobulin G
$I_{0.5}$	:	inhibitor concentration producing 50 % inhibition of enzyme activity
IU	:	international enzyme unit (one micromole substrate transformed per minute)
Ka	:	concentration of effector that produces half maximal activation
Keq	:	equilibrium constant
Ki	:	competitive inhibition constant
Km	:	concentration of substrate that produces half maximal activation
LDH	:	lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase), EC 1.1.1.27
LSD	:	least significant difference
MES	:	(2 [N-morpholino] ethanesulfonic acid)
NAD	:	oxidised nicotinamide-adenine dinucleotide (NAD^+)
NADH	:	reduced nicotinamide-adenine dinucleotide ($NADH + H^+$)
NADP	:	oxidised nicotinamide-adenine phosphate dinucleotide ($NADP^+$)
NADPH	:	reduced nicotinamide-adenine phosphate dinucleotide ($NADPH + H^+$)
ND	:	not determined
ns	:	not significant
OAA	:	oxaloacetate

OPP	:	oxidative pentose phosphate
PAGE	:	polyacrylamide gel electrophoresis
PEP	:	phosphoenolpyruvate
PEPCase	:	phosphoenolpyruvate carboxylase (phosphorylating orthophosphate: oxaloacetate carboxylase), EC 4.1.1.31
PFK	:	6-phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase), EC 2.7.1.11
PFK_c	:	cytosolic PFK
PFK_p	:	plastid PFK
PFP	:	pyrophosphate dependent phosphofructokinase (pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase), EC 2.7.1.90
$PFP_{(total)}$	:	PFP containing both the alpha and beta subunits
PGA	:	phosphoglycerate
PGM	:	phosphoglucomutase (D-glucose-1,6-bisphosphate: α D-glucose-1-phosphate phosphotransferase), EC 2.7.5.1
Pi	:	inorganic phosphate
PK	:	pyruvate kinase (ATP: pyruvate 2-O-phosphotransferase), EC 2.7.1.40
PK_c	:	cytosolic PK
PK_p	:	plastid PK
PMSF	:	phenylmethylsulfonyl fluoride
Ponceau-S	:	3-hydroxy-4-[2-sulfo 4-(4 sulfophenylazo) phenylazo]-2,7-naphthalenedisulfonic acid
PPi	:	inorganic pyrophosphate
rpm	:	revolutions per minute
SD	:	standard deviation
SDS	:	sodium dodecyl phosphate
$S_{0.5}$	:	concentration of substrate needed for half maximum catalytic activity

SPS	:	sucrose phosphate synthase (UDP-D-glucose: D-fructose-6-phosphate 2-α-D-glucosyltransferase), EC 2.4.1.14
Suc	:	sucrose
SuSy	:	sucrose synthase (UDP-D-glucose: D-fructose 2-α-D-glucosyltransferase), EC 2.4.1.13
TBS		tris-buffered saline
TBST	:	tris-buffered saline containing Tween 20
TCA cycle	:	tricarboxylic acid cycle
TEMED	:	N,N,N',N'-tetramethylethylenediamine
TPI	:	triose phosphate isomerase (D-glyceraldehyde-3-phosphate ketol isomerase), EC 5.3.1.11
triose-P	:	triose phosphates
Tris	:	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tween 20	:	polyoxyethylene sorbitan monolaurate
UDP	:	uridine 5'-diphosphate
UDPGlc	:	uridine 5'-diphosphate glucose
UDPGPPase:		uridine 5'-diphosphate-glucose pyrophosphorylase (UTP α-D-glucose-1-phosphate uridylyl transferase), EC 2.7.7.9
UTP	:	uridine 5'-triphosphate

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CHAPTER 1

GENERAL INTRODUCTION

Worldwide, trade of the major crop products is second in value only to petroleum oil, and consequently production of these commodities dominates the local economy in many parts of the world (John, 1992). As a major crop product sucrose is highly valued in the manufactured food industry, and as the primary agricultural source of ethanol production (Hawker, 1985; John, 1992). Almost the entire sucrose yield is derived from sugar beet and sugarcane, with the latter accounting for more than 60 per cent of world production (Hawker, 1985; John, 1992). South Africa is ranked seventh in the top 15 world producers of sucrose from sugarcane (Bremner-Stokes, 1997, pers. comm.*). Hence, sucrose is a valuable source of revenue to South Africa, with the latest provisional net estimate for the 1996 - 1997 season amounting to 3640 million rand (Bremner-Stokes,1997, pers. comm.*). Improved yields through scientific research is thus an important objective of the South African Sugar Industry to maintain international competitiveness.

The utilisation of molecular technology to increase product yield through the manipulation of key enzymes, is expected to dominate the experimental approach undertaken in the future to study aspects of sucrose metabolism (Blakeley and Dennis, 1993; ap Rees, 1995). However, identification of the key regulatory steps and the manipulation of specific enzymes by molecular biology has largely been based on knowledge gained from physiological studies (John, 1992; Stitt and Sonnewald, 1995). Yet, for many of the major crop plants, including sugarcane (Moore, 1995), the biochemical basis for the regulation of product synthesis is not well understood (John,

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1992). Therefore, the identification of regulatory steps controlling product accumulation in sugarcane, and other crop species, should remain an important objective towards developing strategies to improve productivity.

Present understanding of the control of sucrose accumulation within the storage parenchyma of sugarcane internodes is largely based on studies conducted in the 1960s and early 1970s (Glasziou, 1961; Glasziou, 1962; Hatch and Glasziou, 1963; Sacher et al., 1963; Glasziou and Waldron, 1964; Hatch, 1964; Hawker, 1965; Glasziou and Gaylor, 1972). Carbon cycling between sucrose and the hexoses/hexose monophosphates is first described by Sacher and coworkers (1963). Through shifts in the relative rates of sucrose synthesis and degradation, this substrate cycle is presently believed to be primarily responsible for controlling sucrose accumulation (Wendler et al., 1990; Veith and Komor, 1993; Komor, 1994). Sacher et al. (1963) also proposed that vacuolar acid invertase activity, modulated by an auxin-controlled induction system, regulates the accumulation of sucrose in sugarcane internodes. As a consequence, physiological research has largely been focused on investigating the regulatory role of vacuolar acid invertase in internodal tissue (Hatch and Glasziou, 1963; Hatch et al., 1963; Glasziou and Waldron, 1964; Slack, 1965; Glasziou and Bull, 1967; Gaylor and Glasziou, 1972; Batta and Singh, 1986; Singh and Kanwar, 1991; Venkataramana and Naidu, 1993; Dendsay et al., 1995).

The control of sucrose accumulation in sugarcane internodal tissues, as proposed by Sacher and coworkers (1963), is probably an oversimplification for three basic reasons. Firstly, control of sucrose accumulation is primarily ascribed to acid invertase (Sacher et al., 1963). However, recent anatomical studies on sugarcane internodes indicate that a large proportion of sucrose may be imported symplastically (Jacobsen et al., 1992; Welbaum et al., 1992; Walsch et al., 1996). This suggests that both SuSy and neutral invertase may also play a significant role in catalysing sucrose breakdown. Secondly, Sacher et al. (1963) did not characterise carbon partitioning into non-sucrose storage related biosynthetic activity in internodal tissues. In order to identify additional mechanisms which may influence product accumulation in developing storage organs,

a knowledge of developmental changes in carbon partitioning between sucrose storage, polysaccharide synthesis and respiration is a prerequisite (Stitt and Steup, 1985; ap Rees, 1995). Thirdly, it is reported that insignificant randomisation of carbon between the hexose monophosphate and triose-P pools occurs in elongating internodes (Sacher et al., 1963). However PFP activity, responsible for catalysing the reversible interconversion of Fru-6-P and Fru-1,6-P_2 (using PPi as a phosphoryl donor), was unknown during that period.

Following the discovery of PFP (Carnal and Black, 1979), subsequent studies on other plant species have indicated the participation of this enzyme during carbon partitioning, through stimulation of the PPi-dependent sucrose cleavage pathway (Edwards and ap Rees, 1986 a; Edwards and ap Rees, 1986 b; Huber and Akazawa, 1986; Morrel and ap Rees, 1986; Sung et al., 1988; Xu et al., 1989) and rapid cycling of the hexose monophosphate and triose-P pools (Hatzfeld et al., 1990; Hajirezaei et al., 1994; Hill and ap Rees, 1994; Hill and ap Rees, 1995). Coupled to the cycling of carbon between the sugars, triose-P/hexose monophosphate cycling is also considered important in facilitating carbon allocation to various biosynthetic pathways in heterotrophic tissues (Hatzfeld and Stitt, 1990; Hatzfeld et al., 1990; Hill and ap Rees, 1994).

A study of the regulation of sucrose accumulation in sugarcane was only resumed almost two decades after the research of Sacher and contemporaries (Sacher et al., 1963; Hatch, 1964; Hawker, 1965; Glasziou and Gaylor, 1972). Using suspension cells grown in batch culture, Wendler and coworkers (1990) show that rates of sucrose synthesis are in excess of storage at all stages of the cell cycle, thereby confirming the continuous turnover of sucrose. That paper is the first to ascribe importance to SPS and SuSy in controlling sucrose accumulation in sugarcane (Wendler et al., 1990). Additionally, the study is also the first to examine the relationship between respiration and sucrose storage, as well as 'fine'- (metabolite) and 'coarse'- (enzyme activity) regulation of the enzymes catalysing sucrose breakdown and glycolysis (Wendler et al., 1990). Rates of respiration are shown to be both in excess of sucrose storage, and

unchanged during periods of sucrose synthesis and mobilisation. Consequently, no alteration in the fine regulation of glycolysis is reported (Wendler et al., 1990).

Although the rates of sucrose synthesis and O_2 consumption are compared (Wendler et al., 1990), composite changes in hexose monophosphate partitioning between sucrose synthesis, insoluble matter synthesis and total respiration (including anabolism) are not documented. A change in the partitioning of carbon from insoluble matter synthesis and respiration towards increased sucrose storage has been reported in sugarcane cell suspension cultures upon transfer to nitrogen limiting conditions (Veith and Komor, 1993). Since the cells were grown in continuous culture, developmental transitions in the energy requirement would not have been accounted for (Veith and Komor, 1993). Moreover, sugarcane suspension cells are reported to be more closely analogous to the cells of young internodal tissues (Veith and Komor, 1993). Hence, the question which might be raised is whether fine regulation of substrate cycling and respiratory carbon flow in sugarcane suspension cells is comparable to that of the developing stalk.

In developing sugarcane internodal tissue, no attempts have been made to investigate the regulation of sucrose breakdown and glycolysis during carbon partitioning. From physiological studies on sucrose importing tissues from other plant species, carbon partitioning from the central pool of hexose monophosphates may be regulated through several different mechanisms acting on sucrose degradative/synthetic and glycolytic enzyme activity (Dennis and Greyson, 1987; Kruger, 1990; Plaxton, 1996). In some species, where respiration constitutes a major sink for carbon, regulation of respiratory flux is primarily exerted on the rate limiting enzymes catalysing PEP utilisation (PK and PEPCase) and the Fru-6-P/Fru-1,6-P_2 interconversion (PFK and FBPase) respectively (Adams and Rowan, 1970; Kobr and Beevers, 1971; Leegood and ap Rees, 1978; Turner and Turner, 1980; Beaudry et al., 1989; Geigenberger and Stitt, 1991 b; Hatzfeld and Stitt, 1991; Plaxton, 1996). In other tissues, the contribution of the near-equilibrium catalysed reaction sequence from SuSy to PFP during sucrose import, turnover and resultant partitioning to biosynthetic activity (including respiration) has

received much attention (Edwards and ap Rees, 1986 a; Edwards and ap Rees, 1986 b; Morrel and ap Rees, 1986; Sung et al., 1988; Xu et al., 1989; Hatzfeld and Stitt, 1990; Merlo et al., 1993; Hill and ap Rees, 1994; Hill and ap Rees, 1995; Black et al., 1995). Although attention has been focused on studying SuSy activity in sugarcane internodes, where it is suggested that the enzyme may be important in sink strength (Lingle and Smith, 1991; Buczynski et al., 1993; Lingle and Irvine, 1994; Lingle, 1996), PFP activity has only been examined in a single study (Lingle and Smith, 1991). As PFP activity measurements were hampered by extreme variability (Lingle and Smith, 1991), no insight could be gained as to the possible physiological contribution of this enzyme to sucrose metabolism in the developing stalk.

Since significant variation in internodal sucrose content is apparent both in the developing stalk and across the *Saccharum* species, it is envisaged that changing patterns of hexose monophosphate partitioning and flux into the major cellular components (sucrose storage, insoluble matter and respiration) must exist. Fine and coarse control of glycolytic enzyme activity is also proposed to regulate the partitioning of carbon between respiration and sucrose storage in sugarcane internodal tissue.

The present investigation was thus aimed at identifying and characterising regulatory mechanisms which may influence sucrose accumulation in sugarcane internodal tissues. Since little is known about the possible physiological role of PFP during sucrose storage, particular emphasis was placed on examining the regulation of PFP activity (Chapter 4 and Chapter 6). Both developmental and cross-varietal PFP activity and protein concentration profiles were determined to investigate whether PFP is primarily correlated to increased sucrose storage or utilisation (Chapter 4). Based on the inverse relationship between carbon partitioning to sucrose storage and respiration (Chapter 5), and to further clarify the proposed involvement of PFP activity in processes relating to sucrose degradation (Chapter 4), fine regulation of the reactions from SuSy to PK were investigated (Chapter 6). Particular attention was focused on fine control of the regulatory Fru-6-P/Fru-1,6-P_2 interconversion (Chapter 6).

CHAPTER 2

LITERATURE REVIEW

In this chapter, present understanding on control of the sucrose accum lation pathway in sugarcane is reviewed against current knowledge on the regulation of sucrose metabolism derived from studies conducted on other plant species. The review presented is subdivided into two major sections. In the first, aspects of the control of sucrose cleavage and glycolysis are discussed, with particular emphasis on the regulatory Fru-6-P/Fru-1,6-P_2 interconversion. Thereafter, research development towards elucidating the cyclic sugar accumulation path in sugarcane is presented. Included in this section, is the historical based progress in understanding enzyme-mediated control of sucrose accumulation in sugarcane.

2.1 REGULATION OF SUCROSE METABOLISM

2.1.1 SUCROSE AS AN INTEGRAL COMPONENT OF METABOLISM

Sucrose is the most widely abundant oligosaccharide and has a central role in all aspects of plant metabolism (Avigad, 1982; Hawker, 1985). In higher plants, sucrose is both the principle product of photosynthesis and the dominant form in which organic carbon is translocated to heterotrophic sink tissues (Hatch and Glasziou, 1964; Avigad, 1982). As the major transportable metabolite, sucrose is the precursor substrate to structural polysaccharide synthesis, storage polymer synthesis and respiration in recipient sinks (Avigad, 1982; Sung et al., 1989). Additionally, sucrose also comprises the major storage component in several plant species (Avigad, 1982; Hawker, 1985; Stitt and Steup, 1985). Hence, knowledge on the regulation of sucrose degradation and subsequent partitioning to various biosynthetic pathways is considered important

for developing strategies to improve the product yield of commercially important crop plants (Ho et al., 1989; John, 1992; Moore, 1995). Sucrose breakdown and the reactions comprising glycolysis are at the core of carbon allocation to various biosynthetic pathways in plants (Dennis and Greyson, 1987; Blakeley and Dennis, 1993).

2.1.2 MECHANISMS OF CONTROL

The classical scheme of glycolysis is traditionally depicted as the sequence of reactions by which glucose is catabolised anaerobically to pyruvate. Evidence does, however, suggest that both pyruvate and malate can be regarded as the end-products of plant glycolysis (Bryce and ap Rees, 1985; Wiskich and Dry, 1985; ap Rees, 1990). In view of the consideration that sucrose is the major component of imported solutes and the starting point of sink carbon metabolism, it has been proposed that glycolysis be more correctly termed sucrolysis in plant tissues (Sung et al., 1988; Sung et al., 1989). Sucrolysis is composed of irreversible, highly regulated catalysed reactions, as well as reaction steps which operate close to equilibrium under *in vivo* conditions (Davies et al., 1964; Newsholme and Start, 1973; ap Rees et al., 1977; Turner and Turner, 1980; Edwards and ap Rees, 1986 b). The magnitude of metabolic flux is subject to both fine- (metabolite) and long-term, coarse- (enzyme activity) controls.

Fine controls are energetically inexpensive and operate primarily on the rate limiting, enzyme reactions. It has been demonstrated that primary control of respiratory carbon flux is exerted on the enzymes catalysing PEP utilisation, with secondary regulation being exerted at the Fru-6-P/Fru-1,6-P_2 interconversion (Adams and Rowan, 1970; Beaudry et al.,1989; Turpin et al., 1990; Geigenberger and Stitt, 1991 b; Hatzfeld and Stitt, 1991). Although the complete sucrolytic sequence is present in the cytosol (Figure 2.1), stretches of the pathway are sequestrated in the plastids of higher plants (Dennis and Miernyk, 1982). It is notable that due to compartmentation, studies on the regulation of metabolism are hampered by the inability to pinpoint the precise intra-
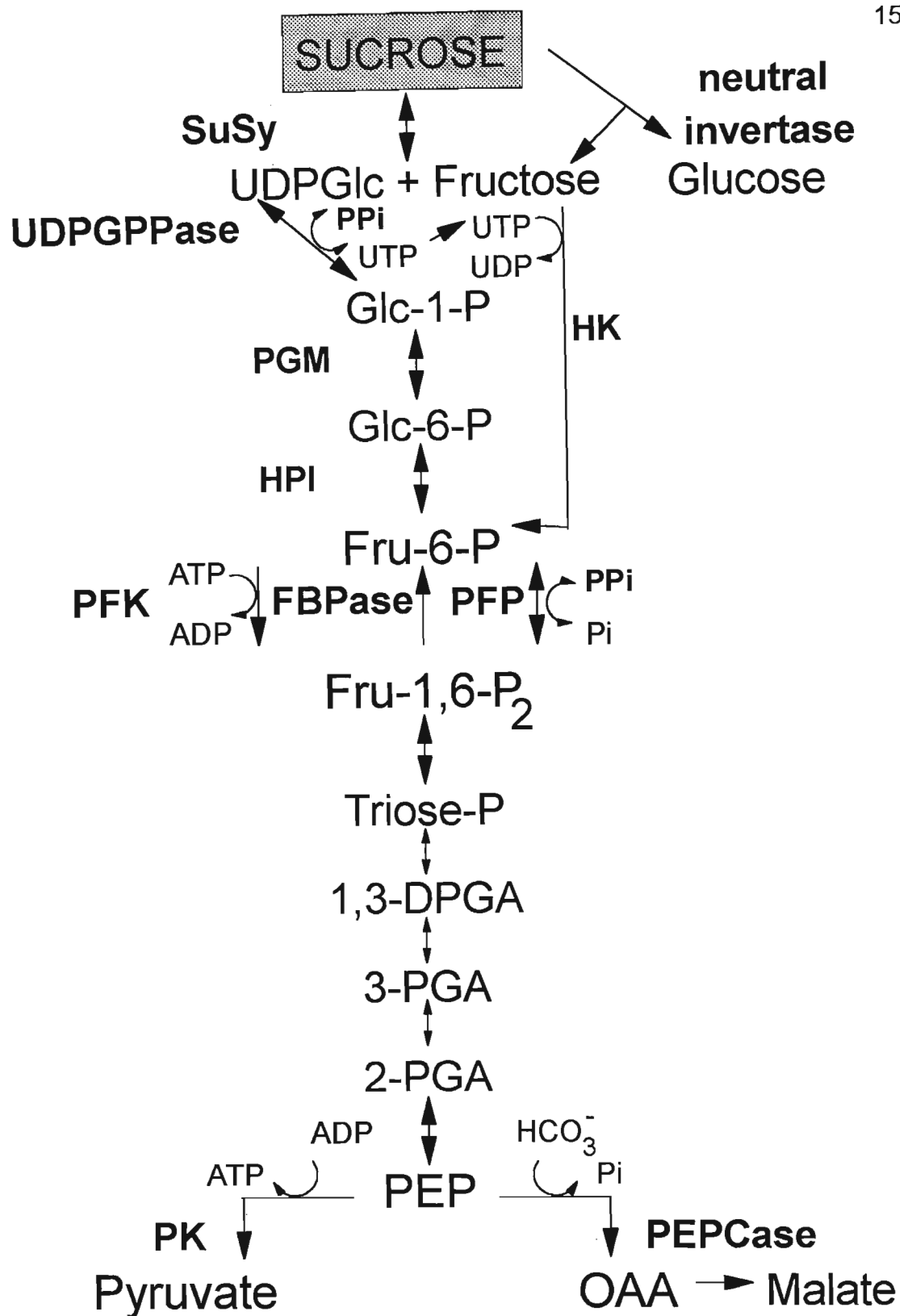
Figure 2.1 The sucrolytic sequence of reactions in the cytosol. Modified from Plaxton (1996).

cellular location of control. A key contributing factor is the presence of plastid and cytosolic isoenzymes which also display distinct differences in kinetic and regulatory properties. In addition, the heterogenous intracellular distribution of many metabolites, which form the substrates of isoenzymes located in the both cytosol and plastid, also complicate understanding of the control of *in vivo* enzyme activity. In a recent review paper by Plaxton (1996), various categories of fine control mechanisms are presented. These include: alteration in substrate (co-substrate) concentration; variation in pH; allosteric regulation; covalent modification (dithiol-disulfide interconversion, phosphorylation-dephosphorylation); subunit association-disassociation and reversible associations of metabolically sequential enzymes. In contrast, coarse metabolic control is achieved through changes in the enzyme concentration which in turn, may be mediated by changes in the rate of transcription, translation and proteolytic turnover (Plaxton, 1996).

2.1.3 REGULATION OF SUCROSE DEGRADATION

2.1.3.1 Enzymes catalysing sucrose breakdown

Sucrose degradation is essential for higher plants to initiate intermediary metabolism. The first step in the breakdown of sucrose is the cleavage of the glycosidic bond by either β -D-fructofuranosidase fructohydrolase (invertase) or SuSy. Plants contain two types of invertase which are distinguishable by the pH optimum for catalysis. Acid invertase has a pH optimum near 5.0 and is located in the vacuole and the cells walls, whilst neutral invertase has a pH optimum of approximately 7.5 and appears to be located in the cytoplasm (Hatch et al., 1963; Gaylor and Glasziou, 1972; Avigad, 1982; Hawker, 1985; Batta and Singh, 1986; Copeland, 1990). The reaction catalysed by invertase is highly exothermic and irreversible under physiological conditions (Turner and Turner, 1980). Resultant phosphorylation of the hexose sugars is catalysed by hexose phosphorylating isoenzymes, which display specificity towards either glucose or fructose (Copeland, 1990).

In contrast, the mass-action ratio of the SuSy catalysed reaction is close to the theoretical equilibrium constant *in vivo* (Avigad, 1982; Geigenberger and Stitt, 1993; Geigenberger et al., 1993). Despite the reversibility of the SuSy reaction, the enzyme is generally considered to catalyse the cleavage of sucrose *in vivo* (Avigad, 1982; Edwards and ap Rees, 1986 b; Morrel and ap Rees, 1986; Sung et al., 1989; Wendler et al., 1990; Sun et al., 1992; Black et al., 1995). SuSy appears to be located exclusively in the cytosol (Macdonald and ap Rees, 1983; Keller et al., 1988) and fine regulation of activity *in vivo* is largely determined by the availability of substrate (Avigad, 1982; Copeland, 1990). From recent studies on the coarse regulation of SuSy, it is proposed that the differential expression of SuSy isoforms (both sugar stimulated and sugar starvation tolerant) is controlled by sucrose availability (Perata and Alpi, 1993; Šebková et al., 1995; Koch et al., 1996; Sturm, 1996).

That SuSy is not subject to stringent fine regulation *in vivo* has led to controversy regarding the role of the enzyme in 'controlling' sink strength, which can be defined as the ability of the sink organ to import assimilate (Ho, 1988; Ho et al., 1989). Support both in favour (Sung et al., 1989; Xu et al., 1989; Sun et al., 1992; Black et al., 1995; Šebková et al., 1995) and against (Geigenberger and Stitt, 1993; Merlo et al., 1993) the role of SuSy in controlling assimilate import prevails. The support in favour of a role for SuSy in sink strength is predominantly based on studies showing induction of increased SuSy activity coinciding with increased storage product synthesis, whilst those against have focused primarily on the aspects of fine control.

2.1.3.2 Pyrophosphate-dependent sucrose cleavage pathway

Huber and Akazawa (1986) proposed a novel pathway for mediating the movement of sucrose cleavage products (UDPGlc and fructose) into the hexose monophosphate pool in the cytosol. This pathway is presently believed to be the major route for the incorporation of translocated sucrose into metabolism in many developing storage organs (Edwards and ap Rees, 1986 b; Morrel and ap Rees, 1986; Sung et al., 1988; Sung et al., 1989; Sun et al., 1992; Black et al., 1995; Šebková et al., 1995; Sturm, 1996). The salient feature of the scheme, proposed by Huber and Akazawa (1986),

is the cycling of uridylates and PPi. According to this model, UDPGPPase catalyses the pyrophosphorolysis of UDPGlc to produce Glc-1-P and UTP (refer to Figure 2.1). The UTP generated is utilised by UTP-dependent fructokinase to phosphorylate the cleavage product fructose, thereby forming UDP for continued SuSy activity. It is postulated that the source of PPi, required for UDPGPPase activity, is produced from PFP catalysing the synthesis of Fru-6-P. In turn, the substrate for the PFP reaction, Fru-1,6-P_2 is proposed to be generated by PFK linking the two enzymes in a substrate cycle (Huber and Akazawa, 1986).

An important contributory factor leading to the design of the model by Huber and Akazawa (1986) was the discovery of a substantial pool of inorganic PPi in plant tissues (Edwards et al., 1984; Smyth et al., 1984). It had widely been accepted that the driving force of polymer synthesis in the plastid was provided from the 'splitting' of nucleoside triphosphates to produce PPi. However, the PPi formed was generally believed to be rapidly hydrolysed by inorganic pyrophosphatase (ap Rees et al., 1985 b). Following the discovery of substantial pools of PPi, the metabolite has since been detected in the tissues from a variety of plant species (Edwards and ap Rees, 1986 b; Botha and Small, 1987; Kesy and Kowalczyk, 1987; Weiner et al., 1987; Nakamura et al., 1992; Geigenberger et al., 1993). As inorganic pyrophosphatase is confined to the plastids (Weiner et al., 1987), the prevailing pool of extractable PPi is proposed to be maintained in the cytosol. The presence of cytosolic UDPGPPase, PFP and a PPi-dependent electro-genic proton pump, capable of utilising/producing PPi (Kruger et al., 1983 a; Macdonald and ap Rees, 1983; Smyth et al., 1984; ap Rees et al., 1985 a; Huber and Akazawa, 1986; Edwards and ap Rees, 1986 b; Rea and Sanders, 1987; Weiner et al.,1987; Sung et al., 1988) and the finding that PPi varies independently of the adenylate level (Dancer et al., 1990 b) collectively supported a revised role for PPi as an autonomous energy donor to the cytosol. In turn, these findings collectively provided support for the PPi-dependent sucrose cleavage pathway, as proposed by Huber and Akazawa (1986).

Additional support for the *in vivo* operation of the model (Huber and Akazawa, 1986)

soon followed from physiological studies. Firstly, the maximum catalytic activity of SuSy and UDPGPPase have been shown to be in excess of the estimated rate of sucrose breakdown in the respective tissues analysed (Edwards and ap Rees 1986 a; Morrel and ap Rees, 1986; Botha et al., 1992). Furthermore, in certain tissues the contribution of invertase (acid and neutral) to sucrose breakdown is reported to be negligible (Edwards and ap Rees 1986 a; Edwards and ap Rees, 1986 b; Morrel and ap Rees, 1986; Sung et al., 1989; Black et al., 1995). Secondly, sufficient concentrations of intermediates to permit the stimulation of the PPi-dependent cleavage pathway have been recorded in various tissues (Edwards and ap Rees, 1986 b; Morrel and ap Rees, 1986; Dancer and ap Rees, 1989). Thirdly, the hexose monophosphates (Glc-1-P, Glc-6-P and Fru-6-P) are in equilibrium both with each other and UDPGlc *in vivo* (ap Rees et al., 1977; Edwards and ap Rees, 1986 b; Weiner et al., 1987). As PFP also catalyses a reaction close to equilibrium (Kruger et al., 1983 a; Weiner et al., 1987), a coupling of UDPGPPase and PFP activity through utilisation/generation of PPi is considered feasible *in vivo* (Edwards and ap Rees, 1986 b; Morrel and ap Rees, 1986; Xu et al., 1986; Black et al., 1987; Weiner et al., 1987; Dancer and ap Rees, 1989).

In rice seedlings grown under oxygen deprivation, the PPi-dependent cleavage pathway is the major route for the entry of carbon into intermediary metabolism (Perata and Alpi, 1993; Guglielminetti et al., 1995). However, in this tissue, the cycling of uridylates is proposed to be mediated by a nucleoside diphosphate kinase (Guglielminetti et al., 1995). Since PFP activity is postulated to catalyse Fru-6-P utilisation under oxygen deprivation (Mertens et al., 1990), the source of cytosolic PPi to stimulate cleavage via UDPGPPase is not known and requires further investigation (Guglielminetti et al., 1995).

2.1.3.3 Central role of hexose monophosphates in metabolism

Following degradation of sucrose, carbon entering the hexose monophosphate pool becomes available for allocation to various carbohydrate sinks (Dennis and Miernyk, 1982; Stitt and Steup, 1985; Dennis and Greyson, 1987; Kruger, 1990). Regulation

of hexose monophosphate utilisation appears to be central in determining carbon distribution between various end-products. The hexose monophosphates not only form common intermediates to pathways of carbohydrate synthesis (sucrose, starch and cell wall polysaccharides) and degradation (catabolism via respiration), but also form the principal site at which these pathways converge and interact with other pathways (Kruger, 1990). In many storage organs the TCA cycle is a major carbohydrate sink during developmental transitions in carbon partitioning (Adams and Rowan, 1971; Giaquinta, 1979; Beaudry et al., 1987; Beaudry et al., 1989; MacRae et al., 1992; Hill and ap Rees, 1994). Intermediates from the TCA cycle are either completely oxidised for the provision of energy (via the mitochondrial electron transport chain) or partially oxidised for incorporation into amino- and organic acid biosynthesis (Lambers, 1985; Stitt and Steup, 1985; ap Rees, 1990; Ireland, 1990; Plaxton, 1996).

2.1.4 REGULATION OF PHOSPHOENOLPYRUVATE UTILISATION

PEP utilisation is an important regulatory reaction step in intermediary metabolism. Plants contain PK, PEPCase (refer to Figure 2.1) and a PEP specific phosphatase capable of catalysing the utilisation of PEP.

PK catalyses the transfer of Pi from PEP to ADP to produce pyruvate and ATP. The mass-action ratio of the reaction has been shown to be significantly displaced from the theoretical equilibrium constant, indicating that PK catalyses a thermodynamically irreversible reaction under physiological conditions (Dixon and ap Rees, 1980 a; Turner and Turner, 1980; Day and Lambers, 1983; Geigenberger et al., 1993). In plant tissue, both plastid and cytosolic isoenzymes of PK have been purified. Studies have shown that the isoforms are immunologically distinct and differ markedly in kinetic and regulatory properties (Ireland et al., 1980; Lin et al., 1989; Podestá and Plaxton, 1991; Sangwan et al., 1992; Plaxton, 1996). The presence of differentially expressed, multiple isoforms of PK_c and PK_p (Plaxton, 1988; Lin et al., 1989; Plaxton et al., 1990; McHugh et al., 1995) indicate distinct specificity to specialised functions carried out

within the cytosol and between different plastid types. In tissues, where the *de novo* synthesis of proteins is dependent on the provision of carbon to the TCA cycle via PK_c, regulation of activity is modulated by levels of amino acids and TCA intermediates (Baysdorfer and Bassham, 1984; Lin et al., 1989; Podestá and Plaxton, 1991). Generally, most studies have indicated fine regulation of both forms of PFK activity by changing pH, substrate availability, and rising ATP levels which act to down regulate activity (Ireland et al., 1980; Baysdorfer and Bassham, 1984; Plaxton, 1988; Lin et al., 1989; Plaxton, 1996).

Adenylate limitation (during Pi deprivation) is postulated to functionally eliminate ADP dependent PK activity (Duff et al., 1989 b; Theodorou and Plaxton, 1993). The *de novo* synthesis of a 'PEP phosphatase' under Pi stress is proposed to by-pass PK activity (Duff et al., 1989 a; Duff et al., 1989 b). PEP phosphatase catalyses the hydrolysis of PEP to produce pyruvate and Pi (Duff et al., 1989 a). Although the role of this enzyme is still highly controversial (Plaxton, 1996), kinetic data have indicated a high substrate specificity towards PEP (Duff et al., 1989 a).

Although PEPCase activity under Pi stress has similarly been reported to circumvent cytosolic PK (Nagano and Ashihara, 1993), PEPCase is still proposed to contribute significantly to PEP utilisation under non-limiting adenylate and Pi levels (Plaxton, 1996). PEPCase is a cytosolic enzyme catalysing the irreversible carboxylation of PEP in the presence of HCO_3^- to yield OAA and Pi. The OAA is subsequently converted to malate (via malate dehydrogenase) which, in turn, enters the mitochondria. Malate in the TCA cycle is replenished through the cytosolic production of OAA from PEP (Dennis and Greyson, 1987; ap Rees, 1990). The significance of this alternative route to metabolism resides in the replenishment of TCA cycle intermediates removed for organic- and amino acid biosynthesis (Wiskich and Dry, 1985; ap Rees, 1990; Schuller et al., 1990; Sangwan et al., 1992; Chollet et al., 1996). Similar to PK, fine controls employed to modulate PEPCase *in vivo* appear to be tissue specific (Chollet et al., 1996). In general, the enzyme is controlled by levels of amino acids and TCA intermediates and is also sensitive to regulation by phosphorylation

(Chollet et al., 1996).

Support for the 'bottom-up' regulation of respiratory carbon flow through glycolysis is based on observations that stimulation of respiration coincides with the decrease in PEP levels, implicating the activation of PEPCase and PK (Adams and Rowan, 1970; Hatzfeld et al., 1990; Turpin et al., 1990; Geigenberger and Stitt, 1991 b; Hatzfeld and Stitt, 1991). In many plant species, increased carbon flow into the TCA cycle is also accompanied by an increase in the extractable activities of the enzymes catalysing PEP utilisation (Sangwan et al., 1992; Mohanty et al., 1993; Nagano and Ashihara, 1993; Podestá and Plaxton, 1994).

Down regulation of PK_c activity alone in transgenic tobacco leaf tissue is shown to have no effect on the rate of respiration (Gottlob-McHugh et al., 1992). That the PEP was transported to the chloroplast and metabolised by PK_p, or that PEP phosphatase and PEPCase acted to circumvent PK_c is also considered a possibility (Gottlob-McHugh et al., 1992). In a recent review article by Plaxton (1996), it is reported that the absence of PK_c activity in the homozygous offspring of those tobacco transformants has a deleterious effect on root development. Furthermore, in the leaves of the transformants PEP phosphatase activity is enhanced, suggesting that this enzyme may be the PK bypass *in vivo* (Plaxton, 1996).

2.1.5 REGULATION OF THE FRUCTOSE-6-PHOSPHATE/FRUCTOSE-1,6-BISPHOSPHATE INTERCONVERSION

The $Fru-6-P/Fru-1,6-P_2$ interconversion is the second key regulatory reaction in intermediary metabolism (Black et al., 1987; Sung et al., 1988; Kruger, 1990). This interconversion is catalysed by PFK (towards glycolysis), FBPase (towards gluconeogenesis) and the reversible enzyme, PFP (refer to Figure 2.1). The presence of the alternative enzyme PFP, and the regulation of the $Fru-6-P/Fru-1,6-P_2$ interconversion by $Fru-2,6-P_2$ is unique to the cytosol.

2.1.5.1 Fructose-2,6-bisphosphate

The significance of Fru-2,6-P_2 to metabolism resides in the allosteric regulation of both PFP and FBPase. Micromolar levels of this regulatory metabolite were discovered in the cytosol of plant tissues (Cséke et al.,1982; Stitt et al., 1983). Levels have been shown to vary markedly in response to changing sucrose contents within leaf tissue (Stitt et al., 1983; Stitt, 1990) and changing biosynthetic (respiratory) activity in heterotrophic tissues (Edwards and ap Rees, 1986 b; Beaudry et al., 1987; Bennet et al., 1987; Sung et al., 1988; Hatzfeld et al., 1990; Mertens et al., 1990; Stitt, 1990; Ashihara and Sato, 1993).

The concentration of Fru-2,6-P_2 is adjusted *in vivo* by the reciprocal activities of two enzymes, namely fructose 6-phosphate 2-kinase and fructose 2,6-bisphosphatase (Cséke et al., 1983; Larondelle et al., 1986). Fructose 6-phosphate 2-kinase is responsible for the synthesis of Fru-2,6-P_2 from Fru-6-P and ATP, whilst fructose 2,6-bisphosphatase catalyses the removal of Pi from Fru-2,6-P_2 to regenerate Fru-6-P (Cséke et al., 1983; Larondelle et al., 1986; Stitt, 1987). The relative activities of the two enzymes are sensitive to regulation by metabolite levels *in vivo*. Fructose-6-phosphate-2-kinase is activated by rising levels of Pi and Fru-6-P and inhibited by increasing triose-P levels (Cséke et al., 1983; Larondelle et al., 1986). In contrast, fructose-2,6-bisphosphatase is inhibited by increasing Pi and Fru-6-P content, and unaffected by changing levels of triose-P (Stitt, 1990).

2.1.5.2 ATP-dependent phosphofructokinase

PFK catalyses the phosphorylation of Fru-6-P by ATP as follows:
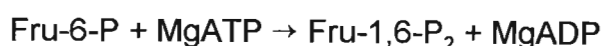
$$Fru\text{-}6\text{-}P + MgATP \rightarrow Fru\text{-}1,6\text{-}P_2 + MgADP$$

The mass-action ratio of the reaction has been shown to be significantly displaced from the theoretical equilibrium constant, indicating that PFK is thermodynamically irreversible and subject to tight regulatory control under physiological conditions (Dixon and ap Rees, 1980 a; Turner and Turner, 1980; Day and Lambers, 1983; Geigenberger

et al., 1993). Both cytosolic and plastid isoenzymes of PFK have been purified to homogeneity from photosynthetic and non-photosynthetic tissues of higher plants (Garland and Dennis, 1980 a; Garland and Dennis, 1980 b; Botha and Small, 1987; Dennis and Greyson, 1987; Häusler et al., 1987; Wong et al., 1987; Botha et al., 1988 b; Cawood et al., 1988). Expression of PFK_c and PFK_p may vary developmentally within the same plant and between species, with the ratio of the two isoenzymes being determined by the relative metabolic activity of the cytosol and plastids (Dennis and Greyson, 1987; Knowles et al., 1990). Hence, it is likely that the relative importance of the two PFK isoenzymes resides in the respiratory provision of carbon from starch (PFK_p) followed by triose-P export to the cytosol, versus respiratory carbon provision from cytosolic sucrose or hexose-monophosphates exported from the plastid (PFK_c). PFK_c and PFK_p are further distinguishable by stability during purification, as well as by differences in immunological and kinetic properties (Garland and Dennis, 1980 a; Garland and Dennis, 1980 b; Dennis and Greyson, 1987; Häusler et al., 1987; Botha et al., 1988 b; Cawood et al., 1988; Plaxton, 1996). However, both isoenzymes are subject to numerous fine controls. Control by changing pH is shown to affect substrate and effector affinity (Garland and Dennis, 1980 a; Isaac and Rhodes, 1986; Botha et al., 1988 b; Cawood et al., 1988), as well as the subunit aggregation (Isaac and Rhodes, 1986; Wong et al., 1987). To varying degrees, both isoenzymes are allosterically activated by Pi and inhibited by 3 PGA and PEP (particularly PFK_p), thereby highlighting the regulatory contribution of the Pi/PEP (or 3 PGA) ratios to the control of *in vivo* PFK activity (Garland and Dennis, 1980 b; Isaac and Rhodes, 1986; Häusler et al., 1987; Wong et al., 1987; Cawood et al., 1988; Plaxton, 1996). Allosteric regulation of PFK activity by PEP *in vivo* is well documented (Adams and Rowan, 1970; Hatzfeld et al., 1990; Turpin et al., 1990; Hatzfeld and Stitt, 1991), and has thereby lent support to the proposal that secondary regulation of plant glycolysis is exerted at Fru-6-P utilisation. Unlike PFK from animal systems, plant PFK is unaffected by the allosteric effector, $Fru-2,6-P_2$ (Sabularse and Anderson, 1981; Cséke et al., 1982; Botha and Small, 1987; Cawood et al., 1988).

The importance of PFK as a regulator of plant glycolysis has been based on several

physiological studies. Reduction of glycolytic flux in low temperature stored potato tubers is primarily attributed to cold-lability of PFK (Dixon and ap Rees, 1980 b; Bredemeijer et al., 1991). Inhibition of PFK activity by elevated CO_2 levels in pear fruit is similarly shown to effect a reduction of respiration (Kerbel et al., 1988). Additionally, stimulation of respiratory carbon flow during the ripening (respiratory climacteric) of certain fruits (Salminen and Young, 1975; Beaudry et al., 1987; Ball et al., 1991) and during nitrogen assimilation (Knowles et al., 1990; Turpin et al., 1990), through activation of PFK, has also lent support to the regulatory role of the enzyme. However, a recent study has indicated that PFK activity does not dominate the control of respiration (Burrell et al., 1994). Those authors demonstrate that a 20-fold increase in PFK activity in transgenic potato tubers has no effect on the respiration rate despite extensive changes in the levels of intermediates. These results suggest that fine regulation can compensate for large changes in enzyme expression (Burrell et al., 1994).

2.1.5.3 Fructose-1,6-bisphosphatase

FBPase catalyses the hydrolysis of Fru-1,6-P_2 as follows:

$$Fru\text{-}1,6\text{-}P_2 \rightarrow Fru\text{-}6\text{-}P + Pi$$
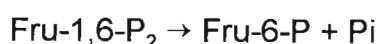
Similar to PFK, the reaction catalysed by FBPase is irreversible under physiological conditions and is tightly regulated *in vivo* (Leegood and ap Rees, 1978; Turner and Turner, 1980; Stitt, 1990). Both plastid and cytosolic isoforms of FBPase have been isolated from a variety of plant species (Herzog et al., 1984; Kruger and Beevers, 1984; Stitt, 1990), although the enzyme does not appear to be ubiquitous to heterotrophic tissues (Entwistle and ap Rees, 1990; Ball et al., 1991). The kinetic properties of FBPase$_c$ are consistent with a regulatory role in carbon partitioning between starch and sucrose biosynthesis. FBPase$_c$ is allosterically inhibited by increasing Fru-2,6-P_2, which acts to reduce the affinity of the enzyme for Fru-1,6-P_2 (Herzog et al., 1984; Kruger and Beevers, 1984; Stitt, 1990). Allosteric inhibition of FBPase$_c$ by Fru-2,6-P_2 is enhanced by AMP (Herzog et al., 1984; Kruger and Beevers,

1984). Increasing levels of the product Pi, further effect inhibition of $FBPase_c$ by the allosteric effectors. In turn, increasing triose-P levels act to stimulate $FBPase_c$ activity (Herzog et al., 1984; Kruger and Beevers, 1984; Stitt, 1990).

The important role of $FBPase_c$ in photosynthetic carbon metabolism has been well documented (Stitt et al., 1983; Herzog et al., 1984; Stitt, 1987; Stitt, 1990). Through allosteric and substrate regulatory control, stimulation of $FBPase_c$ enhances carbon flow from starch breakdown to sucrose biosynthesis, with the reverse existing for $FBPase_c$ inactivation. The preferential participation of FBPase in mediating carbon flow from $Fru-1,6-P_2$ to Fru-6-P is further evident by the low levels of PFP existing in mature leaf tissue (Xu et al., 1989; Stitt, 1990). In certain lipid-rich sink tissues, FBPase (rather than PFP) is also proposed to be important in controlling gluconeogenic carbon flux (Sato and Ashihara, 1992).

2.1.5.4 Pyrophosphate-dependent phosphofructokinase

PFP was initially identified in the specialised microorganisms *Entamoeba histolytica* (Reeves et al., 1974) and *Propionibacterium shermanii* (O'Brien et al., 1975). The isolation of PFP for the first time in higher plants (Carnal and Black, 1979) has been considered one of the most important discoveries affecting the understanding of various aspects of sucrose metabolism (Dennis and Blakeley, 1995; Plaxton, 1996). PFP catalyses the reversible interconversion between Fru-6-P and $Fru-1,6-P_2$, using PPi as a phosphoryl donor in the forward direction and Pi as a phosphoryl acceptor in the reverse direction:

$$Fru-6-P + PPi \leftrightarrow Fru-1,6-P_2 + Pi$$

Unlike PFK and FBPase which are present in both the cytosol and plastids, PFP appears to be located exclusively in the cytoplasm (Sabularse and Anderson, 1981; Cséke et al., 1982; Kruger et al., 1983 a; Kowalczyk et al., 1984; Macdonald and Preiss, 1986; Botha and Small, 1987). That PFP alone is restricted to the cytosol suggests that the enzyme is important in regulating carbon flow in that compartment.

In addition, extractable activities of PFP are generally either comparable to or well in excess of PFK and FBPase (Carnal and Black, 1979; Carnal and Black, 1983; ap Rees et al., 1985 a; Botha and Small, 1987; Xu et al., 1989; Stitt, 1990; Ashihara and Sato, 1993; Enomoto et al., 1994). It is also proposed that the significance of PFP to metabolism resides in the allosteric activation of the enzyme by nanomolar levels of the regulatory metabolite, Fru-2,6-P_2 (Stitt, 1987; Sung et al., 1988). Hence, the reaction catalysed by PFP is unusual in that it does not uphold the widely recognised view that equilibrium catalysed reactions are not subject to stringent regulatory control (Stitt, 1990). The near equilibrium reaction catalysed by PFP *in vivo* (Kruger et al., 1983 a; Edwards and ap Rees, 1986 b; Weiner et al., 1987; Geigenberger et al., 1993) therefore responds to cellular metabolism in a very flexible but 'regulated' manner. Additionally, PFP utilises PPi which is an important alternative energy source (Dancer et al., 1990 b).

Molecular and kinetic properties of PFP

In most plants examined, PFP contains two immunologically distinct subunits (Kruger and Dennis, 1987; Botha et al., 1988 a; Carlisle et al., 1990; Theodorou and Plaxton, 1996), namely the larger α subunit (65 - 68 kD) and smaller β subunit (60 - 64 kD) respectively (Yan and Tao, 1984; Kruger and Dennis, 1987; Botha et al., 1988 a; Botha et al., 1989; Botha and Botha, 1991; Blakeley et al., 1992; Theodorou et al., 1992; Botha and Botha, 1993 b; Nielson, 1994; Podestá and Plaxton, 1994; Theodorou and Plaxton, 1996). Different levels of subunit aggregation, which include the heterotetramer (Yan and Tao, 1984; Bertagnolli et al., 1986; Kruger and Dennis, 1987; Botha et al., 1988 a; Theodorou et al., 1992; Nielson, 1994; Podestá and Plaxton, 1994), homodimer (Yan and Tao, 1984; Kruger and Dennis, 1987; Wong et al., 1990) and singlet (Wong et al., 1990) forms have been purified from various plant species. Recent evidence has also demonstrated that PFP exists as a heterooctomer (Nielson, 1994; Theodorou and Plaxton, 1996) and that reversible covalent modification (disulfide-dithiol interconversion) constitutes an important mechanism of fine control in maintaining the 460 kD heterooctomeric form in potato (Plaxton, 1996).

Control of subunit association-dissociation by Fru-2,6-P$_2$ and PPi has previously been proposed to regulate the level of subunit aggregation (Wu et al., 1983; Wu et al., 1984; Black et al., 1987; Kruger and Dennis, 1987; Enomoto et al.,1991). Here, rising levels of Fru-2,6-P$_2$ are postulated to stabilise the tetrameric form of the enzyme against dissociation effects by PPi and promote catalysis in the forward direction, whilst the dimeric form of PFP and its activity in the reverse direction is proposed to be regulated by PPi (Wu et al., 1983; Wu et al., 1984; Black et al., 1987; Kruger and Dennis, 1987; Enomoto et al., 1991). However, this mechanism of modulating preferential glycolytic versus gluconeogenic activity has been complicated by the discovery of the heterooctomeric form. Furthermore, subunit association-dissociation studies on PFP were conducted *in vitro*, and more experimentation is required to determine whether this mechanism of fine control modulates PFP activity *in vivo* (Plaxton, 1996).

The structural features of the α subunit of PFP are consistent with a regulatory function involved in the binding of the allosteric activator Fru-2,6-P$_2$, whilst the β subunit contains the catalytic site (Yan and Tao, 1984; Carlisle et al., 1990; Cheng and Tao, 1990; Botha and Botha, 1991; Theodorou et al., 1992; Podestá and Plaxton, 1994). Although both subunits are expressed in most tissues, further characterisation of multiple molecular forms has revealed both α- (Fru-2,6-P$_2$ sensitive) and β subunit (less sensitive to Fru-2,6-P$_2$) enriched isoforms (Yan and Tao, 1984; Cheng and Tao, 1990; Wong et al., 1990; Botha and Botha, 1991; Podestá and Plaxton, 1994). It is notable that the existence of isoforms differentially enriched for the two subunits in an enzyme requiring both subunits for maximal catalytic activity is not clearly understood (Dennis and Blakeley, 1995).

Kinetic studies on PFP purified from a variety of different tissue sources, have indicated that the enzyme is highly regulated (Sabularse and Anderson, 1981; van Schaftigen et al., 1982; Kombrink et al., 1984; Bertagnolli et al., 1986; Botha et al., 1986; Wong et al., 1988; Mahajan and Singh, 1989; Stitt, 1989; Nielson, 1994). Since PFP catalyses an equilibrium reaction *in vivo*, net flux is dependent on and subject to modulation by changes in the allosteric activator, Fru-2,6-P$_2$ and relative concentrations of Fru-6-P,

Fru-1,6-P$_2$, Pi and PPi (Sabularse and Anderson, 1981; Cséke et al., 1982; Kruger et al., 1983 a; Botha et al., 1986; Stitt, 1989; Stitt, 1990).

The major effect of Fru-2,6-P$_2$ is to activate PFP activity in both the forward (glycolytic) and reverse (gluconeogenic) directions by increasing the catalytic affinity for Fru-6-P and Fru-1,6-P$_2$ (Sabularse and Anderson, 1981; van Schaftigen et al., 1982; Kombrink et al., 1984; Bertagnolli et al., 1986; Botha et al., 1986; Macdonald and Preiss, 1986; Stitt, 1989). In most plant tissues, the *in vivo* concentration of Fru-2,6-P$_2$ is considered sufficient to fully activate PFP (Stitt, 1990). However, the proposal by Stitt (1987) that a significant proportion of Fru-2,6-P$_2$ may be allosterically bound to enzymes has recently been supported by Nielson and Wischmann (1995). These authors show that the free concentration of Fru-2,6-P$_2$ in developing barley leaves is not sufficient to fully activate PFP. Furthermore, elevated activities of fructose-6-phosphate 2-kinase, and hence increased Fru-2,6-P$_2$ levels, in transgenic tobacco leaves have a major effect on carbon partitioning to starch synthesis via modulation of PFP activity (Scott and Kruger, 1995). This result further casts doubt as to whether Fru-2,6-P$_2$ is sufficient to fully activate PFP *in vivo* (Scott and Kruger, 1995). Recent evidence has corroborated earlier proposals (Stitt and Vasella, 1988) by showing that Fru-1,6-P$_2$ can also substitute Fru-2,6-P$_2$ as an allosteric activator of PFP (Nielson, 1995). This observation has interesting implications for *in vivo* modulation of activity (in the reverse direction) in certain tissue-types (Nielson, 1995). Additionally, a very recent report has similarly highlighted the importance of Pi concentration as an allosteric activator of glycolytic PFP activity during Pi deprivation in *Brassica nigra* cell suspension cultures (Theodorou and Plaxton, 1996).

Differences in the kinetic properties of PFP are apparent in different plant species and presumably stem from differences in enzyme form (Botha et al., 1986; Wong et al., 1988; Stitt, 1989; Wong et al., 1990; Theodorou et al., 1992). These structural differences, as well as the differential or coordinated expression of the two subunits during ontogenetic development (Botha et al., 1991; Blakeley et al., 1992; Nielson, 1994; Podestá and Plaxton, 1994) may be indicative of differences in the proposed role

of the enzyme in these respective tissues.

Physiological roles of PFP in sucrose metabolism

Although both PFK and PFP mediate entry of carbon into glycolysis, it is proposed that in young biosynthetically active tissues, PFP provides additional metabolic capacity to the conversion of Fru-6-P to Fru-1,6-P_2 (Ashihara and Horikosi, 1987; Dennis and Greyson, 1987; Kerbel et al., 1988; Enomoto et al., 1990; Tobias et al., 1992; Ashihara and Sato, 1993; Enomoto et al., 1994; Podestá and Plaxton, 1994). The presence of increased extractable PFP activities, higher PFP/PFK activity ratios and increased Fru-2,6-P_2 levels, coinciding with increased respiratory carbon flux, has collectively suggested that in certain tissue types PFP activity may play an important role in catalysing carbon flow to respiration (ap Rees et al., 1985 b; Bennet et al., 1987; Botha and Small, 1987; Duff et al., 1989 b; Mertens et al.,1990; Mertens, 1991; Tobias et al., 1992; Ashihara and Sato, 1993; Bogatek, 1995; Hill and ap Rees, 1995).

The potential advantage for the use of PFP over PFK in certain tissues is suggested to reside in increasing the ATP yield (Mertens et al., 1990; Enomoto et al., 1994). In turn, the hydrolysis of PPi instead of ATP during the conversion of Fru-6-P to Fru-1,6-P_2 also promotes a favourable equilibrium for biosynthetic activity (Stitt, 1990; Enomoto et al., 1994). Although studies have shown that there is no obligatory link between increased Fru-2,6-P_2 levels and respiration (Hatzfeld et al., 1990; Hatzfeld and Stitt, 1991), Fru-2,6-P_2 stimulation of PFP activity has been shown to be correlated with the stimulation of respiratory glycolytic carbon flow in ripening pear fruit (Bennet et al., 1987) and during cyanide-mediated dormancy removal in apple seed embryos (Bogatek, 1995).

PFP is also reputed to perform an important role in catalysing the conversion of Fru-6-P to Fru-1,6-P_2 under stress conditions, including nutritional Pi deficiency, hypoxia (reduced O_2 concentration) and anoxia (anaerobiosis) (Duff et al., 1989 a, Duff et al., 1989 b; Mertens et al., 1990; Theodorou and Plaxton, 1993; Hill and ap Rees, 1995). Nutritional Pi deficiency effects a reduction in the adenine nucleoside levels (Dancer

et al., 1990 b; Theodorou and Plaxton, 1993), which are the substrates of both PFK and PK activity. Since PPi is an autonomous energy donor and levels are unaffected by Pi deprivation (Dancer et al., 1990 b), PFP activity is considered an important adenylate bypass of PFK activity during Pi deficiency (Duff et al., 1989 b; Theodorou and Plaxton, 1993; Plaxton, 1996). Unlike PFP activity in *Brassica nigra* cell suspension cultures under Pi limitation (Duff et al.,1989 b), enzyme activity in *Catharanthus roseus* suspension cells is not consistent with a role as a 'Pi starvation inducible, glycolytic by-pass' (Nagano and Ashihara, 1993). However, in contrast to *Brassica nigra*, *Catharanthus roseus* cell suspension cultures derived a significant proportion of respiratory substrates from an internal supply of amino acids (Duff et al., 1989 b; Nagano and Ashihara, 1993). Increased PFP activity and Fru-2,6-P_2 levels are also induced in rice seedlings grown under anoxic conditions (Mertens et al., 1990; Perata and Alpi, 1993). In those tissues, PFP is proposed to catalyse Fru-6-P utilisation to conserve ATP levels. Additional support for this physiological role during oxygen deprivation is based on the observation that the intracellular PPi pool is unaltered (Dancer and ap Rees, 1989; Mohanty et al., 1993). In a recent article by Hill and ap Rees (1995), the importance of PFP activity in catalysing Fru-6-P utilisation has been highlighted by showing that under hypoxia, PFK activity is insufficient to sustain flux.

The occurrence of increased PFP activity during gluconeogenic carbon flux in certain CAM species (Fahrendorf et al., 1987) and germinating lipid-rich seeds (Kruger et al., 1983 a; Botha et al.,1989; Botha and Botha, 1991) suggested a role for PFP in gluconeogenesis. More convincing evidence for this role was provided when it was shown that FBPase activity in *Citrullus lanatus* is probably insufficient to sustain gluconeogenesis at the prevailing Fru-2,6-P_2 concentration (Botha and Botha, 1993 a). The potent allosteric activation of barley leaf PFP by Fru-1,6-P_2 (Nielson, 1995) also provides new support for the gluconeogenic role of PFP during sucrose synthesis in young leaf tissue, where FBPase activity is reportedly insufficient (Collis and Pollock, 1991; Nielson, 1992)

The regulation of the cytosolic PPi levels by PFP (Dancer and ap Rees, 1989; Stitt, 1989) is consistent with the involvement of the enzyme during sucrose cleavage. In certain tissues engaged in starch biosynthesis, PFP (acting in the gluconeogenic direction) is proposed to generate PPi as an energy source to stimulate the PPi-dependent sucrose cleavage pathway by SuSy and UDPGPPase (Edwards and ap Rees, 1986 b; Huber and Akazawa, 1986; Macdonald and Preiss, 1986; Morrel and ap Rees, 1986; Xu et al., 1986; Dancer and ap Rees, 1989). Regeneration of $Fru-1,6-P_2$ as a substrate for PFP is suggested to occur via PFK, thereby linking the two enzymes in a substrate cycle (Huber and Akazawa, 1986; Morrel and ap Rees, 1986; Black et al., 1987; Xu et al., 1989; Stitt, 1990).

As discussed in section 2.1.3.2, evidence supporting the physiological involvement of PFP in the PPi-dependent sucrose cleavage pathway is based on studies of metabolite levels, which show that the reactions catalysed by SuSy, UDPGPPase, HPI and PFP are in equilibrium *in vivo* (ap Rees et al., 1977; Edwards and ap Rees, 1986 b; Weiner et al., 1987; Merlo et al., 1993). Additionally, both substrate and effector concentrations are reported to be sufficiently high to support PFP activity in the reverse direction (Edwards and ap Rees, 1986 b; Morrel and ap Rees, 1986; Xu et al., 1986; Mahajan and Singh, 1989). Increased activities of PFP have similarly lent support to the involvement of the enzyme during sucrose breakdown (Edwards and ap Rees, 1986 a; Xu et al., 1989; Botha et al., 1992; Merlo et al., 1993). Although PFP activity is significantly higher than PFK in sucrose importing tissues (Huber and Akazawa, 1986; Morrel and ap Rees, 1986; Xu et al., 1989; Botha et al., 1992), sufficient activity of the latter is a prerequisite to sustain $Fru-1,6-P_2$ synthesis for catalysis by PFP. In developing maize endosperm, PFK activity is considered inadequate in sustaining the provision of $Fru-1,6-P_2$ to support the proposed involvement of PFP activity (reverse direction) in sucrose degradation (Doehlert et al., 1988).

As with SuSy, PFP activity has similarly been suggested to play an important role in controlling sink strength within certain plant species (Xu et al., 1989; Black et al., 1995). Black and coworkers (1995) further proposed that sucrose may act as a signal

molecule to induce expression of both SuSy and PFP. The mode of action is, however, yet to be discovered (Black et al., 1995). As discussed previously (refer to section 2.1.3.1), it is also perceived that sink strength is controlled by sucrose utilisation, where SuSy and PFP merely form part of a coordinated response to cellular metabolism (Geigenberger and Stitt, 1993; Merlo et al., 1993).

It is notable that although the role of PFP in maintaining PPi levels *in vivo* cannot be negated, the near removal of PFP in developing potato tubers had no effect on sucrose mobilisation via SuSy and UDPGPPase (Hajirezaei et al., 1994). If PFP is crucial in regulating PPi concentration during sucrose cleavage, then removal of PFP would be expected to lead to an accumulation of UDPGlc and a reduced level of hexose monophosphates due to a restriction on the reaction catalysed by UDPGPPase. However, no significant difference in the PPi, UDPGlc and/or the hexose monophosphate content is evident between the wild-type and transgenic tubers (Hajirezaei et al., 1994). This study, which is the first to employ a direct approach to determine the role of PFP *in vivo*, suggests that PFP is not essential in regulating the PPi concentration during sucrose degradation (Hajirezaei et al., 1994).

A substrate cycle, involving PFP and FBPase activity, has also been proposed to facilitate removal of PPi during biosynthetic activity (Black et al., 1987; Stitt, 1990). This proposal was corroborated from studies on cold stored potato tubers (Claassen et al., 1991) and developing pollen grains (Nakamura et al., 1992). In those tissues removal of PPi by PFP, is suggested to facilitate the formation of UDPGlc by UDPGPPase (synthetic direction) during sucrose and structural polysaccharide biosynthesis. Although regulation of PPi levels is postulated to occur through a coupling of the PFP and UDPGPPase catalysed reactions, PFP activity is primarily stimulated in the forward direction to produce Fru-1,6-P_2 (Claassen et al., 1991; Nakamura, 1992). In both tissue sources, FBPase activity is reportedly sufficient to mediate conversion of Fru-1,6-P_2 to Fru-6-P for catalysis by PFP (Claassen et al., 1991; Nakamura et al., 1992).

From investigations on PFP antisense potato and tobacco plants, there is also no evidence to suggest that PFP plays a crucial role in facilitating the removal of PPi during sucrose synthesis (Hajirezaei et al., 1994). The near removal of PFP activity in cold-sweetened and sprouting tubers did not lead to an accumulation of hexose monophosphates and UDPGlc, or an inhibition of sucrose synthesis which would be expected if decreased expression of PFP disturbed regulation of PPi turnover (Hajirezaei et al., 1994). Additionally, investigations carried out on transgenic tobacco leaves also indicated that near removal of PFP activity has no effect on either carbon flux to sucrose or PPi turnover during photosynthetic sucrose synthesis (Paul et al., 1995).

Additional functions attributed to PFP include the equilibrium of the triose-P/hexose monophosphate pools by rapid substrate cycling (Hatzfeld et al., 1990; Hill and ap Rees, 1994). Although the present understanding of the control of sucrose partitioning in heterotrophic tissues is more rudimentary, there is support for the operation of a triose-P/hexose monophosphate substrate cycle (Hatzfeld and Stitt, 1990; Hatzfeld et al., 1990; Hajirezaei et al., 1994; Hill and ap Rees, 1994). In that substrate cycle hexose monophosphates are converted to triose-P, and subsequently converted back to hexose monophosphates again. Combined with the cycle of simultaneous sucrose synthesis and degradation (Dancer et al., 1990 a; Hubbard et al., 1990; Wendler et al., 1990; Geigenberger and Stitt, 1991 a), this substrate cycle is proposed to regulate carbon partitioning between net storage and catabolism. In both *Chenopodium rubrum* cell suspension cultures (Hatzfeld et al., 1990) and ripening banana fruit (Hill and ap Rees, 1994; Hill and ap Rees, 1995), recycling of triose-P is primarily attributed to PFP since FBPase is either limiting (Hatzfeld et al., 1990) or absent (Ball et al., 1991), respectively.

Down-regulation of PFP activity (by more than 90 %) in transgenic potato tubers is shown to decrease the rate of triose-P recycling (Hajirezaei et al., 1994). The reduced triose-P/hexose monophosphate ratio effects a slight change in carbon partitioning towards increased sucrose and decreased starch synthesis, respectively (Hajirezaei

et al., 1994). This is the first major perturbation which arises from reduced PFP activity (Hajirezaei et al., 1994). Similarly, in transgenic tobacco leaves containing elevated levels of Fru-2,6-P_2, the rate of starch degradation in the dark is decreased (Scott and Kruger, 1995). It is argued that the decrease in starch breakdown is caused by an increase in unidirectional starch synthesis. These metabolic changes are suggested to have arisen due to an increase in the triose-P to hexose monophosphate ratio. The perturbation in the hexose monophosphate to triose-P ratio is attributed to an increased stimulation of PFP (operating in the glycolytic direction) in response to the elevated Fru-2,6-P_2 levels (Scott and Kruger, 1995). PFP activity is also proposed to convert Fru-1,6-P_2 to Fru-6-P in transgenic potato tubers over-expressing PFK activity (Burrell et al., 1994).

It is notable that although the near-removal of PFP effected a slight change in the partitioning between sucrose and starch (Hajirezaei et al., 1994), it is apparent that no effect on vegetative growth could be observed in both transgenic potato (Hajirezaei et al., 1994) and tobacco (Paul et al., 1995). However, the importance of PFP activity to metabolism can only be irrefutably demonstrated upon complete elimination of the enzyme (Blakeley and Dennis, 1993). Additionally, from all the physiological studies presented in this review, it would appear that the role of PFP varies according to tissue-type. Evidently, PFP is an 'adaptive enzyme' (Black et al., 1987), and an understanding of its effect on metabolism will require investigation on a tissue-by-tissue basis.

2.2 SUCROSE METABOLISM IN SUGARCANE

The product yield of field-grown crops is potentially regulated at the level of both the source and sink (Gifford et al., 1984). However, selection for increased productivity through conventional plant breeding has not produced cultivars with an improved net photosynthesis rate per unit leaf area (Gifford et al., 1984). Rather, successes have largely been based on the selection of cultivars with improved carbon allocation to storage products (Gifford et al., 1984; Ho et al., 1989; Sturm, 1996). In the *Saccharum*

species sucrose accumulation is suggested to be primarily regulated within the translocation system and/or at the level of the sink (Moore, 1995). To elucidate the biochemical basis for increased sucrose accumulation within the storage parenchyma, research emphasis on sugarcane has been directed almost exclusively to studying expression levels of the enzymes responsible for catalysing sucrose turnover.

2.2.1 THE SUGAR ACCUMULATION CYCLE AND ENZYME-MEDIATED CONTROL

The earliest description of sugar interconversion and accumulation in excised sugarcane internodal tissues is presented by Glasziou (1961). From radiolabelling studies, salient findings include an 'inner space compartment' (vacuole) spatially distinct from the 'outer space compartment' (cytosol), in which sucrose is synthesised from glucose and fructose (Glasziou, 1961). Since sucrose accumulation occurs against a concentration gradient, Glasziou (1960) proposed that sucrose is directly activated in the outer space compartment prior to storage. Breakage of the activated derivative of sucrose (termed 'sucrose-X' by Glasziou, 1960) is hypothesised to provide the energy required to deposit the synthesised sucrose in the inner space (Glasziou, 1961). Due to a higher uptake rate of ^{14}C derived from glucose, the latter is postulated to be the preferred precursor hexose of sucrose-X (Glasziou, 1961). Within the inner space, inversion of sucrose to produce fructose and glucose is reported. Any movement of sugars (sucrose, glucose or fructose) from either the inner or outer space compartments, respectively, is proposed to occur via diffusion (Glasziou, 1961). The inversion mechanism in the inner space was later attributed to acid invertase, shown to occur for the first time in immature sugarcane internodal tissue by Glasziou (1962).

The cyclic nature of the sucrose accumulation process in immature sugarcane internodal tissues is clearly shown for the first time in 1963 by Sacher and coworkers (Figure 2.2).
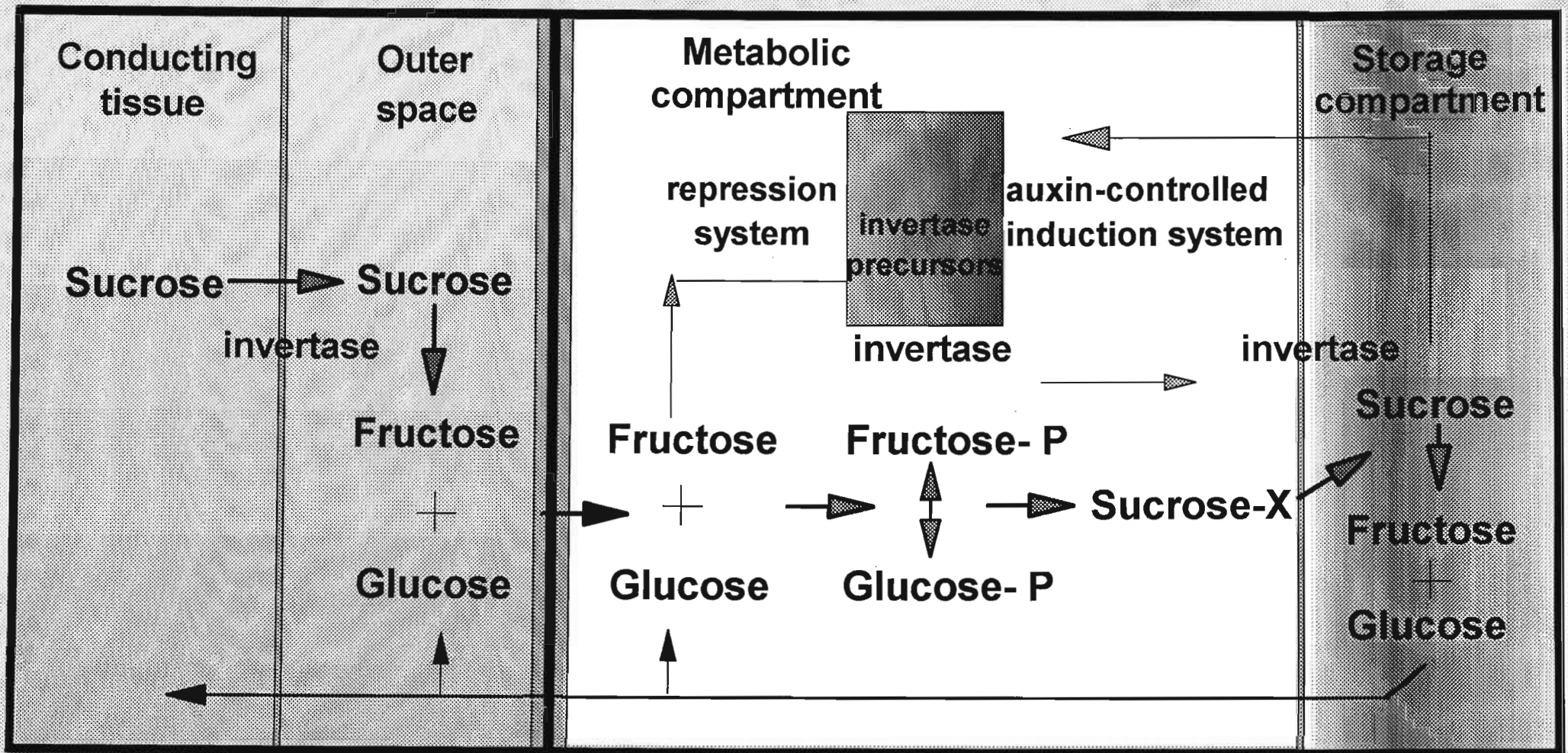
Figure 2.2 Schematic representation of the sugar accumulation cycle in sugarcane storage tissue as illustrated by Sacher et al. (1963).

Based on radiolabelling studies and enzyme measurements on excised tissue slices, five new experimental findings could be added to the original scheme of Glasziou (1961). Firstly, the presence of three distinct compartments in the storage parenchyma, namely the 'outer space' (apoplast and cell walls), 'metabolic compartment' (cytosol) and the 'storage compartment' (vacuole) are recognised. A distinction is further made between the storage parenchyma and conducting phloem. Secondly, acid invertase is shown to be located in the outer space (Hatch et al., 1963; Sacher et al., 1963). Based on this observation, the inversion of imported sucrose prior to uptake was believed to be an integrated feature of the sugar accumulation path (Sacher et al., 1963). Thirdly, the concept of direct sucrose conversion to sucrose-X is modified in favour of hexose phosphorylation and the resultant synthesis of the sucrose-X (Sacher et al., 1963). Fourthly, substantial levels of soluble acid invertase activity are shown to exist in the storage compartment (Sacher et al., 1963). Hence, the extent of sucrose cycling between sucrose and the hexose sugars (hexose monophosphates) is proposed to be largely dependent on the presence of invertase activity. Invertase activity, in turn, is postulated to be modulated by an auxin-controlled induction and glucose repressor system, respectively. Lastly, no randomisation of carbon between the phosphorylated hexose sugars and metabolites down-stream (triose-P) is reported to occur (Sacher et al., 1963). Hence, carbon cycling according to Sacher et al. (1963) is exclusive to sucrose and the hexose sugars/hexose monophosphates.

Although cell wall invertase is suggested to control the overall rate of sucrose entering the cell, 'inner space' invertase activity predominated (Sacher et al., 1963). Subsequent studies also lent support for the regulatory role of the inner space acid invertase during sucrose accumulation. Those studies show that vacuolar invertase is subject to regulation, with extractable activity levels being closely correlated to internodal elongation rates (Hatch and Glasziou, 1963; Hatch et al., 1963; Glasziou and Waldron, 1964; Slack, 1965). Stimulation of vacuolar acid invertase in elongating internodes by auxin lent support to the probable function of the enzyme in catalysing sucrose mobilisation to support growth (Glasziou and Waldron, 1964). The role of the

repressor system is based on the regulatory effect of glucose in inhibiting formation of invertase no longer required to sustain growth (Sacher et al., 1963). Additional support for the regulatory role of vacuolar acid invertase in the sucrose accumulation cycle, as proposed by Sacher et al. (1963), was derived from physiological studies on the *Saccharum* species (Bull and Glasziou, 1963; Hatch and Glasziou, 1963). Here, higher activity levels are shown to occur in the mature internodes of certain low sucrose storing *Saccharum spontaneum* species, whilst higher activity levels are reported in the *Saccharum officinarum* (Bull and Glasziou, 1963; Hatch and Glasziou, 1963).

In another study conducted by Hawker and Hatch (1965), it is proposed that neutral invertase (in the metabolic compartment) replaced the storage compartment invertase in mediating sucrose breakdown in mature internodal tissues. The measurement of significant levels of sucrose in both the outer space (water phase of cell walls and intercellular space of the metabolic compartment) and inner space (vacuole), led Hawker (1965) to conclude that diffusion processes may also contribute to the movement of sucrose in mature tissues. The relative contribution of sucrose diffusion versus active uptake of synthesised sucrose-X, in mature tissues, is proposed to be both dependent on and determined by the extent of cell wall acid- and neutral invertase activity (Hawker, 1965).

With the discovery of an irreversible UDPGlucose-fructose-6-P transcosylase (SPS) reaction in sugarcane (Hatch et al., 1963), Hatch (1964) described sucrose synthesis in the metabolic compartment in greater detail. Similar to the findings of Sacher et al. (1963), sucrose imported from the phloem appeared to be inverted prior to uptake. Phosphorylation of the hexose sugars is also proposed to occur in the metabolic compartment, where the phosphorylated hexose sugars are in equilibrium both with each other and UDPGlc. However, the distinguishing finding is the replacement of sucrose-X, with sucrose phosphate, synthesised from UDPGlc and Fru-6-P (Hatch, 1964). Although not recognised at the time, confirmatory evidence for the presence of a specific sucrose phosphate phosphatase was subsequently provided by Hawker and Hatch (1966). Despite the increasing support for the role of the invertases in

regulating sucrose storage, the importance of SPS in sucrose accumulation is first recognised by Hatch (1964). However, difficulties relating to the measurement of SPS through interference by hydrolytic enzyme activity precluded further verification of the role of SPS in sucrose accumulation. Hatch (1964) further proposed that UDPGlucose-fructose transcosylase (sucrose synthetase or SuSy) detected in sugarcane (Hatch et al., 1963) could potentially replace the role of the invertases in mediating sucrose breakdown. However, as with SPS, definitive evidence at the time was lacking.

Control of cyclic sucrose accumulation was revised almost two decades later using sugarcane suspension cells grown in batch culture (Wendler et al., 1990). Wendler and coworkers (1990) show that sucrose synthesis exceeds net storage, implicating the presence of a rapid simultaneous cycle of sucrose synthesis and breakdown. Shifts in carbon partitioning between the net storage and mobilisation of sucrose are interpreted to reflect shifts in the relative rates of sucrose synthesis and degradation (Wendler et al., 1990). A diagrammatic representation of sucrose cycling in sugarcane is shown in Figure 2.3.

With improved assaying techniques, the role of SPS in catalysing sucrose synthesis was confirmed (Wendler et al., 1990). Activity levels of SPS in sugarcane cell suspension cells are shown to be well in excess of the rates of sucrose synthesis (Wendler et al., 1990; Goldner et al., 1991). Despite the recorded presence of SuSy in sugarcane internodal tissues (Hatch et al., 1963; Hawker and Hatch, 1965), the study by Wendler et al. (1990) is the first to ascribe importance to the role of SuSy. Carbon cycling between sucrose and the hexoses/hexose monophosphates is presently considered an important control mechanism facilitating switches in carbon allocation between product accumulation and mobilisation in the cytosol of both sucrose and starch accumulating tissues (Dancer et al., 1990; Hubbard et al., 1990; Wendler et al., 1990; Geigenberger and Stitt, 1991 a; MacRae et al. 1992; Veith and Komor, 1993). Both metabolite levels and glycolytic enzyme activity, indicate that in sugarcane cell suspension cultures, sucrose accumulation is not driven by a restriction of respiration
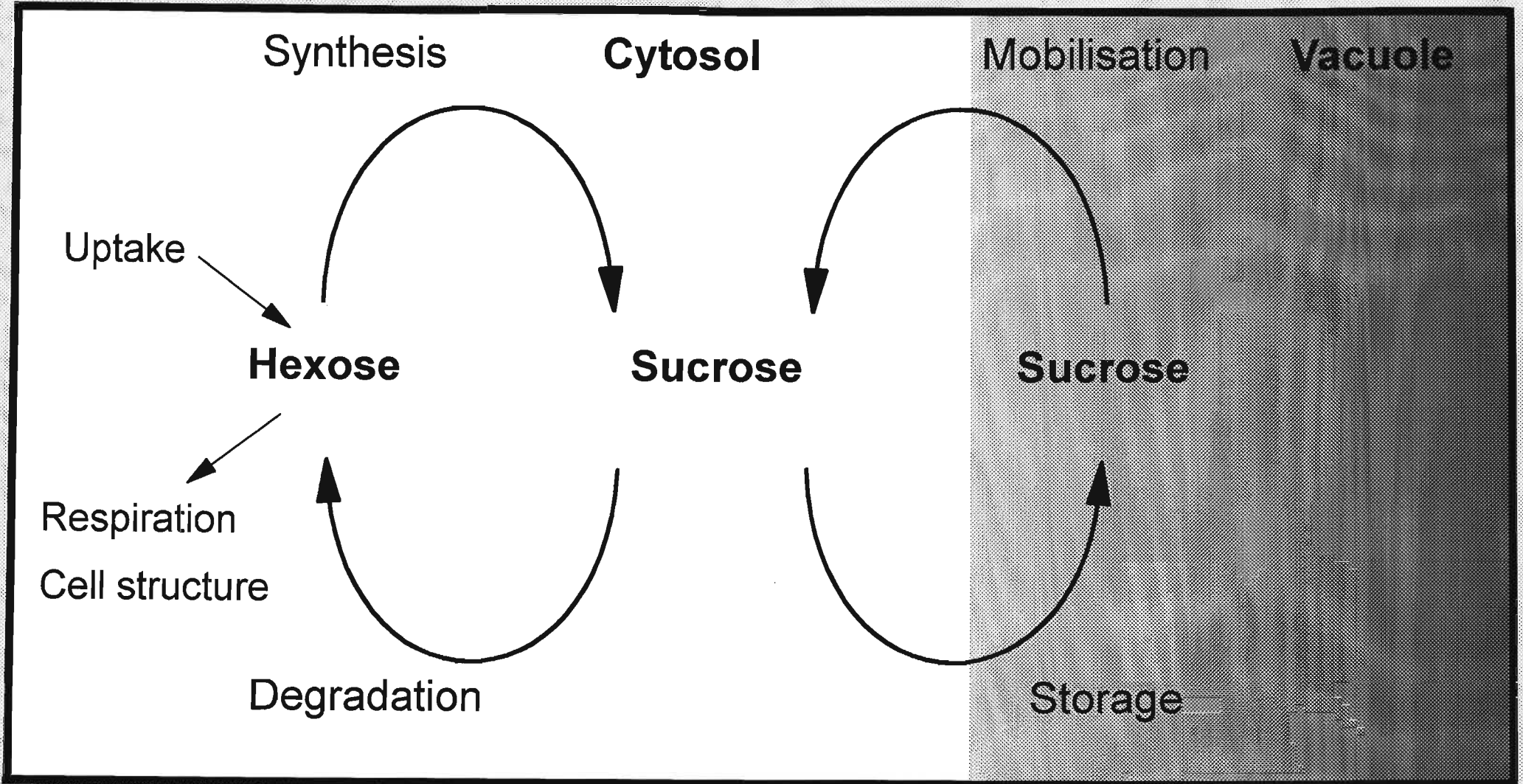
Figure 2.3 The cycle of sucrose synthesis and degradation in sugarcane sink tissues showing the input and output pathways. Drawn from Komor (1994).

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(Wendler et al., 1990). The respiration rate is approximately 2.7 fold higher than the net rate of sucrose storage, suggesting that a relatively small shift in carbon partitioning from utilisation to storage would result in marked changes in the rate of storage (Wendler et al., 1990).

A cycle of sucrose turnover is also proposed to regulate storage in excised internodal tissues (Komor, 1994). In contrast to the role of SuSy in suspension cells (Wendler et al.,1990), this enzyme does contribute towards sucrose synthesis in immature internodes (Komor, 1994). However, from a very recent paper (Komor, 1996) soluble acid invertase in the vacuole is considered largely responsible for catalysing the higher rates of sucrose degradation in the substrate cycle. A decline in substrate cycling is reported to coincide with increased partitioning of carbon into sucrose accumulation in ripening internodes (Komor et al., 1996). A redirection of carbon partitioning into sucrose storage from respiration and insoluble matter synthesis in maturing sugarcane internodal tissues has similarly been shown to occur (Botha et al., 1996), but without the decline in soluble acid invertase. As with sugarcane cell suspension cultures, the net rate of respiration exceeds the rate of sucrose storage (Botha et al., 1996; Komor et al., 1996).

The role of vacuolar acid invertase in controlling sucrose degradation in sugarcane, as originally proposed by Sacher et al. (1963), has continued to be widely supported from inverse correlations between sucrose content and enzyme activity (Batta and Singh, 1986; Goldner et al., 1991; Lingle and Smith, 1991; Singh and Kanwar, 1991; Venkataramana and Naidu, 1993; Dendsay et al., 1995; Zhu et al., 1996). The absolute requirement for sucrose hydrolysis by cell wall acid invertase prior to uptake in the storage parenchyma has, however, been challenged (Lingle, 1989; Thom and Maretzki, 1992). Experiments showing the uptake of a synthetic analogue of sucrose, which cannot be hydrolysed by invertase, indicated that hydrolysis of sucrose is not a prerequisite for uptake (Thom and Maretzki, 1992). The insignificant randomisation of labelled carbon in the hexose moieties of asymmetrically labelled sucrose taken up by tissue discs (Lingle, 1989), further suggested that extracellular inversion is not

obligatory. The lack of randomisation (Lingle, 1989) does not negate the presence of intracellular sucrose cycling, as it is probable that the duration of labelling was insufficient to have detected turnover. The fact that earlier investigators always reported sucrose hydrolysis prior to uptake is primarily attributed to the experimental technique employed, which included a pre-labelling wash of the tissue slices in water (Sacher et al., 1963; Hatch, 1964). This is proposed to have inactivated the sucrose transporter. Although it is not known to what extent sucrose is taken up intact, a significant proportion of imported sucrose is suggested to be hydrolysed (Komor, 1994; Komor et al., 1996).

Recent studies have shown that the cell walls of the storage parenchyma lignify with maturation (Jacobsen et al., 1992). In addition, suberisation between the vascular bundles and storage parenchyma suggest that an increasing proportion of sucrose uptake by the storage tissues may occur through the plasmodesmata (Jacobsen et al., 1992). Welbaum et al. (1992) similarly proposed that sucrose enters the storage parenchyma via plasmodesmata interconnections, with subsequent extrusion into the apoplast which is also shown to contain appreciable levels of sucrose (Hawker, 1965; Welbaum and Meinzer, 1990). These findings have been corroborated by Walsh et al. (1996), who described the physical path of sucrose unloading in the sugarcane stem. Results indicate that sucrose unloaded from the phloem passes through a fibre sheath into the storage parenchyma cells, which in turn are connected by plasmodesmata (Walsh et al., 1996). Collectively, these findings suggest that both SuSy and neutral invertase may also play a significant role in catalysing the cytosolic breakdown of imported sucrose. Based on reports of appreciable levels of both neutral invertase (Lingle and Smith, 1991; Singh and Kanwar, 1991; Venkataramana and Naidu, 1993; Dendsay et al., 1995) and SuSy (Lingle and Smith, 1991; Buczynski et al., 1993; Lingle and Irvine, 1994; Lingle, 1996) activity in the cytosol of sugarcane storage tissue, these enzymes are also likely to contribute to cytosolic sucrose turnover. The relative contribution of neutral invertase and SuSy to sucrose breakdown in the storage parenchyma is, however, not known (Komor, 1994; Moore, 1995). Tight regulation of sucrose degradation versus synthetic activity is likely since the rates of

extractable neutral invertase and SuSy are in excess of the net sucrose synthesis rate *in vivo* (Botha et al., 1996). A positive correlation between SuSy activity and sucrose accumulation in sugarcane has also led to the formulation of a working hypothesis that SuSy activity may be positively associated with increased sink strength (Lingle, 1996).

In a single study, the 'glycolytic' enzymes, PFP and PFK were measured in the developing stalk of an interspecific hybrid variety (Lingle and Smith, 1991). As variable trends in activity were recorded, no insight could be gained on glycolytic activity during sucrose storage (Lingle and Smith, 1991). In sugarcane internodal tissue, no attention has been focused on the fine regulation of sucrose breakdown and glycolysis, which is considered the primary pathway in plant metabolism (Blakeley and Dennis, 1993; Plaxton, 1996).

CHAPTER 3

GENERAL MATERIALS AND METHODS

Only the materials and methods which are applicable to more than one experimental chapter are included in Chapter 3. Additional materials and methods will be reviewed in the relevant chapters.

3.1 MATERIALS

3.1.1 BIOCHEMICALS

All auxiliary enzymes, cofactors and substrates used for enzyme assays and metabolite determinations were from either the Sigma Chemical Company or Boehringer Mannheim. Prepacked Presto desalting (cross-linked dextran) 5 mL columns were obtained from Pierce. The sucrose / D-glucose / D-fructose biochemical analysis kit was purchased from Boehringer Mannheim. [U-^{14}C]glucose was supplied by Amersham International. Sep-Pak 1 mL (100 mg) Alumina A cartridges and the Sugar-Pak column (6.5 mm x 460 mm) were obtained from Waters Chromatography. The Millex-GV$_4$ (0.22 μm) filter units were purchased from Millipore and the Ultima Gold XR quench resistant scintillation cocktail from Packard. All other solvents and biochemicals were of analytical grade.

3.1.2 PLANT MATERIAL

Mature, non-flowering, field-grown plants were sampled. Stalks from separate plants with approximately 20 above-ground internodes were randomly selected and harvested in the morning. The first leaf with the uppermost visible dewlap was defined as number
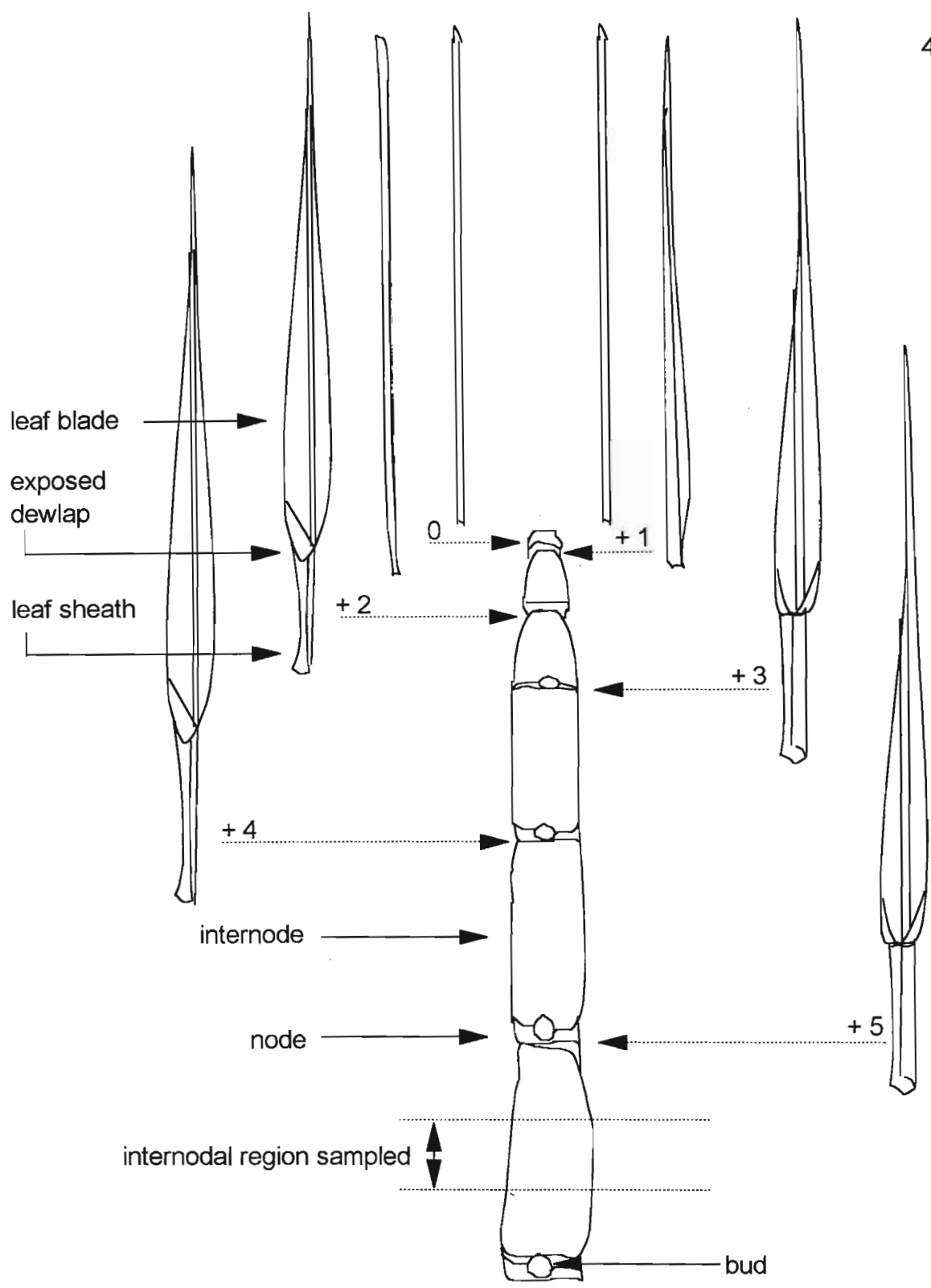
Figure 3.1   The upper section of a sugarcane stalk showing internodes +1 to +5. Leaves are numbered according to the system of Kuijper.   Leaf +1 represents the first unfolded leaf with a visible dewlap. The older leaves are consecutively numbered. Internodes attached to the respective leaves carry the same number. Adapted from van Dillewijn (1952).

1. The internode immediately below the point of leaf attachment was designated by the same number (Figure 3.1), according to the system of Kuijper (van Dillewijn, 1952). Identification of the different varieties sampled, is included in the respective experimental chapters.

3.2 METHODS

3.2.1 SAMPLE PREPARATION

In the laboratory, internodes selected for analysis were excised from the stalk and the rind carefully removed. The underlying tissue, spanning the core to the periphery, was rapidly sliced and frozen in liquid nitrogen. Samples were stored at -80°C until further use.

3.2.2 SUCROSE EXTRACTION AND MEASUREMENT

For the measurement of sucrose, internodal samples (± 2.0 g) were powdered in liquid nitrogen and extracted according to Ball and ap Rees (1988). The frozen samples were boiled for 5 min in 15 mL (v/v) 25 mM Tris-Cl (pH 7.8) and 50 % (v/v) ethanol, and then cooled on ice. Extracts were centrifuged at 4000*g* for 15 min (4°C) and the supernatant dried down in a rotary evaporator at 40 - 50°C. Dried samples were resuspended in 1 mL deionised, distilled H_2O and treated with activated charcoal to remove colouration. All internodal samples were extracted and measured in triplicate.

Sucrose was determined enzymatically using the Boehringer Mannheim sugar food analysis kit according to the manufacturer's instructions. The principle of the analysis was described by Bergmeyer and Bernt (1974). Glucose concentration was measured prior to and after the hydrolysis of sucrose by invertase at pH 4.6. Glucose was determined in a coupled assay system which involved the addition of HK to produce Glc-6-P. The Glc-6-P was subsequently converted to gluconate-6-phosphate and

NADPH following the addition of Glc-6-P dehydrogenase. The NADPH formed from this reaction was measured at 340 nm using a Beckman DU 7500 spectrophotometer. NADPH production was stoichiometric with the amount of glucose present. Sucrose content was calculated from the difference in glucose concentration before and after enzymatic conversion. The extraction efficiency was determined from the recovery of exogenously added sucrose at a similar concentration to that present in the tissues. The extraction efficiency was 94.7 %.

3.2.3 INSOLUBLE MATTER DETERMINATION

Insoluble matter was extracted according to a modification of Anon (1987). Internodal tissues (2.0 - 5.0 g) were powdered in liquid nitrogen. The ground samples were suspended in 100 mL H_2O for a minimum of 5 h to solubilise the cellular constituents. Thereafter, the insoluble matter was thoroughly washed under partial vacuum with additional volumes of H_2O to remove residual water-soluble material. The washed insoluble matter was then oven-dried at 80°C for 12 h and weighed. All internodal samples were extracted in triplicate.

3.2.4 DRY MASS DETERMINATION

Excised internodes (minus the rind) were sliced and the fresh mass determined. Tissue slices were oven dried at 80°C for 12 h and reweighed. Dry mass was expressed as a percentage of the fresh mass.

3.2.5 ENZYME EXTRACTION AND MEASUREMENT

The extraction procedure for the measurement of enzymes was carried out at 4°C according to a modification of the method by Lingle and Smith (1991). Crude extracts were prepared by grinding internodal tissues to a fine powder with liquid nitrogen. The ground tissue was suspended in ice-cold extraction buffer in a buffer volume to tissue mass ratio of 2:1. The standard extraction buffer contained 100 mM Tris-Cl (pH 7.5),

2 mM MgCl$_2$, 1 mM EDTA, 2 mM DTT, 2 mM PMSF and 10 % glycerol. After filtering through nylon, the extracts were centrifuged for 5 min (4°C) at 10,000g. The supernatants were retained and 1 mL volumes were desalted using the prepacked 5 mL desalting (cross-linked dextran) columns equilibrated with extraction buffer. Three to six replicate extractions were made for each of the enzymes assayed.

All enzyme assays were carried out at 25°C in a total volume of 1 mL. Activity was measured by following the oxidation of NADH or reduction of NAD/NADP (at 340 nm), using a Beckman DU 7500 spectrophotometer.

3.2.5.1 PFP activity

PFP activity was measured in the glycolytic (forward) direction. The standard reaction contained 100 mM Tris-Cl (pH 7.5), 1 mM MgCl$_2$, 10 mM Fru-6-P, 0.1 mM NADH, 10 µM Fru-2,6-P$_2$, 1 IU aldolase, 10 IU TPI and 1 IU glycerol-3-P dehydrogenase. Activity was initiated by the addition of 1 mM PPi (Kruger et al., 1983 a).

3.2.5.2 PFK activity

The reaction mixture was the same as for PFP, except that PPi was replaced with 1 mM ATP, Fru-2,6-P$_2$ was omitted and 100 mM Na-Hepes (pH 7.2) was used (Botha et al., 1988 b). The reaction was initiated with Fru-6-P.

3.2.6 PROTEIN MEASUREMENTS

Protein was measured on desalted extracts according to the method of Bradford (1976), using gamma globulin as a standard.

3.2.7 ^{14}CARBON LABELLING STUDIES

[U-^{14}C]glucose was used in the labelling studies at a concentration of 2.0 GBq mmol^{-1}. The labelling protocol and extraction techniques described in this chapter were employed for all labelled sugars used in the following experimental chapters.

3.2.7.1 Tissue preparation and labelling

Internodal tissues from separate plants were labelled, extracted and fractionated in triplicate. Transverse sections of tissue (0.75 - 1.5 g) from each internode were excised, sliced (approximately 0.5 mm thick) and washed four times in 50 mL 25 mM K-Mes (pH 5.7), containing 250 mM mannitol and 1 mM $CaCl_2$, for a period of 15 min (Lingle, 1989). The labelled substrate was supplied to the respective tissues in 2 mL 25 mM K-Mes (pH 5.7) containing 250 mM mannitol (Lingle, 1989). The tissue slices, contained in buffer, were incubated in airtight containers (500 mL) with a centre well containing 500 µL 12 % (m/v) KOH. Prior to incubation, the labelled substrate was vacuum-infiltrated into the tissue slices for 1 min. Thereafter, tissues were incubated at 28°C in a Labcon rotary shaking incubator (175 rpm) for 5.5 h. The $^{14}CO_2$ evolved was collected in the KOH solution. Preliminary trials indicated that the incorporation of label was linear over the uptake period ($r^2 = 0.991$). After labelling, the tissue slices were washed in 15 mL 25 mM K-Mes (pH 5.7), containing 250 mM mannitol and 1 mM $CaCl_2$ for 30 min (Lingle, 1989).

3.2.7.2 Tissue extraction

The tissues were powdered in liquid nitrogen and extracted in 20 mL methanol:chloroform:water (12:5:3 v/v) according to a modification of Dickson (1979). After centrifugation at 4000g for 10 min, the supernatant was removed and both the pellet and supernatant fractions retained. The supernatant was further fractionated by the addition of 5 mL chloroform and 4 mL deionised H_2O to separate the lipid-soluble and water-soluble components. The pellet fraction was thoroughly washed four times in 30 - 40 mL H_2O to remove unincorporated label. $^{14}Carbon$ in the various chemical components was determined by liquid scintillation spectroscopy (Packard Tricarb 1900 TR). Quench resistant Ultima-Gold XR scintillation cocktail (5 mL) was added to each sample aliquot prior to measurement.

3.2.7.3 Fractionation of the neutral water-soluble component

Samples from the water-soluble component were dried down in a rotary evaporator at 40 - 50°C, and the dried samples dissolved in HPLC grade H_2O. Thereafter, the

samples were passed through Sep-Pak Alumina A cartridges (prepared by washing with 5 volumes of deionised distilled H_2O) and then filtered (0.22 µm Millex-GV$_4$ filters). The sugars (20 µL injection volume) were fractionated by HPLC (Spectra-Physics 8800 ternary pump and Waters 410 differential refractometer detector) using the Sugar-Pak I column. Sugars were separated over 15 min with HPLC grade H_2O containing 1.33 mM EDTA (disodium calcium salt) at a flow rate of 0.5 mL min^{-1}. Fractions (0.5 mL) were collected over the 15 min period. The extraction efficiency for the uniformly labelled glucose exceeded 92 %. Peak areas of the sucrose, glucose and fructose, fractionated by HPLC, were integrated using the computer package Autochrom (Apex Chromotography). The concentration of each sugar was calculated from the slopes of linear calibration curves ($r^2 = 0.99$, for 0 - 1000 nmol sucrose, glucose and fructose, respectively).

3.2.8 STATISTICAL ANAYLSIS

All data was expressed as the mean ± SD. The application of other statistical methods is described in the respective chapters.

CHAPTER 4

COARSE CONTROL OF PYROPHOSPHATE: D-FRUCTOSE-6-PHOSPHATE 1-PHOSPHOTRANSFERASE IN SUGARCANE HYBRID VARIETIES

4.1 INTRODUCTION

The regulation of hexose monophosphate utilisation is important in determining carbon distribution between various end-products (Blakeley and Dennis, 1993). One of the key reactions is the Fru-6-P/Fru-1,6-P_2 interconversion (Black et al., 1987), catalysed by PFK (towards glycolysis), FBPase (towards gluconeogenesis), and the reversible enzyme, PFP. The reaction velocity of the PFP catalysed reaction *in vivo* is subject to both 'fine-' (metabolite concentration) and 'coarse-' (enzyme expression) control.

Available evidence suggests that PFP catalyses a near-equilibrium reaction *in vivo* (Cséke et al.,1982; Kruger et al., 1983 a; Weiner et al., 1987), and is therefore able to respond to cellular metabolism in a very flexible manner (Edwards and ap Rees, 1986 b; Stitt, 1989). Much evidence for coarse mediated control of PFP during tissue differentiation and long-term adaptive changes, is derived from studies on developing and germinating seeds, cell cultures and storage organs. Major functions assigned to PFP in these tissues have included roles in glycolysis (Duff et al., 1989 b; Mertens et al., 1990; Mertens, 1991; Mohanty et al., 1993; Perata and Alpi, 1993), gluconeogenesis (Kruger et al., 1983 a; Botha et al., 1989; Botha and Botha, 1993 a), and the rapid equilibrium of the hexose monophosphate and triose-P pools (Dennis and Greyson, 1987; Hajirezaei et al., 1994). PFP may also be important in the maintenance of cytosolic PPi, particularly under conditions of active growth-related

biosynthetic activity (ap Rees et al., 1985 a; ap Rees et al., 1986 b; Ashihara and Horikosi, 1987; Botha and Small, 1987; Tobias et al., 1992; Ashihara and Sato, 1993; Enomoto et al., 1994) and sucrose cleavage, via SuSy and UDPGPPase (Edwards and ap Rees, 1986 a; Huber and Akazawa, 1986; Morrel and ap Rees, 1986; Dancer and ap Rees, 1989; Xu et al., 1989; Botha et al., 1992). Consistent with the proposed participation of PFP during sucrose cleavage, it has been shown that PFP activity is positively associated with increased sink strength (Xu et al., 1989; Black et al., 1995).

In most storage sinks, where PFP has been well studied, sucrose cleavage activity is a prerequisite to mediate carbon flux into the hexose monophosphate pool for subsequent utilisation by both storage polymer synthesis and non-storage related biosynthetic activity. This finding has led to the proposal that the PPi-dependent sucrose cleavage pathway may be used as a determinant of sink strength (Xu et al., 1989; Black et al., 1995). In high sucrose-storing internodal tissues of sugarcane, the extent to which imported sucrose is either hydrolysed or cleaved *in vivo* prior to storage has not been elucidated (Moore, 1995). It has, however, recently been shown that SuSy activity is correlated to the rate of sucrose accumulation (Lingle, 1996), implying that cleavage may be positively associated with assimilate import in elongating sugarcane internodes. Previous investigations on sugarcane internodal tissue have similarly suggested that SuSy activity may be associated with sink strength (Lingle and Smith, 1991; Buczynski et al., 1993). In sugarcane suspension cells, increased activities of SPS, SuSy and to a lesser extent alkaline invertase and PFP, have all been shown to coincide with increased sucrose storage (Wendler et al., 1990). A role for these enzymes during the simultaneous synthesis and degradation of sucrose during accumulation is implicated (Wendler et al., 1990).

Coarse control of PFP during sucrose storage in developing sugarcane internodes is inconclusive (Lingle and Smith, 1991) and has never been investigated across hybrid varieties with significant variation in sucrose content. Control of PFP in sugarcane stem tissue warrants investigation for several reasons. Firstly, with the exception of fructokinase, all enzyme catalysed reactions from SuSy to PFP are in apparent

equilibrium (ap Rees et al., 1977; Edwards and ap Rees, 1986 b; Geigenberger and Stitt, 1993; Geigenberger et al., 1993; Merlo et al., 1993). Hence, if sucrose cleavage contributes towards increased sucrose import (sink strength) and resultant storage in sugarcane, then it is feasible that PFP activity may similarly be correlated to sucrose storage. Secondly, PFP activity has been shown to contribute significantly to triose-P recycling (Hatzfeld et al., 1990). In addition to stimulating PPi-dependent sucrose cleavage, substrate cycling between the hexose monophosphate and triose-P pools (catalysed by PFP and PFK) is further poised to facilitate switches in carbon partitioning from net storage to catabolism (Hatzfeld and Stitt, 1990; Hatzfeld et al., 1990). Thus, increased PFP activity could potentially impact negatively on sucrose accumulation in sugarcane through increased cytosolic substrate cycling which could facilitate carbon partitioning to non-sucrose storage related biosynthetic activity. The present study was therefore aimed at investigating developmental and cross-varietal PFP activity and protein concentration profiles, with the objective of assessing whether or not PFP is positively correlated to sucrose storage in sugarcane internodal tissue.

The developing sugarcane stalk is particularly suited to such metabolic analysis since a gradient of sucrose content exists between the younger and mature internodes (Rosenfeld, 1956). The approach taken in the present study was to compare PFP activity to sucrose accumulation. In addition, developmental profiles of PFP activity were compared to PFK, since the latter is proposed to regulate changes in respiratory glycolytic flux (Dixon and ap Rees, 1980 b; Dennis and Greyson, 1987; Kerbel et al., 1988; Stitt, 1990; Tobias et al., 1992; Plaxton, 1996).

In addition to the sucrose gradient which has been shown to exist down the stalk of sugarcane, significant variation in internodal sucrose content between sugarcane hybrid varieties is also evident (Alexander, 1973). Sucrose storage between high and low sucrose storing varieties is not reflected by differences in source activity (net photosynthesis per unit area) (Irvine, 1975), but is proposed to reflect differences in sink activity (Moore, 1995). Hence, interspecific hybrids could serve as useful tools for further analysis of the relationship between PFP activity (and PFK activity) and

sucrose storage. This formed the basis for incorporating an analysis between PFP activity and sucrose in several hybrid varieties in the present study. Coarse regulation of PFP was also analysed to determine whether PFP protein expression may act to control sugarcane PFP activity, as has been demonstrated for other plant species (Spilatro and Anderson, 1988; Botha et al., 1989; Botha and Botha, 1991; Blakeley et al., 1992; Botha and Botha, 1993 b; Nielson, 1994; Podestá and Plaxton, 1994).

Here it is reported that PFP activity is not positively correlated to sucrose storage in sugarcane internodal tissue. Differences in PFP activity, both within the developing stalk and between the varieties, are reflected by PFP protein concentration.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

4.2.1.1 Biochemicals

The purified potato $PFP_{(total)}$ IgG was the same as previously described (Botha et al. 1988 a). The anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate and the ECL protein blotting detection reagents were supplied by Amersham International. Partially purified PFP from potato tuber, the standard protein molecular weight markers and insoluble Protein A (10 % m/v non-viable *Stafylococcus aureus* cells) were from the Sigma Chemical Company.

4.2.1.2 Plant material

Seven *Saccharum* species hybrid varieties, N24, N19, N14, NCo376, Co331, CP66-1043 and US6656-15 were sampled. Details pertaining to the maturity status and sampling of the plants are recorded in section 3.1.2.

A subset of 70 clones were also sampled from a total of 150 clones in a F1 segregating population (N14 X 79F2011), grown at Pongola in Northern KwaZulu-Natal. The plants were approximately nine months-old and had 10 to 15 above-ground internodes.

4.2.2 METHODS

4.2.2.1 Sample preparation

Preparation of sampled plants is reviewed in section 3.2.1.

4.2.2.2 PFP and PFK extraction and measurement

PFP and PFK were extracted and measured as described in sections 3.2.5, 3.2.5.1 and 3.2.5.2.

To determine whether differential losses of PFP occurred, recovery experiments were conducted in high and low sucrose storing varieties and within the developing stalk of NCo376. Partially purified PFP (0.03 IU) from potato was included during extraction and the percentage recovery determined. Across the five hybrid varieties, the percentage recovery of the partially purified potato PFP was determined in extracts from internode 7. Within the developing stalk of NCo376, recovery measurements were made in samples extracted from internodes 3 through to 10. Recovery values of 98.0 ± 1.1 % (N24), 103.0 ± 5.5 % (N19), 93.6 ± 1.5 % (NCo376), 95.7 ± 4.5 % (Co331) and 100.0 ± 4.7 % (US6656-15) were obtained for the potato PFP. The percentage recovery of exogenously added potato PFP exceeded 90.0 % in extracts from internodes 5 to 10. In sample extracts of internodes 3 and 4, there was a 74.8 ± 4.8 % and 80.9 ± 7.0 % recovery of PFP, respectively. The correction factor was taken into consideration when expressing enzyme activity in these internodes.

The treatment of a few crude extracts with a broad spectrum of protease inhibitors which included: antipain dichloride (50 μg mL^{-1}), APMSF (20 μg mL^{-1}), aprotinin (1 μg mL^{-1}), bestatin (40 μg mL^{-1}), chymostatin (100 μg mL^{-1}), E-64 (0.75 μg mL^{-1}), EDTA-Na$_2$ (0.5 μg mL^{-1}), leupeptin (0.5 μg mL^{-1}), pepstatin (0.7 μg mL^{-1}), and phosphoramidon (120 μg mL^{-1}) had no effect on enzyme recovery.

PFP stimulation by Fru-2,6-P$_2$ was investigated in the standard reaction (refer to section 3.2.5.1) after acid-treating Fru-6-P to remove contaminating Fru-2,6-P$_2$ (Kruger et al., 1983 b). The commercial Fru-6-P preparation was incubated with 200 mM HCl for 30 min at 25°C, and then neutralised with 1 M Tris-acetate. PFP activity was initiated with Fru-2,6-P$_2$. In all varieties, inclusion of Fru-2,6-P$_2$ resulted in a 17.5 fold stimulation of PFP activity.

Since it has been shown that commercial ATP and Fru-6-P may be contaminated with PPi and Fru-2,6-P$_2$, respectively (Kruger, 1995), it was important to confirm that PFP was not a source of interference in the PFK activity assay. PFK activity from different varieties was measured in the presence of acid-treated Fru-6-P (to remove Fru-2,6-P$_2$)

and compared to activity initiated with untreated Fru-6-P. PFK activity, assayed in the presence of treated Fru-6-P, was 106.0 ± 11 % of the activity measured with untreated Fru-6-P. Since sugarcane PFP is highly Fru-2,6-P$_2$ dependent, it was evident that there was no interference from PFP in the PFK activity assay.

The possible presence of PFP and PFK activators and inhibitors was tested by preparing a series of extracts, each containing two internodal tissue combinations from either the different varieties or from internodes within the developing stalk (Botha and Small, 1987). PFP and PFK activities in a series of combined extracts from the developing stalk were 108.0 ± 14.0 % and 109.0 ± 19.0 %, respectively, of that when the tissues were extracted separately. Similarly, between the different varieties, the measured PFP and PFK activities in combined tissue extracts were 98.1 ± 4.1 % (PFP) and 102.0 ± 10.0 % (PFK), respectively, of that when the tissues were extracted separately.

4.2.2.3 Protein determination
This procedure is described in section 3.2.6.

4.2.2.4 SDS PAGE and protein blotting
Protein (25 µg) from desalted internodal extracts was precipitated with 90 % acetone. The precipitates were dried and solubilised in 70 mM Tris-Cl (pH 6.7) containing 3 % (m/v) SDS, 100 mM DTT and 8.0 M urea. Polypeptides were resolved in a 12 % polyacrylamide gel with a 6 % polyacrylamide stacking gel, according to a modified method of Moorhead and Plaxton (1991). The separation gel contained 200 mM Tris-Cl (pH 8.8), 100 mM glycine, 12 % (m/v) polyacrylamide, 0.12 % (m/v) N'N'-methylene bisacrylamide, 5 % (v/v) glycerol, 0.4 % (m/v) SDS, 0.1 % (m/v) APS and 0.1 % (v/v) TEMED), whilst the stacking gel contained 70 mM Tris-Cl (pH 6.8), 6 % (m/v) polyacrylamide, 0.06 % (m/v) N'N'-methylene bisacrylamide, 5 % (v/v) glycerol, 0.1 % (m/v) SDS, 4 mM EDTA, 0.1 % (m/v) APS and 0.1 % (v/v) TEMED. Polyacrylamide gels were run at 200 V in a slab electrophoresis unit (SE 250 Mighty Small II) containing 100 mM Tris-Cl, 150 mM glycine and 0.1 % (m/v) SDS in the upper

electrophoresis tank. A two-fold dilution of the gel running buffer was included in the lower electrophoresis tank. The separated polypeptides were transblotted onto nitrocellulose in 25 mM Tris-Cl, containing 192 mM glycine and 10 % (v/v) methanol. Following transfer at 75 Volts for 1 h, the blots were stained in Ponceau-S (0.2 % [m/v] Ponceau-S, 3 % [m/v] trichloroacetic acid), and the standard molecular weight bands marked.

The blots were then thoroughly rinsed in TBST and blocked overnight in 3 % (m/v) fat-free milk powder in TBST (10 mM Tris-Cl [pH 7.8], 150 mM NaCl, 1 % [v/v] Tween 20) at 4°C. All subsequent steps in the procedure were carried out at room temperature. Blots were incubated in TBS containing 3 % (m/v) BSA and purified potato $PFP_{(total)}$ IgG (1:750 dilution) for 1 h. Following incubation, the blots were washed five times in TBST for 5 min. Blots were then incubated for 45 min in TBST containing 3 % (m/v) milk powder and anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate (1:1000 dilution). The blots were subsequently washed for 10 min in TBST containing 1 % (m/v) SDS, and a further four times in TBST for 5 min. The ECL kit was used as a detection system and cross-reacting polypeptides were identified on hyper film-ECL. Western blots were scanned (Scanman Colour, Logitech) onto computer and the images processed using Photo Touch Colour (Logitech). The relative intensity of the polypeptide(s) cross-reacting with the antibody, as well as the molecular mass of the polypeptide(s) were determined with the computer package, Wincam II (Cybertech).

4.2.2.5 Immuno-inactivation of PFP activity

The effect of potato $PFP_{(total)}$ IgG on sugarcane PFP activity was determined in a reaction mixture containing 75 µL crude extract (0.012 - 0.015 units PFP activity) and 2.5 to 50 µL antiserum. After incubation for 45 min at 22°C, 10 µL insoluble Protein A was added, and the extract incubated for a further 30 min (Botha et al., 1989). The sample was then centrifuged and residual PFP activity measured as in section 3.2.5.1. Percentage inactivation by the antibody was expressed as a percentage of the PFP activity in extracts which were similarly incubated, but treated with insoluble Protein A only. The immuno-inactivation studies were performed in duplicate.

4.2.2.6 Sucrose and insoluble matter determinations

Determination of sucrose and insoluble matter and tissue dry mass are described in sections 3.2.2, 3.2.3 and 3.2.4, respectively.   Measurements for the rate of internodal development of NCo376 (Inman-Bamber, 1994) at the time of sampling were generously supplied by Dr NG Inman-Bamber, and used to calculate the rates of insoluble matter and sucrose accumulation.

Insoluble matter and recoverable sucrose were also analysed in stalk sections by the Plant Breeding Department (excluding the immature top section and the base), according to standards set by the Sugar Industry Central Board (SCIB)(Anon, 1987). Sucrose and insoluble matter measurements were comparable to those obtained in the smaller scale preparations, described in sections 3.2.2 and 3.2.3.

4.2.2.7 Statistical analysis

The PFP activity data were analysed for variance using the statistical computer programme Lotus 1-2-3, and the *LSD* calculated.   Correlations between PFP activity, sucrose and the insoluble matter were calculated by linear regression.   Changes in PFP activity levels and PFP protein concentration were similarly correlated by linear regression.

4.3 RESULTS

4.3.1 ENZYME ACTIVITY, SUCROSE AND INSOLUBLE MATTER IN THE DEVELOPING STALK

4.3.1.1 Protein content and specific PFP activity

Specific PFP activity and soluble protein content were measured in internodes 3 to 10 from stalks of NCo376, sampled over three growing seasons. Soluble protein content, expressed on both a dry and fresh mass basis decreased from internodes 3 to 5 (Table 4.1). On a fresh mass basis, protein content remained constant between internodes 5 and 10 (Table 4.1), whilst a decrease was evident when soluble protein was expressed as function of the dry mass. To better represent the cytosolic (metabolic) compartment in which PFP is located, activity was expressed on a soluble protein basis (Table 4.1).

The developmental trend in specific PFP activity was similar between internodes 3 to 10 over the different seasons (Table 4.1). An analysis of variance (anova) was conducted using the PFP data from internodes 3 to 10 from the respective seasons. From this analysis, PFP activity in internodes 6, 7 and 8 was significantly higher than in the remaining internodes ($p = 0.005$), whereas activity in internodes 3, 4 and 10 (and 9 in the first two seasons) was significantly lower.

4.3.1.2 Comparison of specific PFP and PFK activity, sucrose, insoluble matter, and the rate of sucrose and insoluble matter accumulation

To ensure accurate comparisons between PFP and PFK activity, and rates of insoluble matter and sucrose accumulation, 12 stalks were harvested. Internodes 2 to 10 were excised from each of the 12 stalks and the respective internodes bulked for analysis. Comparison of all parameters was made on a soluble protein basis. The complication with evaluating metabolism and gene expression expressed on a mass basis is the large increase in the contribution of sucrose to total dry mass (a large part of which is

Table 4.1 Measurements of soluble protein content on a dry and fresh (in parenthesis) mass basis and specific PFP activity in internodes 3 to 10 of NCo376 over three different growing seasons.

Internode number	Season 1	Season 2	Season 3
		Protein content	
		(mg gram dry mass^{-1} / [gram fresh mass^{-1}])	
3	14.01 ± 2.6 (1.94)	18.35 ± 2.8 (1.85)	22.00 ± 3.3 (2.28)
4	8.65 ± 1.1 (1.47)	12.30 ± 1.3 (1.40)	11.61 ± 1.3 (1.37)
5	4.65 ± 0.5 (0.90)	7.05 ± 1.3 (0.95)	10.55 ± 0.6 (1.32)
6	4.60 ± 0.6 (0.92)	6.84 ± 1.2 (0.99)	7.89 ± 0.6 (1.20)
7	3.83 ± 0.4 (0.85)	5.54 ± 0.6 (0.85)	6.43 ± 0.3 (1.20)
8	3.25 ± 0.3 (0.78)	4.75 ± 0.7 (0.88)	5.67 ± 0.4 (1.16)
9	3.31 ± 0.5 (0.79)	3.74 ± 0.4 (0.82)	5.45 ± 0.2 (1.20)
10	2.71 ± 0.4 (0.67)	3.96 ± 0.5 (0.89)	5.29 ± 0.6 (1.24)
		PFP activity	
		(nmol min^{-1} mg protein^{-1})	
3	23.2 ± 3.1	19.1 ± 3.1	27.5 ± 8.0
4	22.5 ± 5.7	23.0 ± 1.7	22.4 ± 3.4
5	29.1 ± 2.8	30.7 ± 2.8	29.6 ± 2.0
6	34.9 ± 6.7	34.5 ± 6.7	43.6 ± 2.7
7	40.8 ± 5.1	38.2 ± 5.1	47.6 ± 5.0
8	30.5 ± 5.0	28.1 ± 5.0	41.1 ± 1.8
9	25.2 ± 4.5	25.4 ± 5.0	42.0 ± 3.6
10	24.8 ± 6.5	25.8 ± 6.8	34.0 ± 7.0

-Each value is the mean ± SD of three to six separate internodal extractions.

stored in the vacuole and apoplast) as the internodes mature (Hawker, 1965; Glasziou and Gaylor, 1972). The proportion of sucrose to total dry mass increased from 14.6 % (internode 2) to 50.1 % (internode 10) in NCo376. Expression of cytosolic constituents on a dry mass basis would therefore provide a very skewed picture. As a result, soluble protein was selected as the basis on which to express enzyme activity, carbon flux and metabolite levels (in subsequent chapters) in order to better reflect metabolism in the cytosolic compartment.

Sucrose content increased from internodes 3 to 7 (Figure 4.1 A). Insoluble matter content increased from internodes 3 to 5, after which there was no further significant increase (Figure 4.1 B). From the sucrose and insoluble matter data (Figure 4.1 A and Figure 4.1 B), accumulation rates of sucrose and insoluble matter synthesis were calculated for the top ten internodes. Sucrose and insoluble matter reflect end-product accumulation, hence synthesis of these components could be expressed as a function of internodal age.

When calculating the rate of sucrose accumulation, the assumption made in this study was that incoming sucrose would have been cleaved/hydrolysed and resynthesised in the cytosolic compartment. Sucrose and insoluble matter accumulation rates were calculated using total content as a function of total age. The time interval taken for the development of each successive internode ranged between 16 days (internodal growth during the summer months) and 26 days (internodal growth during the winter months). These growth rates are typical for field-grown plants of the variety NCo376 at SASEX. It is likely that actual accumulation rates of sucrose and insoluble matter accumulation fell within the range of values calculated using the two approaches.

Sucrose accumulated at an increasing rate (sucrose/total internodal age) from internodes 2 to 10 (Figure 4.1 C). The mean rate of insoluble matter synthesis (total insoluble matter/total time) was unchanged (Figure 4.1 D). The trend in specific PFP was reviewed in section 4.3.1.1. PFK activity was relatively constant (Figure 4.1 F).
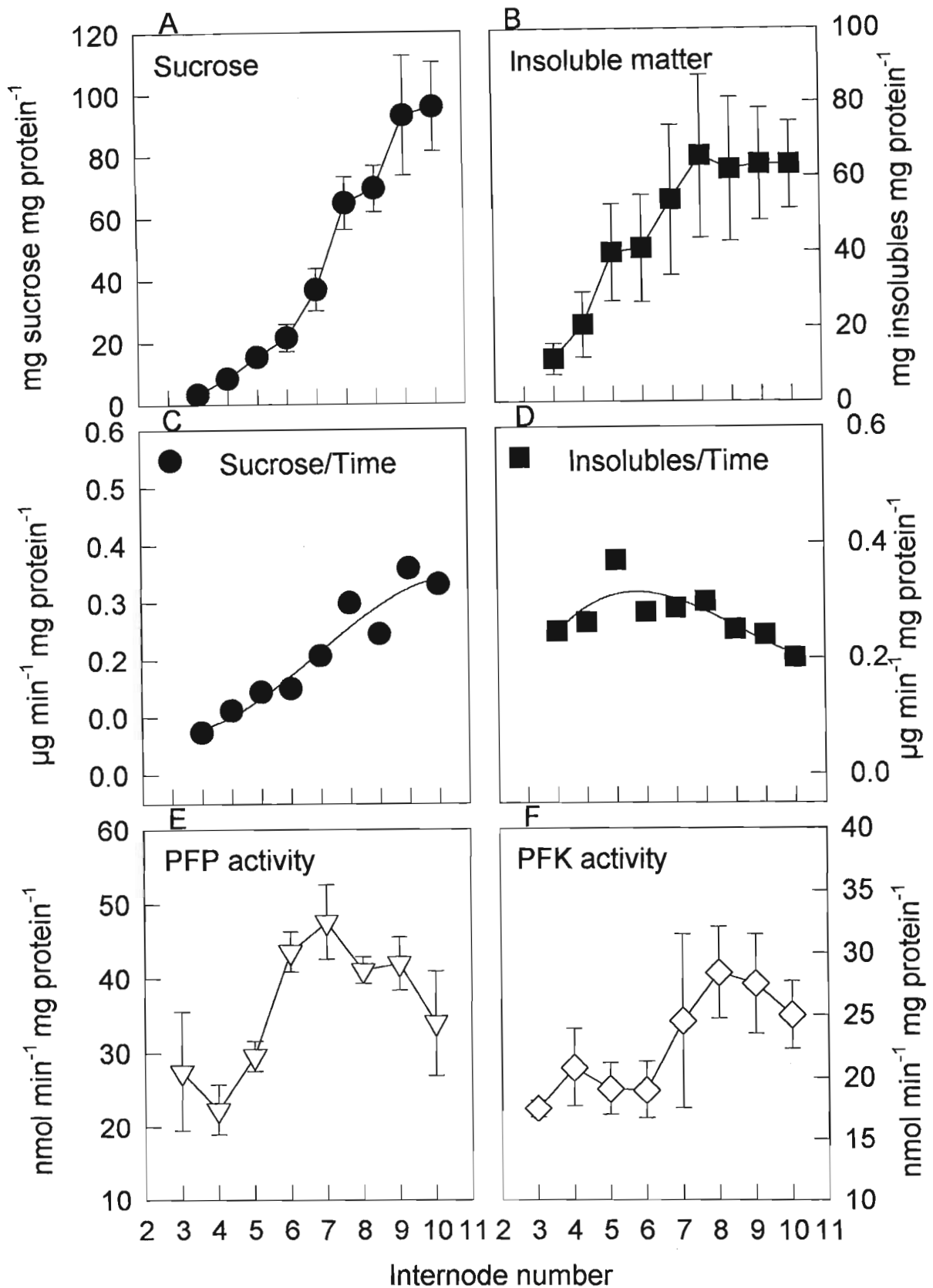
Figure 4.1 Changes in (A) sucrose, (B) insoluble matter, (C) the rate of sucrose and (D) insoluble matter accumulation, (E) PFP and (F) PFK activity in the developing stalk of NCo376.

PFP and PFK activity measurements were not made for internode 2 due to sample browning upon excision of the internodes. There was no correlation between the developmental specific PFP activity profile and sucrose content expressed as a function of total sucrose/total internodal age (r^2 = 0.476, ns). Similarly, no relationship between PFP activity and the insoluble matter content was evident (total insoluble matter/total internodal age, r^2 = 0.115, ns). Additionally, PFK activity was also not correlated to either the rate of insoluble matter synthesis or sucrose synthesis.

4.3.2 ENZYME ACTIVITY, SUCROSE AND INSOLUBLE MATTER BETWEEN HYBRID VARIETIES

4.3.2.1 Specific PFP and PFK activity

To investigate coarse control of PFP between varieties, four additional interspecific hybrids with known differences in maximal sucrose content were selected. The specific PFP activity was determined in internodes, 6, 7 and 8 (Table 4.2) as these contained the highest developmental mean PFP activities (refer to section 4.3.1.1). Differences in the level of PFP activity between the varieties was evident (Table 4.2). An analysis of variance (anova) was conducted using all the PFP data (internodes 6 to 8) from each variety. A LSD value of 7.2 was calculated and PFP activity (internodes 6 to 8) between the varieties was analysed for significance. From this analysis, PFP activity in US6656-15 and Co331 was found to be significantly different from each other and from the remaining three varieties. There was no significant difference in the PFP activity between N19 and N24, and between N19 and NCo376. PFP activities in internode 18 (fully elongated) were approximately 80.0 ± 12 % of that recorded for internode 7, indicating that for each variety, PFP activity did not decline substantially below the mean level observed for internode 7.

PFK activities, measured in internode 7, were comparable between the five varieties (Table 4.3). The PFP/PFK ratio increased from 0.9 (N24), 1.2 (N19, NCo376), 1.8 (Co331) to 2.0 (US6656-15). Similarly, for NCo376 and US6656-15 sampled over a different season, the PFP/PFK ratio in internode 7 was 1.4 and 2.0, respectively.

Table 4.2 Specific PFP activity between internodes 6 and 8 in N24, N19, NCo376, Co331 and US6656-15.

Variety	Internode 6	Internode 7	Internode 8
		PFP activity	
		$(nmol\ min^{-1}\ mg\ protein^{-1})$	
N24	26.6 ± 4.1	25.5 ± 3.1	23.6 ± 4.1
N19	31.1 ± 2.4	32.8 ± 3.5	25.4 ± 3.2
NCo376	34.6 ± 6.5	40.8 ± 5.1	31.0 ± 5.1
Co331	52.0 ± 5.3	47.2 ± 6.3	44.0 ± 3.0
US6656-15	61.5 ± 7.0	69.9 ± 7.4	56.5 ± 3.1

-Each value is the mean ± SD from six separate plants.

Table 4.3 Specific PFK activity in internode 7 of N24, N19, NCo376, Co331 and US6656-15.

Variety	PFK activity
	$(nmol\ min^{-1}\ mg\ protein^{-1})$
N24	27.8 ± 1.1
N19	27.8 ± 6.0
NCo376	33.0 ± 10
Co331	26.8 ± 5.8
US6656-15	33.2 ± 6.4

-Each value is the mean ± SD of four separate plants.

4.3.2.2 Relationship between PFP activity and sucrose/insoluble matter

Between the different varieties, specific PFP activity of internode 7 was correlated to sucrose and insoluble matter content, expressed as a percentage of the dry mass. The average dry mass percentage of the total fresh mass did not vary significantly (29.0 ± 1.5 %) across the varieties sampled. Two additional hybrid varieties, N14 and CP66-1043, were included to extend the range of sucrose/insoluble matter and PFP activity data points. A positive relationship between PFP activity and the insoluble matter was evident in the seven *Saccharum* species hybrids ($r^2 = 0.86$, $p < 0.001$; $F = 246$, $p < 0.005$) (Figure 4.2 A). In contrast, PFP activity was inversely related to sucrose ($r^2 = 0.82$, $p < 0.001$; $F = 182$, $p < 0.005$) (Figure 4.2 B).

4.3.2.3 Relationship between PFP activity and sucrose/insoluble matter content in a segregating population

To determine whether PFP activity was linked to sucrose and insoluble matter content, PFP activity was measured in internode 7 of 70 clones from a segregating population. These measurements were correlated to sucrose and insoluble matter, analysed from stalk sections of duplicate clones. Sucrose and insoluble matter content were expressed as a percentage of the dry mass content of the stalk section. The variation in the dry mass percentage of the total fresh mass across the 70 clones was 23.7 ± 2.7. Results showed a positive relationship between insoluble matter content and PFP activity (Figure 4.3 A), and an inverse relationship between PFP activity and sucrose (Figure 4.3 B). Although correlation coefficients for the linear regression between PFP activity and insoluble matter ($r^2 = 0.22$, $p < 0.001$; $F = 18.6$, $p < 0.005$) and between PFP activity and sucrose ($r^2 = 0.30$, $p < 0.001$; $F = 30$, $p < 0.005$) were low, correlations were significant (Figures 4.3 A and 4.3 B).
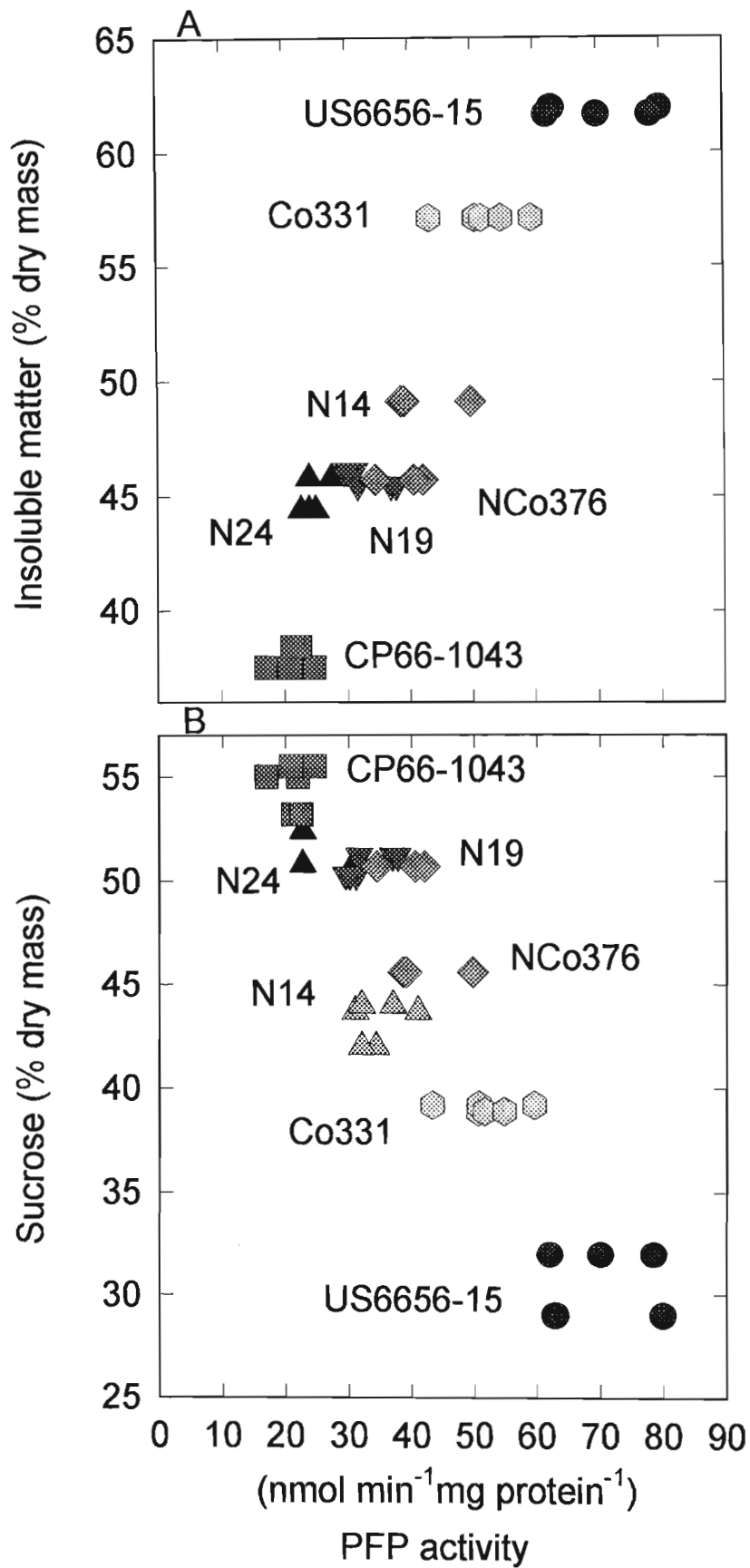
Figure 4.2 The relationship between peak specific PFP activity and (A) insoluble matter and (B) sucrose in N24, N19, N14, NCo376, Co331, CP66-1043 and US6656-15.
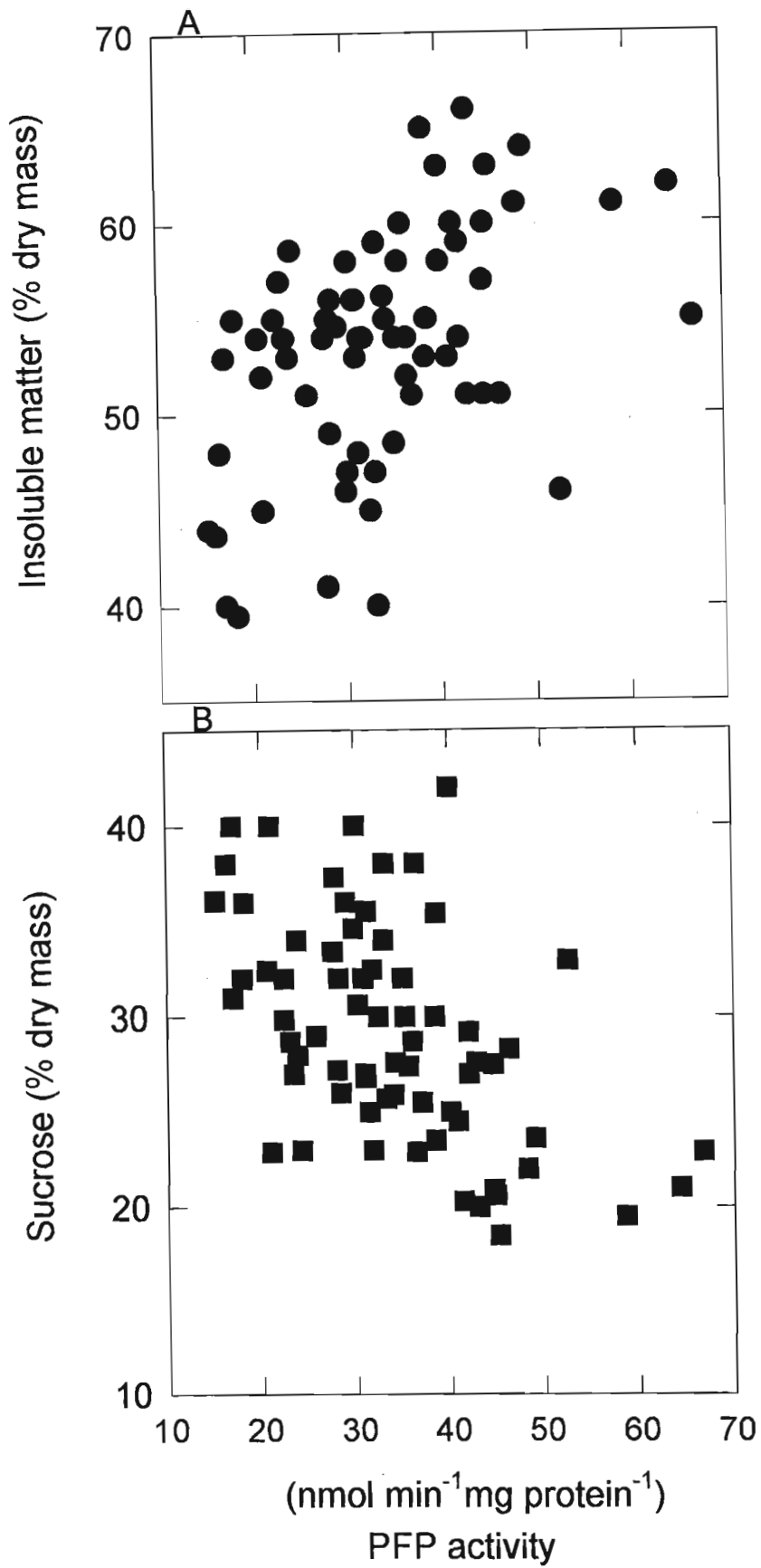
Figure 4.3 The relationship between specific PFP activity in internode 7 and stalk (A) insoluble matter and (B) sucrose content in 70 clones of a F1 segregating population.

4.3.3 PFP PROTEIN STUDIES

4.3.3.1 Protein expression

A polyclonal antibody raised against potato $PFP_{(total)}$ was used to establish whether changing levels of PFP activity were reflected by differences in the relative PFP protein content. Protein blots of internodes 3 to 10 from NCo376, and internode 7 across the five hybrid varieties, revealed a single cross-reacting protein band with a molecular mass of approximately 72 kD (Figures 4.4 and 4.5). The 72 kD polypeptide(s) were quantified and there was a positive relationship between the changes in PFP activity and the level of PFP protein both within the stalk ($r^2 = 0.93$, $p < 0.01$) and between the five varieties ($r^2 = 0.94$, $p < 0.01$). The observed difference in the expression of PFP protein was also evident for the elongating internodes and a subset of hybrid varieties grown over a different season (Figures 4.4 B and 4.5 B).

4.3.3.2 Immuno-inactivation of PFP activity

The potato anti-$PFP_{(total)}$ was effective at immuno-removing sugarcane PFP activity in extracts of NCo376 (Figure 4.6). Over 80.0 % of the PFP activity in internode 7 was precipitated with 20 µL antiserum (Figure 4.6). The anti-$PFP_{(total)}$ was also effective at removing more than 80.0 % (20 µL volume antiserum) of the PFP activity in extracts from internode 7 between sugarcane hybrid varieties.
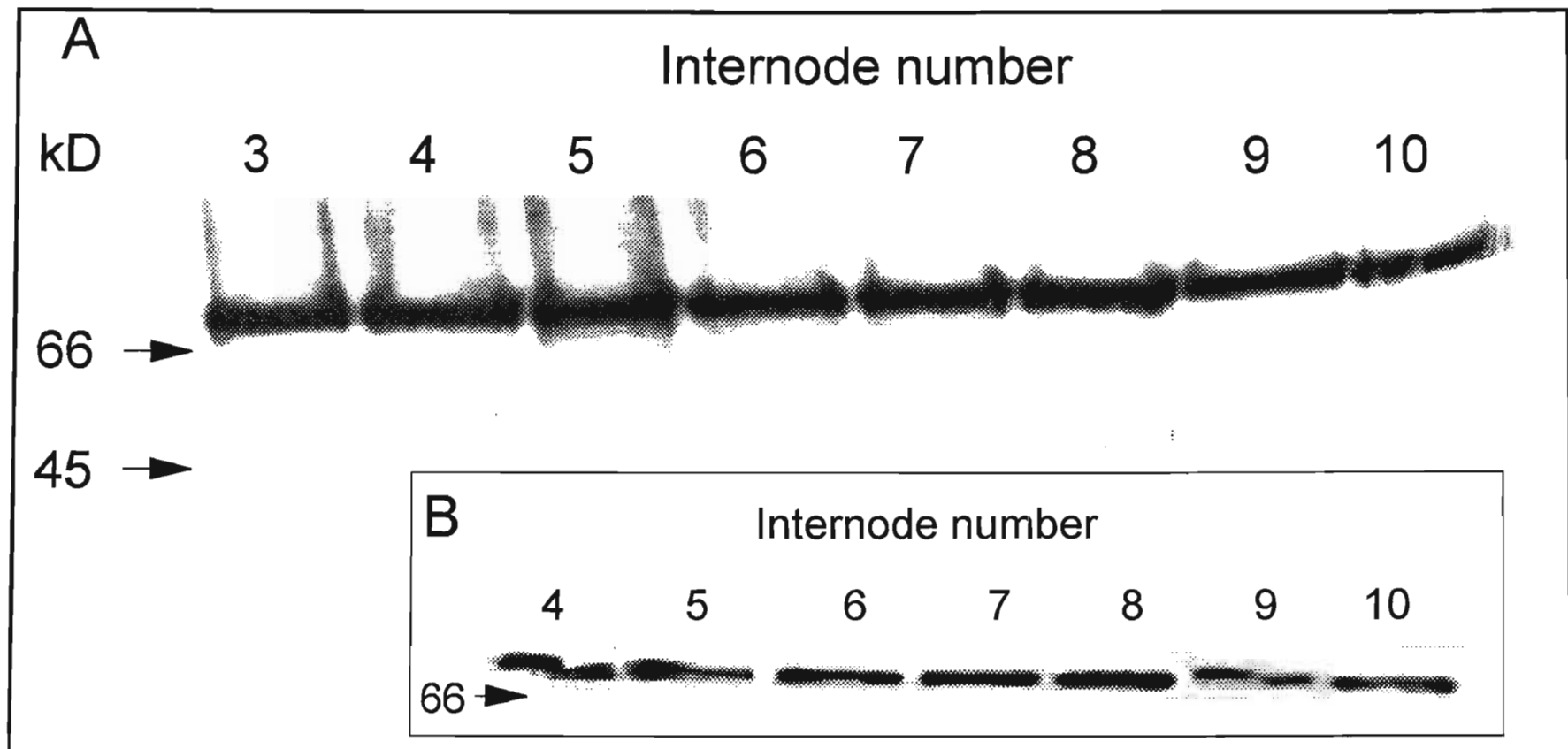
Figure 4.4 Protein blot analysis of PFP (total) content in (A) internodes 3 to 10 of NCo376 and (B) internodes 4 to 10 of NCo376 grown over a different season.   Each sample contained 20 µg protein.
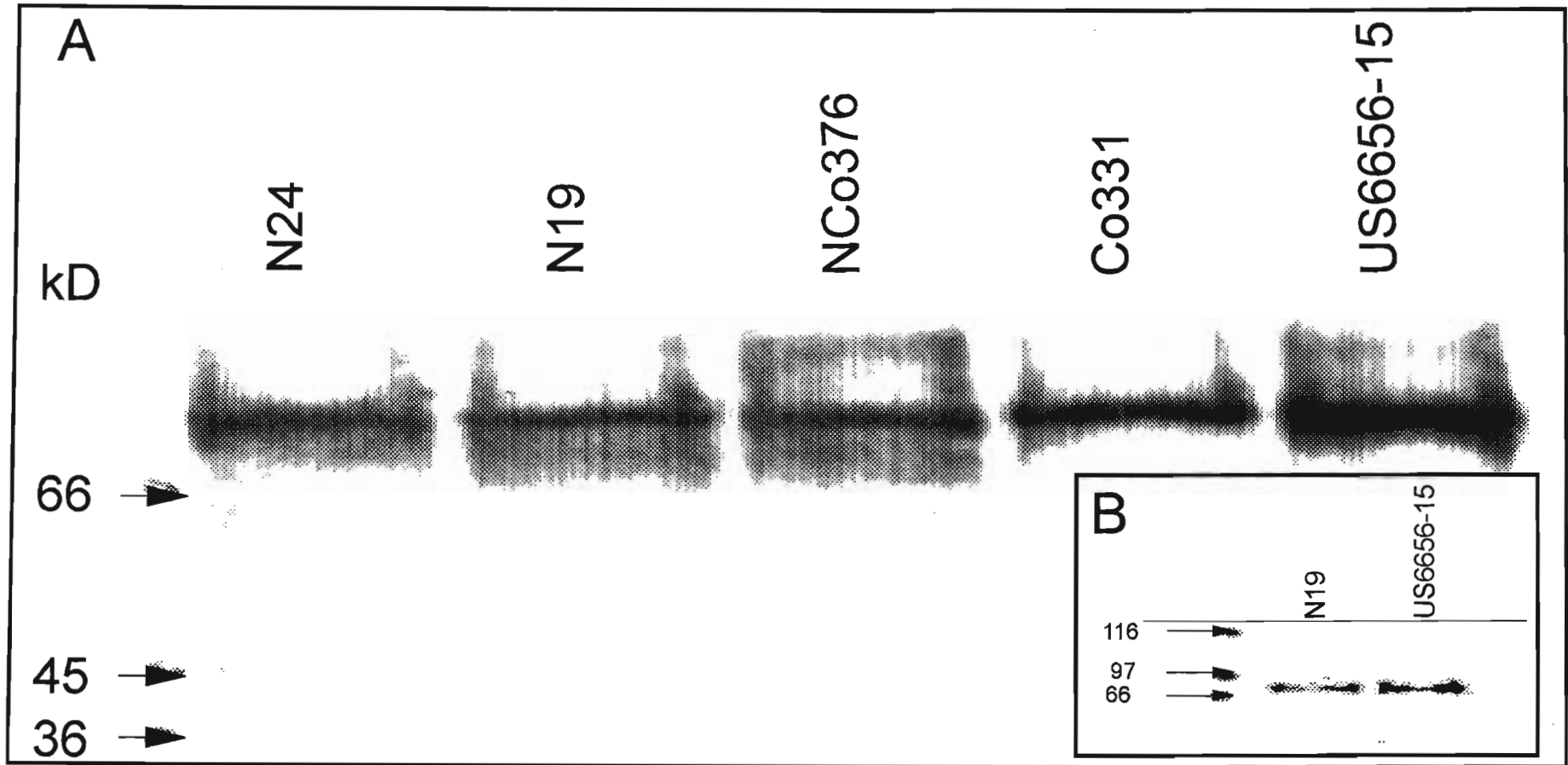
Figure 4.5 Protein blot analysis of PFP (total) content in (A) internode 7 from N24, N19, NCo376, Co331 and US6656-15, and (B) internode 7 from N19 and US6656-15 grown over a different season. Each sample contained 20 µg protein.
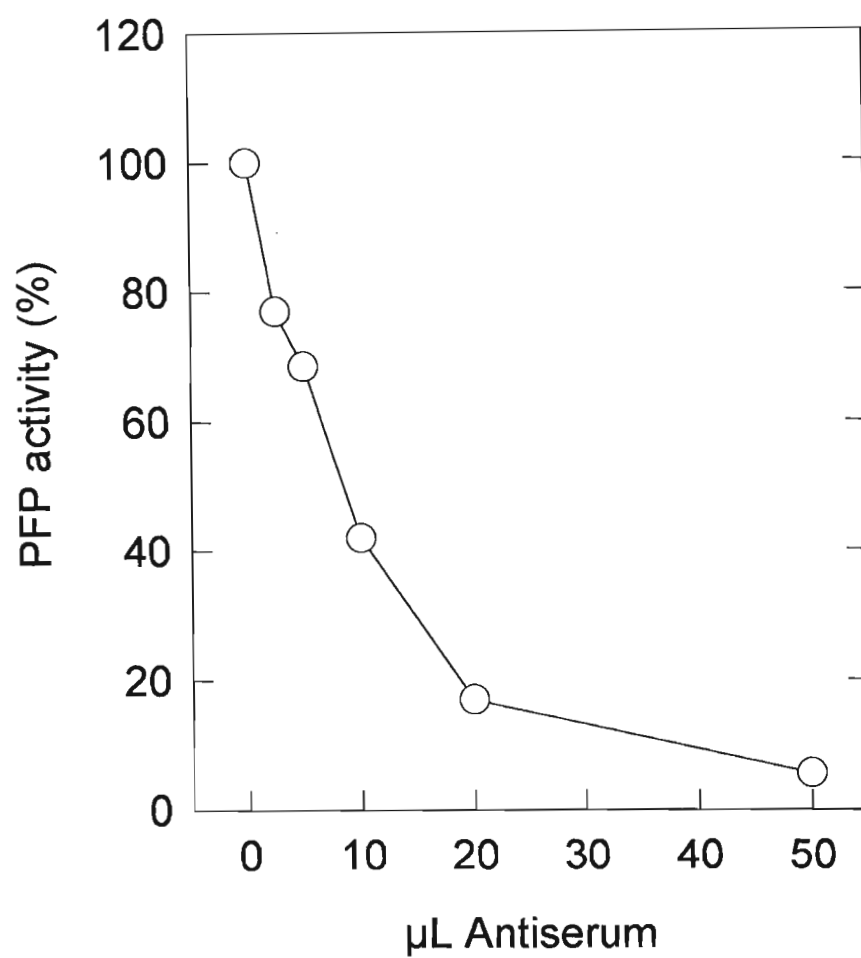
Figure 4.6 Percentage inactivation of PFP activity (internode 7 of NCo376) by potato anti-PFP$_{(total)}$.

4.4 <u>DISCUSSION</u>

It is evident that in sugarcane internodal tissues, PFP activity varies both developmentally and across interspecific hybrids. Both the recovery experiments and lack of inhibitors demonstrate that PFP activity losses during extraction and measurement did not occur. The differences in PFP activity, both within the developing stalk and between the varieties, are reflected by corresponding differences in the relative level of PFP protein. This demonstrates that PFP in sugarcane, as in other species (Spilatro and Anderson, 1988; Botha et al., 1989; Botha and Botha, 1991; Blakeley et al., 1992; Botha and Botha, 1993 b; Nielson, 1994; Podestá and Plaxton, 1994), is in part modulated by coarse regulation of transcriptional and/or translational factors.

Results from the present study are not supportive of a positive relationship between coarse mediated control of PFP and increased sucrose storage in sugarcane. The most convincing observation is the inverse correlation between PFP activity/expression levels and the prevailing sucrose content between hybrid varieties. In sugarcane, the induction of higher expression levels of PFP in the lower sucrose storing hybrids, and the positive relationship between PFP activity and the insoluble matter content, may be related to a long-term requirement for sucrose utilisation rather than storage at any particular stage during development. The manner in which PFP may function to meet the demand for increased sucrose utilisation requires further definitive evidence. In other sink tissues, higher levels of PFP activity have coincided with the increased utilisation of imported sucrose for the biosynthesis of cellular intermediates such as organic acids, amino acids and structural polysaccharides, as well as storage polymer (starch) synthesis (ap Rees et al., 1985 a; ap Rees et al., 1985 b; Ashihara and Horikosi, 1987; Dennis and Greyson, 1987; Enomoto et al., 1990; Hatzfeld et al., 1990; Nakamura et al., 1992; Tobias et al., 1992; Ashihara and Sato, 1993; Enomoto et al., 1994).

is required or where adenylate control may limit glycolytic flux to anabolic respiration (Dennis and Greyson, 1987; Tobias et al., 1992; Ashihara and Sato, 1993; Enomoto et al., 1994; Plaxton 1996). A higher PFP/PFK activity ratio has also been observed during periods of increased PPi-dependent sucrose cleavage (Huber and Akazawa, 1986; Xu et al.,1989; Botha et al., 1992).

There also does not appear to be a positive relationship between coarse mediated control of PFP and sucrose within the developing stalk. The rate of sucrose accumulation between internodes 3 and 10 in the developing stalk is not correlated to the PFP activity rate. Between internodes 3 and 10, the rate of insoluble matter synthesis is constant, whilst the rate of sucrose synthesis increases. It is thus possible that the requirement for energy and cellular intermediates to sustain the combined rates of both insoluble matter synthesis and sucrose accumulation necessitate the induction of PFP activity measured in that region of the stalk. In sugarcane suspension cultures, the presence of appreciable levels of both PFP and PFK activity during net sucrose storage has lent support to the proposal that sucrose storage was not driven by a restriction in respiration (Wendler et al., 1990).

Although differences in the magnitude of the PFP/PFK ratio is reported for other plant species, a similar developmental profile for both enzymes has been suggested to reflect changing biosynthetic activity (ap Rees et al., 1985 a; Ashihara and Horikosi, 1987; Botha and Small, 1987; Wendler et al., 1990; Hagen and Muneta, 1993; Podestá and Plaxton, 1994). The ratio of PFP to PFK activity in developing sugarcane internodal tissues is on average lower (less than two times) than that in other biosynthetically active tissues (Ashihara and Horikosi, 1987; Xu et al., 1989; Ashihara and Sato, 1993; Enomoto et al., 1994). The occurrence of appreciable PFK activities, relative to that of PFP, in both sugarcane and certain other species (Ashihara and Stupavska, 1984; Botha and Small, 1987; Podestá and Plaxton, 1994; Bogatek, 1995), suggest a significant contribution of both enzymes to the regulation of Fru-6-P utilisation.

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From the present study, it is evident that significant levels of PFP activity remain in sugarcane internodes following the cessation of growth. Numerous reports have shown a substantial decline in extractable PFP activity in fully differentiated tissues which are no longer engaged in growth-related biosynthetic activity (ap Rees et al., 1985 a; ap Rees et al., 1985 b; Botha and Small, 1987; Xu et al., 1989; Stitt, 1990; Ashihara and Sato, 1993; Enomoto et al.,1994; Podestá and Plaxton, 1994). Since coarse metabolic control of PFP (and other enzymes) is energetically expensive (Plaxton, 1996), and not a viable option if no further function is served, the present results have interesting implications for metabolism. If carbon partitioning into non-sucrose storage related biosynthetic activity is substantially lower, then the presence of significant levels of PFP activity in the fully elongated, mature internodes (80.0 % that recorded for internode 7) exceeding one year in age may reflect futile substrate cycling. The presence of high levels of SPS (FC Botha, unpublished results) and SuSy (Buczynski et al., 1993) in both elongating and fully elongated internodes strengthens this proposal. Simultaneous cycles of sucrose synthesis and degradation during net mobilisation and storage have been documented for both sugarcane cell suspension cultures and internodal tissues (Sacher et al., 1963; Wendler et al., 1990; Komor, 1994). As the reactions from SuSy to PFP are proposed to be in equilibrium *in vivo* (ap Rees et al., 1977; Kruger et al., 1983 a; Edwards and ap Rees, 1986 b; Weiner et al., 1987; Stitt, 1989; Geigenberger et al., 1993), PFP could conceivably stimulate PPi-dependent cleavage via SuSy and UDPGPPase. In cell suspension cultures of *Chenopodium rubrum*, a substrate cycle between the triose-P and hexose monophosphates, catalysed by PFP (reverse direction) and PFK (forward direction), is considered important in providing PPi for the PPi-dependent sucrose cleavage

pathway and in facilitating carbon partitioning between various biosynthetic activities (Hatzfeld et al., 1990). In sugarcane, cycling of carbon between the triose-P and hexose monophosphates (catalysed by PFP and PFK) may similarly be a focal point in facilitating carbon partitioning into non-storage related biosynthetic activity. This, however, remains to be investigated. The role of PFP activity in catalysing triose-P recycling has recently been demonstrated in transgenic potato tubers, where down-regulation of activity resulted in reduced triose-P recycling and a slight alteration in carbon flux to starch biosynthesis (Hajirezaei et al., 1994).

The specific PFP activities in sugarcane are comparable to other plant species engaged in active biosynthesis (Smyth et al., 1984; Mahajan and Singh, 1989; Xu et al., 1989; Hatzfeld et al., 1990; Wendler et al., 1990; Botha et al., 1992; Mohanty et al., 1993). Developmental profiles of specific PFP activity in sugarcane are consistent over different seasons and contrast with Lingle and Smith (1991), who showed inconsistent seasonal trends and extreme variation in PFP activity measurements in four elongating and fully elongated internodes, respectively. The source of this discrepancy is not known. Specific PFP activity levels reported for NCo376 in the present study, are also approximately two-fold lower than that of the high sucrose storage cultivar used by Lingle and Smith (1991). The lower levels of PFP activity measured in this study could be related to the overall age of the experimental material, which had been growing for approximately 13 months as opposed to the 3 months in the study of Lingle and Smith (1991).

The antigenic relatedness of sugarcane PFP and that of other plant species was investigated from both Western blotting and immunoprecipitation studies. It is evident that the anti-potato PFP$_{(total)}$ serum cross-reacts with a sugarcane protein after immunoblotting, indicating antigenic similarity in the primary structure between the sugarcane PFP and that of other dicotyledonous plant species probed with the antibody (Botha et al., 1988 a; Botha et al., 1989). However, substantial differences in the primary structure are shown to exist. Firstly, a single band cross-reacting with the anti-potato PFP$_{(total)}$ is resolved across the interspecific sugarcane varieties. This is

contrary to previous observations which show cross-reactivity between two distinctive polypeptides, comprising the larger α- and smaller β subunit, respectively (Botha et al., 1988 a; Botha et al., 1989; Botha and Botha, 1991). Secondly, the molecular mass of the sugarcane PFP polypeptide(s) is approximately 72 kD, which is substantially larger than the 65 - 68 kD (α subunit) and 60 - 64 kD (β subunit) polypeptides resolved for other dicotyledonous (Kruger and Dennis, 1987; Botha et al., 1988 a; Botha et al., 1989; Carlisle et al., 1990; Cheng and Tao, 1990; Botha and Botha 1991; Botha and Botha 1993 b; Podestá and Plaxton, 1994) and monocotyledonous (Yan and Tao, 1984; Nielson, 1994) species.

Despite differences in the primary structure, authenticity of the cross-reacting 72 kD polypeptide(s) as PFP is provided by the immunoprecipitation studies. The anti-potato PFP$_{(total)}$ is shown to be effective at precipitating over 80 % of the PFP activity in crude extracts across the interspecific hybrid varieties investigated. Not only does this result suggest that most of the measurable PFP activity is associated with 72 kD polypeptide(s), but also indicates immunological similarity between sugarcane PFP and that of other plant species with respect to catalysis.

The presence of the single cross-reacting band does not negate the existence of the two subunits, which in sugarcane may be of a similar size, as has been demonstrated for mung bean (Cheng and Tao, 1990) and carrot (Wong et al., 1988). The difference in the molecular weight between the sugarcane PFP polypeptide(s) and those of other species may reflect differences in the length of the coding sequence of the PFP gene or in the amino acid sequence. The physiological significance of the larger polypeptide and the possible effect on the kinetic properties of sugarcane PFP will require further investigation.

Conclusion

From the present investigation it is evident that coarse mediated control of PFP activity

is not correlated to sucrose storage. Specific PFP activity trends within the developing stalk and across interspecific hybrids lend support to this proposal, highlighting the importance of considering both developmental and cross-varietal tissue comparisons when investigating coarse control of an enzyme to a particular trait. In sugarcane, the levels of PFP activity appear to be controlled by PFP protein expression. The primary structure of the sugarcane PFP protein differs from that of other species in that the polypeptide(s) are resolved as a single band with a molecular mass of approximately 72 kD. PFP activity in sugarcane is comparable to that of other plant species engaged in biosynthetic activity. It is hypothesised that PFP activity in internodal tissues is likely to be associated with metabolic processes involving sucrose degradation (and utilisation) rather than increased storage. The manner in which PFP may function to meet such a requirement in sugarcane necessitates further investigation. However, it is possible that PFP activity may reflect an increased contribution to non-sucrose storage related biosynthetic activity, either directly by providing additional capacity for Fru-6-P utilisation or as a consequence of substrate cycling between the triose-P and hexose monophosphates.

CHAPTER 5

CARBON PARTITIONING IN THE DEVELOPING STALK AND ACROSS DIFFERENT VARIETIES

5.1 INTRODUCTION

From differences in specific PFP activity, both within the developing stalk and between interspecific hybrids, it was proposed that PFP activity is likely to be associated with metabolic processes involving sucrose degradation (and utilisation) rather than increased storage (refer to Chapter 4). To test this proposal, and to further identify physiological processes which may influence sucrose accumulation in sugarcane, changes in carbon partitioning between storage, polysaccharide synthesis and respiration require characterisation.

In an attempt to understand the regulation of sucrose accumulation in sugarcane, research emphasis has largely been placed on studying the turnover of sucrose by cycles of simultaneous synthesis and degradation during net storage (Sacher et al., 1963; Glasziou and Gaylor, 1972; Wendler et al., 1990; Veith and Komor, 1993; Komor, 1994). Carbon cycling within the sugar pool is suggested to facilitate switches in carbon partitioning between product accumulation and mobilisation in sink tissues (Dancer et al., 1990 a; Hubbard et al., 1990; Wendler et al., 1990; Geigenberger and Stitt, 1991 a; MacRae et al., 1992; Geigenberger and Stitt, 1993). Despite proposals that cycles of sucrose synthesis and degradation are likely to play a role in regulating sucrose storage in sugarcane internodes (Sacher et al., 1963; Komor 1994), the partitioning of incoming carbon between different carbohydrate pathways remains to be characterised.

In sugarcane internodal tissue, knowledge on the partitioning of incoming carbon between different cellular fractions is largely limited to the constituents of the sugar pool (Sacher et al., 1963; Hatch, 1964; Glasziou and Gaylor, 1972; Batta and Singh, 1986; Lingle, 1989). The proportion of carbon either metabolised directly by non-sucrose storage related biosynthetic activity or lost from the sugar pool has not been further characterised (Sacher et al., 1963; Hatch, 1964; Bowen and Hunter, 1972; Glasziou and Gaylor, 1972; Batta and Singh, 1986; Komor et al., 1996).

As growth and sucrose storage occur concomitantly in developing sugarcane internodes (Fernandes and Benda, 1985), a coordinated control of carbon flux between different metabolic pathways must exist. Hence, there is a requirement for more extensive characterisation of carbon partitioning between sucrose storage and non-sucrose storage related biosynthetic activity. As respiration comprises several highly regulated pathways including glycolysis, the OPP pathway, the TCA cycle and electron transport (Lambers, 1985; Wiskich and Dry, 1985; ap Rees, 1990; Plaxton, 1996), which in turn may influence sucrose turnover in sugarcane internodal tissues, carbon partitioning into respiration warrants further investigation. In particular, glycolytic enzyme activity, including PFP, has been considered important in mediating carbon partitioning and flux to meet respiratory demands in other tissues (Dennis and Greyson, 1987; Plaxton, 1996).

In heterotrophic tissues, the provision of energy is solely dependent on the catabolic role of respiration, which is characterised by the complete oxidation of carbohydrate to generate reducing equivalents for ATP production. The energy requirement derived from catabolic respiration (measured either by CO_2 production or O_2 consumption) for both pre-storage related biosynthetic activity, and the synthesis, accumulation and remobilisation of storage products, appears to vary according to tissue type (Stitt and Steup, 1985). In sugarcane suspension cells grown in continuous culture, carbon partitioning into respiration (measured from O_2 consumption) and insoluble matter synthesis decreases with a concomitant increase in the sucrose content when cells are transferred to nitrogen limiting conditions (Veith and Komor, 1993). The rate of

respiration is similarly shown to decrease (Veith and Komor, 1993). However, since the cells were maintained under continuous culture, the change in respiratory carbon partitioning could not have accounted for *in vivo* developmental changes in the energy requirement relating to growth and storage. In contrast, developmental transitions in sucrose synthesis and mobilisation in sugarcane suspension cells, grown under batch culture, are shown to occur without any change in the rate of respiration (Wendler et al., 1990). Composite changes in the partitioning of carbon between sucrose storage, structural polysaccharide synthesis and O_2 production are not documented for sugarcane cells grown under batch culture (Wendler et al., 1990). From a very recent paper, it appears that sucrose accumulation coincides with a decrease in partitioning and flux of carbon into the 'growth and energy use component' (collectively measured as respiration) in developing internodal sugarcane tissues (Komor et al., 1996).

The catabolic role is not the sole function of respiration. A significant proportion of partially oxidised TCA intermediates are utilised in the biosynthesis of numerous cellular intermediates. Partitioning of carbon into anabolism (particularly amino and organic acid biosynthesis) is reported to be higher in immature sugar and starch sinks (Giaquinta, 1979; Stitt and Steup, 1985; ap Rees, 1990; Wendler et al., 1990; Komor, 1994). Partitioning and carbon flux into the anabolic respiratory component can not be accurately determined from measurements of O_2 consumption or CO_2 release.

The aim of the present study was to characterise changes in carbon partitioning, both within the developing sugarcane stalk and across different varieties, in relation to sucrose content. Particular emphasis was placed on investigating developmentally related changes in both carbon partitioning and flux into respiratory processes. The objective was to identify prospective metabolic pathways, which potentially influence partitioning, and to clarify the proposed involvement of PFP in processes of sucrose degradation. The approach taken in the present study was to examine carbon partitioning from distribution patterns of radiolabelled carbon after supplying uniformly labelled sucrose, glucose and fructose, and specifically labelled glucose to internodal tissue slices.

It is reported that maturation in the developing stalk coincided with a redirection of carbon from the insoluble matter and total respiration into sucrose. Between sugarcane varieties with significant variation in sucrose content, an inverse relationship exists between carbon allocated to sucrose storage and respiration. Expressed on a protein basis, changing patterns of carbon partitioning into respiration are also reflected by changes in the rate of carbon flux.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

5.2.1.1 Biochemicals

[U-^{14}C]sucrose, [U-^{14}C]fructose, [1-^{14}C] and [6-^{14}C]glucose were supplied by Amersham International.   Cation (Dowex AG 50W, 200 to 400 mesh, hydrogen form) and anion (Dowex AG 1-X8, 100 to 200 mesh, chloride form) exchange resins were from Bio-Rad. Acid invertase was purchased from the Sigma Chemical Company.

5.2.1.2 Plant material

For radiolabelling studies on the developing stalk, the commercial variety NCo376 was sampled.   The interspecific hybrid US6656-15 and the *S. spontaneum* Coimbatore, were included for cross-varietal comparisons.   Details pertaining to the sampling of the plants are recorded in section 3.1.2.

5.2.2 METHODS

5.2.2.1 Sucrose and insoluble matter determinations

The excision of internodal material is reviewed in section 3.2.1.   Sucrose was extracted and measured as recorded in section 3.2.2.   Sucrose and insoluble matter content were expressed on a tissue dry mass basis.   Determination of insoluble matter and tissue dry mass are described in section 3.2.3 and section 3.2.4, respectively.

5.2.2.2 Protein measurements

The methods for extraction and measurement of soluble protein are recorded in section 3.2.5 and section 3.2.6.

5.2.2.3 Extraction and measurement of PFP and PFK activity

PFP and PFK were extracted and measured as described in sections 3.2.5, 3.2.5.1 and

3.2.5.2.

5.2.2.4 ^{14}Carbon labelling studies

[U-^{14}C]sucrose, [U-^{14}C]glucose, [U-^{14}C]fructose, [1-^{14}C] and [6-^{14}C]glucose were used in the labelling studies at a concentration of 2.0 GBq mmol^{-1}.

Tissue preparation, labelling and extraction

Preparation of internodal tissues for labelling is reviewed in section 3.2.7.1. As with the [U-^{14}C]glucose labelling, tissues supplied with [U-^{14}C]sucrose and fructose were incubated at 28°C on a rotary shaking incubator at 175 rpm for either 5 h or 5.5 h. Tissues supplied with [1-^{14}C]glucose and [6-^{14}C]glucose were prepared similarly, but incubated for both 45 min and 3 h periods. The method for the extraction of labelled tissues is presented in section 3.2.7.2.

Ion exchange separation of the water-soluble fraction

Fractionation of the water-soluble fraction was conducted according to Dickson (1979). Ion exchange columns were prepared in 2 mL syringes. Dowex cation (AG 50W-X4) and anion (AG 1-X8) exchange resins (loaded with H+ and COOH$^-$) in 80 % (v/v) ethanol were added to a final bed length of 2 cm and washed to neutral pH with 80 % (v/v) ethanol. The water-soluble fraction was loaded onto the cation exchange column, the eluent collected and reloaded a further two times. The final eluent was then loaded onto the anion exchange column in a similar manner. A tandem column was prepared by connecting the cation exchange column over the anion exchange column. The neutral fraction was eluted from the tandem column with 80 % (v/v) ethanol and 60 mL eluate collected. After separating the columns, the amino acids were eluted from the cation exchange column with 30 mL 4 M NH$_4$OH in 80 % (v/v) ethanol. Organic acids were eluted similarly from the anion exchange column with 30 mL 6 M HCOOH in 80 % (v/v) ethanol. After removal of the organic acids, phosphorylated intermediates were eluted from the anion exchange column with 20 mL 2 M HCl. Radioactivity in the samples was determined as in section 3.2.7.2

Fractionation of the neutral water-soluble component

This method is described in section 3.2.7.3. Labelling of the glucosyl and fructosyl moieties of sucrose was determined by boiling the sucrose for 2 h in 75 mM citric acid and then separating the hexose sugars by HPLC.

5.3 RESULTS

5.3.1 METABOLISM IN THE DEVELOPING SUGARCANE STALK

5.3.1.1 Dry mass, sucrose, protein and PFP activity

The percentage moisture content of the total internodal fresh mass decreased from internodes 2 to 10, and this was reflected by an increase in the tissue dry mass (Table 5.1). Soluble protein content decreased from internodes 2 to 10 (Table 5.1). Internodal sucrose content increased to form a higher proportion of the dry mass (Table 5.1). Within the top ten internodes, the percentage sucrose composition of the dry mass reached a maximum by internode 7 (43.1 ± 5.5 %) and showed no further increase (Table 5.1). Due to the sharp sucrose gradient, internodes 2 to 7 were selected to study sink carbon partitioning since any changes in metabolism associated with increasing sucrose storage are most likely to be detected in these internodes. As reported previously (4.3.1.2), to better represent the contribution of the cytosolic compartment to metabolism, all measurements of ^{14}C incorporation into the cellular fractions were expressed on a protein basis. Specific PFP activities in internodes 3, 5 and 7 ranged from 25.0 ± 4.0 to 29.7 ± 6.0 and 36.0 ± 2.0 nmol min^{-1} mg protein^{-1}, respectively. PFK activity was not determined in internode 3, but was 26.3 ± 5.0 nmol min^{-1} mg protein^{-1} in internode 5, and 28.3 ± 6.0 nmol min^{-1} mg protein^{-1} in internode 7.

5.3.1.2 Carbon partitioning of [U-^{14}C]sucrose

As sucrose is the source substrate translocated to the stem tissues in vivo, carbon allocation into the cellular components was first investigated by supplying tissue slices with [U-^{14}C]sucrose. Of the labelled sucrose supplied, 45.0 % (internode 2), 28.0 % (internode 3), 11.4 % (internode 5) and 7.7 % (internode 7) was taken up by a standard 1.5 g tissue over 5.5 h. Following labelling, a higher extracellular sucrose concentration was detected in the washing medium containing internode 7 tissue slices (3.0 ± 0.52 µmol gram fresh mass^{-1}) than that containing internode 2 tissues (0.84 ± 0.20 µmol gram fresh mass^{-1}). Although sucrose uptake by the younger internodes

Table 5.1 Dry mass, sucrose and soluble protein content in internodes 2 to 10 of NCo376.

Internode number	Tissue dry mass	Sucrose content	Protein content
	(%)	*(mmol gram dry mass^{-1})*	*(mg gram dry mass^{-1})*
2	7.59 ± 0.78	0.42 ± 0.03	29.52 ± 8.17
3	9.19 ± 0.68	0.59 ± 0.17	15.50 ± 2.83
5	11.89 ± 0.89	0.83 ± 0.13	7.96 ± 1.76
7	16.50 ± 1.20	1.26 ± 0.16	6.02 ± 1.12
10	23.20 ± 2.27	1.35 ± 0.20	4.18 ± 0.28

-Each value is the mean ± SD of three separate samples.

was higher, the increased apoplastic sucrose concentration in internode 7 would likely have contributed to a lower uptake of labelled substrate. The total ^{14}C recovered in the cellular components of extracted tissues was 92.2 %, 106.0 %, 112.0 % and 111.0 % for internodes 2, 3, 5 and 7, respectively. The percentage distribution of ^{14}C in sucrose increased from internodes 2 to 7 (Table 5.2). Conversely, there was a decreased percentage ^{14}C allocated to non-sucrose storage related biosynthetic activity (Table 5.2). This included allocation to the insoluble matter, the acidic (organic acids and phosphorylated intermediates)/basic (amino acids) water-soluble component, and CO_2 production. $^{14}Carbon$ partitioning into the lipid-soluble fraction was negligible in all internodes investigated (Table 5.2).

5.3.1.3 Metabolism of [U-^{14}C]glucose

Carbon partitioning

To characterise the partitioning of carbon between net sucrose synthesis and remaining biosynthetic activity, tissue slices were labelled with [U-^{14}C]glucose. Viola (1996) reported that radiolabelled hexose precursors, supplied to excised tissue discs, can be converted to sucrose prior to uptake. Direct incorporation of labelled carbon into sucrose through isotopic equilibrium (via SuSy) was reported to be extensive when fructose was used as a precursor, whereas the metabolism of labelled glucose prior to uptake was minimal (Viola, 1996). In the present study, the effect of possible extracellular metabolism of [U-^{14}C]glucose (particularly sucrose synthesis) on the pattern of carbon partitioning in excised sugarcane internodal tissues was investigated. Due to the storage of higher levels of sucrose, tissue discs from internode 7 were selected for analysis. The inclusion of 200 IU acid invertase in the labelling medium to prevent apoplastic synthesis of sucrose, had no effect on the percentage ^{14}C allocation to the sugars (Table 5.3). Sucrose comprised 90.0 % of the total sugar pool in the samples analysed. The percentage carbon allocation to the insoluble matter and the respiratory components was comparable between tissue slices incubated in either the absence or presence of acid invertase (Table 5.3).

Table 5.2 Incorporation of ^{14}C and percentage distribution (in parenthesis) into the cellular components of tissue slices from elongating internodes of NCo376, incubated with [U-^{14}C]sucrose for 5.5 h.

Cellular components	Internode 2	Internode 3	Internode 5	Internode 7
	(kBq mg protein^{-1})			
CO_2	1.67 ± 0.35	0.80 ± 0.26	0.42 ± 0.02	0.09 ± 0.03
	(6.75)	**(4.08)**	**(3.54)**	**(1.19)**
Water-soluble				
-sucrose	7.58 ± 1.12	8.51 ± 3.89	7.86 ± 0.35	5.43 ± 1.60
	(30.63)	**(43.23)**	**(66.16)**	**(72.02)**
-glucose	0.81 ± 0.09	1.04 ± 0.26	0.54 ± 0.07	0.38 ± 0.04
	(3.27)	**(5.31)**	**(4.55)**	**(5.04)**
-fructose	0.64 ± 0.06	0.66 ± 0.16	0.45 ± 0.07	0.20 ± 0.05
	(2.59)	**(3.37)**	**(3.79)**	**(2.65)**
-acidic/basic component	5.75 ± 1.05	5.38 ± 1.15	1.78 ± 0.80	0.95 ± 0.01
	(23.23)	**(27.45)**	**(14.98)**	**(12.60)**
Water-insoluble	8.00 ± 0.86	3.00 ± 0.52	0.71 ± 0.17	0.42 ± 0.26
	(32.32)	**(15.31)**	**(5.98)**	**(5.57)**
Lipid-soluble	0.30 ± 0.11	0.21 ± 0.10	0.12 ± 0.03	0.07 ± 0.01
	(1.21)	**(1.07)**	**(1.01)**	**(0.98)**
Total uptake	24.75 ± 3.64	19.60 ± 6.34	11.88 ± 1.51	7.54 ± 2.00

-The acidic/basic component comprises the amino acids, organic acids and phosphorylated intermediates.

-Each value is the mean ± SD of three separate samples.

Table 5.3 Percentage distribution of ^{14}C from uniformly labelled glucose into the cellular components of tissue slices from internode 7 of NCo376, in the absence and presence of 200 IU acid invertase. The acid invertase was included in the incubation medium during the 5.5 h labelling period.

Cellular constituents	Internode 7 (- invertase)	Internode 7 (+ 200 IU invertase)
	(%)	
Sugars	65.68	65.32
CO_2, acidic/basic and lipid-soluble components	15.51	14.74
Insoluble matter	18.81	19.95

-The percentage distribution values for each treatment are from a single labelling experiment using a bulked tissue sample from three separate plants.

Tissues slices, excised from internodes 2, 3, 5 and 7, were labelled with [U-^{14}C]glucose. The uptake of [U-^{14}C]glucose was 61.4 % (internode 3), 51.6 % (internode 5) and 45.5 % (internode 7). Recovery of the ^{14}C taken up by the tissue slices was 103.0 %, 98.8 %, 95.0 % and 99.6 % for internodes 2, 3, 5 and 7, respectively. In all internodes investigated, more than 90.0 % of the incorporated [U-^{14}C]glucose was metabolised (Table 5.4). The carbon metabolised was defined as the total ^{14}C recovered in cellular components other than in glucose at the end of the 5.5 h labelling period. The increased percentage of ^{14}C recovered in sucrose from internode 2 to 7 reflected an increased partitioning of hexose monophosphates into sucrose synthesis. The pattern of carbon partitioning into sucrose was similar in tissues labelled with either sucrose or glucose (Tables 5.2 and 5.4). Less carbon was allocated to the insoluble matter and respiration in the more mature internodal tissues (Table 5.4).

Respiratory substrate in plants is divided between the provision of carbon for the complete oxidation to CO_2 and intermediates for biosynthesis. The ^{14}C recovered in CO_2 production (catabolic respiration), the amino acids, organic acids and lipids (anabolic respiration) were summed as an indicator of the total carbon partitioned into the respiratory pathway. Of the ^{14}C-glucose metabolised, 27.1 %, 18.5 % and 14.9 % entered respiration in internodes 2, 3 and 7, respectively (Table 5.4). CO_2 production as a percentage of total respiration, was comparable in internodes 2 (32.7 %), 5 (24.2 %) and 7 (27.3 %). In combination, the insoluble matter and non-sugar, water-soluble components were the dominant competitors for incoming carbon in younger internodes, since more than 90.0 % of the increase in sucrose content during maturation was ascribed to a reduction in the labelling of these two pools (Tables 5.2 and 5.4).

There was no further increase in the percentage carbon (from ^{14}C-glucose) allocated to sucrose storage in the fully elongated internode 18 (Table 5.5). Similarly, the partitioning of [U-^{14}C]glucose into the insoluble matter and respiratory components was comparable in tissue slices from internodes 7 and 18 (Table 5.5). Although at the time

Table 5.4 Incorporation of ^{14}C and percentage distribution (in parenthesis) into the cellular components of elongating internodal tissue slices from NCo376, supplied with [U-^{14}C]glucose for 5.5 h.

Cellular components	Internode 2	Internode 3	Internode 5	Internode 7
	(kBq mg protein^{-1})			
CO$_2$	3.82 ± 0.1 **(8.55)**	2.47 ± 0.1 **(5.60)**	2.56 ± 0.3 **(4.39)**	1.78 ± 0.3 **(3.82)**
Water-soluble				
-Sugars				
-sucrose	12.70 ± 1.4 **(28.43)**	21.50 ± 7.2 **(48.76)**	28.60 ± 9.8 **(49.05)**	30.10 ± 6.1 **(64.66)**
-glucose	1.51 ± 0.7 **(3.38)**	2.01 ± 0.9 **(4.56)**	2.62 ± 0.8 **(4.49)**	2.87 ± 1.0 **(6.17)**
-fructose	1.02 ± 0.3 **(2.28)**	0.79 ± 0.30 **(1.79)**	0.83 ± 0.4 **(1.42)**	0.77 ± 0.4 **(1.65)**
-acidic/basic		4.03 ± 0.80 **(9.14)**		
-amino acids	1.83 ± 0.2 **(4.10)**	ND	1.77 ± 0.6 **(3.04)**	0.97 ± 0.2 **(2.08)**
-organic acids	5.71 ± 2.2 **(12.78)**	ND	5.40 ± 1.1 **(9.26)**	2.83 ± 0.5 **(6.08)**
-phosphorylated intermediates	1.36 ± 0.5 **(3.04)**	ND	0.99 ± 0.10 **(1.70)**	0.30 ± 0.10 **(0.64)**
Water-insoluble	16.40 ± 0.2 **(36.71)**	12.80 ± 2.4 **(29.03)**	14.70 ± 2.4 **(25.21)**	5.99 ± 2.0 **(12.87)**
Lipid-soluble	0.32 ± 0.2 **(0.72)**	0.49 ± 0.10 **(1.11)**	0.83 ± 0.10 **(1.01)**	0.94 ± 0.2 **(2.02)**
Total uptake	44.67 ± 5.8	44.09 ± 11.8	58.30 ± 15.6	46.55 ± 9.2

-Each value is the mean ± SD of three separate samples.

Table 5.5   The percentage distribution of ^{14}C in the cellular components of tissue slices from elongating (internode 7) and fully elongated (internode 18) internodes of NCo376, supplied with $[U-^{14}C]$glucose for 5 h.

Cellular component	Internode 7	Internode 18
	(%)	
CO_2	5.87 ± 0.41	5.29 ± 0.96
Water-soluble		
sucrose	59.27 ± 3.00	59.85 ± 5.99
glucose	3.77 ± 0.25	4.43 ± 0.41
fructose	0.86 ± 0.13	1.11 ± 0.17
acidic/basic	11.50 ± 0.75	13.07 ± 4.39
Water-insoluble	17.45 ± 1.19	14.28 ± 1.26
Lipid-soluble	1.28 ± 0.71	1.98 ± 0.09

-The acidic/basic component comprises the amino acids, organic acids and phosphorylated intermediates.

-Each value is the mean ± SD of three separate samples.

of sampling, the sucrose content in internodes 7 and 18 was 44.0 ± 4.0 % gram dry mass^{-1} and 50.9 ± 3.0 % gram dry mass^{-1}, respectively, once sucrose content (per gram dry mass) was closer to the maximum, the proportion of carbon allocated to other biosynthetic activity remained constant. The total uptake of ^{14}C- glucose into tissue slices of internode 18 was 80.3 % of that recorded for internode 7. The percentage ^{14}C incorporation into respiration in internode 7 tissue slices (Table 5.5) was slightly higher than that recorded previously (Table 5.4) and this was attributed to seasonal variation.

Relative contribution of SPS and SuSy to sucrose labelling

To attempt to separate the relative contribution of SuSy to sucrose labelling, tissue slices were supplied with [U-^{14}C]glucose. The approach used is based on the analysis of the intramolecular labelling pattern of sucrose. If it is assumed that glucose labelling leads to rapid isotopic equilibrium of the hexose monophosphates, then sucrose synthesis from SPS will result in both moieties of sucrose being equally labelled. In contrast, it is likely that any movement of label into sucrose via SuSy will lead to a higher proportion of labelling in the glucosyl moiety of the sucrose molecule (Geigenberger and Stitt, 1993). According to Geigenberger and Stitt (1993), the relative contribution of SuSy to sucrose synthesis can be estimated as follows:

$$\% \text{ SuSy} = \left\{ 1 - \frac{^{14}\text{C in fructosyl moiety of sucrose}}{^{14}\text{C in glucosyl moiety of sucrose}} \right\} \times 100$$

Comparable amounts of label in the glucosyl and fructosyl moieties of sucrose were evident in internodes 6 to 18 (Table 5.6). A dissimilar ratio of label in the glucosyl to fructosyl moiety of sucrose was evident in the younger internodes (Table 5.6). Using the above mentioned equation, the contribution of SuSy to unidirectional sucrose was estimated and shown to decrease from approximately 48.0 % (internode 2), 37.8 % (internode 3) to 7.4 % (internode 6).

Although results suggest that SuSy does make an important contribution to sucrose synthesis in the younger internodes, the percentage values calculated (Table 5.6) likely

Table 5.6 Incorporation of ^{14}C into the glucosyl and fructosyl moieties of sucrose in internodal tissue slices of NCo376 supplied with [U-^{14}C]glucose for 5 h. Values are the ratio of ^{14}C in the glucosyl moiety/fructosyl moiety of sucrose.

Ratio	Internode 2	Internode 3	Internode 5	Internode 6	Internode 7	Internode 18
^{14}C-Glc / ^{14}C-Fru in extracted sucrose	1.94	1.63	1.38	1.08	1.17	1.07

-Each ratio is representative of a single labelling experiment using a bulked tissue sample from three separate plants.

reflect an underestimation. The experimental rationale depends on there being negligible recycling of label from [U-^{14}C]glucose to the endogenous fructose pool (Geigenberger and Stitt, 1993). Yet, it is evident that fructose does become labelled over the experimental period (Table 5.4). The extent to which the specific activity of the fructose pool approaches that of the hexose monophosphate/UDPGlc pool is a measure of the extent to which the percentage contribution of SuSy (to sucrose synthesis) will become increasingly underestimated (Geigenberger and Stitt, 1993). Since the neither the concentration of the hexose monophosphate/UDPGlc pool or the uptake of ^{14}C into the latter was measured, the specific activity could not be determined and compared to the specific activity of the fructose pool. Additionally, the lack of suitable techniques to quantify the subcellular sugar (and ^{14}C-sugar) distribution also precluded absolute measurements of recycling in the cytosolic compartment. As a result, it was not possible to assess the magnitude by which SuSy activity was underestimated in the tissues of maturing internodes.

Respiratory flux

Incorporation of ^{14}C into CO_2 production and anabolism (organic acid, amino acid and lipid synthesis) decreased with tissue maturation (Table 5.4). To estimate the potential flux from ^{14}C-glucose into respiration, the specific activity of the endogenous glucose pool at the end of the 5.5 h [U-^{14}C]glucose pulse was calculated. The assumption made was that glucose was located exclusively in the cytoplasm and that the ^{14}C-glucose was equally distributed. From glucose concentrations of 4.30 ± 0.56 (internode 2), 3.70 ± 0.48 (internode 5) and 5.31 ± 0.70 μmol mg protein^{-1} (internode 7), and the ^{14}C recovered in glucose (Table 5.4), the specific activity of the endogenous glucose pool in internodes 2, 5 and 7 was calculated as follows:

Specific activity = $\dfrac{^{14}\text{C in Glc (kBq mg protein}^{-1})}{\text{Glc concentration (μmol mg protein}^{-1})}$

Using the specific activity of the endogenous glucose pool (Table 5.7), the rate of glucose flux into CO_2 production and anabolism was estimated accordingly:

$$\text{Flux rate} = \frac{^{14}\underline{C \text{ metabolised into respiratory components (kBq mg protein}^{-1})}}{\text{specific activity (kBq } \mu\text{mol}^{-1}) \times \text{time (min}^{-1})}$$

^{14}Carbon flux into CO_2 production and the combined pool of amino acids, organic acids and lipids decreased from internodes 2 to 7 (Table 5.7). This potentially reflected a decrease in glycolysis, TCA cycle and/or OPP pathway activity.

5.3.1.4 Respiratory metabolism of [U-^{14}C] fructose

Similar to the [U-^{14}C]glucose labelling data, when tissues of internodes 2 and 7 were supplied with [U-^{14}C]fructose, there was a decreased allocation of carbon to CO_2 production and the anabolic respiratory fraction (organic acids, amino acids and lipids) in internode 7 (Table 5.8). To estimate a potential flux through respiration, the specific activity of the endogenous fructose pool in internodes 2 and 7 (Table 5.8) was calculated from the fructose concentration and the amount of ^{14}C recovered in fructose following the 5.5 h labelling period. A fructose concentration of 3.90 ± 0.50 μmol mg protein^{-1} (internode 2) and 8.17 ± 1.80 μmol mg protein^{-1} (internode 7) was measured. ^{14}Carbon recovered in fructose was 1.08 ± 0.29 and 2.38 ± 0.51 kBq mg protein^{-1} in internodes 2 and 7, respectively. Estimated ^{14}C flux into CO_2 production and the anabolic respiratory fraction was higher in internode 2 than in internode 7 (Table 5.8).

Table 5.7 The estimated carbon flux into CO_2 production and the combined lipid, organic and amino acid pool in tissue slices of internodes 2, 5 and 7 of NCo376. The flux was calculated using the ^{14}C incorporation values from the glucose labelling experimental data recorded in Table 5.4.

Internode number	Specific activity of the endogenous glucose pool	Flux into CO_2 production	Flux into lipids, organic and amino acids
	$(kBq\ \mu mol^{-1})$	$(nmol\ min^{-1}\ mg\ protein^{-1})$	
2	0.35 ± 0.16	33.10 ± 0.87	68.00 ± 22.4
5	0.44 ± 0.12	17.60 ± 2.07	55.00 ± 12.3
7	0.54 ± 0.15	9.99 ± 1.67	26.60 ± 5.04

Table 5.8 Incorporation of ^{14}C and percentage distribution (in parenthesis) of [U-^{14}C]fructose into CO_2 production and the combined pool of amino acids, organic acids and lipid-soluble components, of internode 2 and 7 tissue slices of NCo376, labelled for 5.5 h. The ^{14}C incorporation data was used to calculate flux rates using the specific activity of the endogenous fructose pool.

Internode number	^{14}C incorporation into CO_2	^{14}C incorporation into lipids, amino and organic acids	Specific activity of endogenous fructose pool	Flux into CO_2	Flux into lipids, amino and organic acids
	$(kBq\ mg\ protein^{-1})$		$(kBq\ \mu mol^{-1})$	$(nmol\ min^{-1}\ mg\ protein^{-1})$	
2	4.00 ± 0.74 **(9.00)**	9.33 ± 2.79 **(23.09)**	0.28 ± 0.08	43.30 ± 8.01	101 ± 30.20
7	0.74 ± 0.22 **(2.98)**	3.47 ± 0.89 **(13.92)**	0.29 ± 0.06	7.62 ± 2.29	36.20 ± 9.30

-Each value is the mean ± SD of three separate samples.

5.3.1.5 Carbon partitioning of [1-^{14}C]glucose and [6-^{14}C]glucose

To estimate the relative contributions of glycolysis and the OPP pathway to glucose catabolism ,^{14}CO$_2$ production was measured from tissues specifically labelled with [1-^{14}C]glucose and [6-^{14}C]glucose. The extent to which CO$_2$ evolution from carbon 1 exceeded that from carbon 6 was used as an indicator of increased OPP pathway activity. The reverse was taken as an indicator of increasing glycolytic activity, i.e. C-6 /C-1 ratio near unity. After a 45 min labelling period, the C-6/C-1 ratio exceeded unity in internode 3, suggesting the contribution of pentan synthesis to CO$_2$ production (Table 5.9). Due to the probable release of CO$_2$ from carbon 6 during pentan synthesis, it was not possible to estimate the relative activities of glycolysis and the OPP pathway to glucose catabolism, even though the C-6/C-1 ratio decreased below unity in internodes 5 to 7 (Table 5.9). Similar C-6/C-1 ratios were obtained after a 3 h labelling period (Table 5.10). The greatest contribution to CO$_2$ release appeared to be from carbon 6 (Table 5.10).

An increased contribution of carbon 1 over that of carbon 6 was evident in the insoluble matter for all internodes investigated (Table 5.10). The overall decreasing percentage distribution of label into the insoluble matter was similar to that observed in the presence of [U-^{14}C]glucose (Compare Table 5.10 to Table 5.4). If the assumption is made that the dissimilar labelling pattern of the insoluble matter by C-1-labelled and C-6-labelled glucose solely reflects cell wall polysaccharide synthesis, then the difference in incorporation from [6-^{14}C]glucose (cellulose synthesis + other) from that of [1-^{14}C]glucose (cellulose + pentan synthesis + other) can be attributed to pentan synthesis. From this crude estimation, pentan synthesis accounted for approximately 20 to 40 % of total cell wall polysaccharide synthesis.

Since the pentans comprise 33 % of the cell wall polyssacharides (Rosenfeld, 1956), the estimate calculated is comparable with the proportion of pentan in the cell walls. It is, however, notable that the following factors require consideration before an accurate flux rate into pentan synthesis can be calculated. Firstly, the extent to which sugarcane PFP activity in the different internodes equilibrates the triose-P and hexose

Table 5.9 Production of $^{14}CO_2$ in internodal tissues of NCo376 supplied with specifically labelled glucose for 45 min. Values are the ratio of $^{14}CO_2$ released from [6-^{14}C]glucose/[1-^{14}C]glucose.

	Internode 3	Internode 5	Internode 7
C-6 / C-1 ratio	2.13 ± 0.3	0.80 ± 0.2	0.65 ± 0.1

-Each ratio calculated is the mean ± SD of three independent labelling experiments.

Table 5.10 Incorporation of ^{14}C and percentage distribution (in parenthesis) in CO_2 production and the insoluble matter of internodal tissue slices from NCo376, supplied with [1-^{14}C]glucose and [6-^{14}C]glucose for 3 h.

Internode	CO_2	CO_2		Insoluble matter	
	C-6/C-1	C-1	C-6	C-1	C-6
	ratio	*(kBq mg protein^{-1})*			
2	1.53	4.00	6.10	20.48	17.68
		(9.64)	**(14.21)**	**(49.36)**	**(41.18)**
3	2.08	2.99	6.23	15.37	12.28
		(8.21)	**(15.52)**	**(42.25)**	**(30.59)**
5	1.53	2.78	4.26	16.07	11.80
		(5.27)	**(7.14)**	**(30.46)**	**(19.76)**
7	0.60	4.56	2.75	11.98	8.17
		(8.82)	**(4.75)**	**(23.19)**	**(14.00)**

-Each value is representative of a single labelling experiment using a bulked tissue samples from three separate plants.

monophosphate pools requires investigation. The higher the randomisation of label between C-1 and C-6 over the 5.5 h period, the larger the underestimation of flux to pentan synthesis will be. Secondly, the extent to which the OPP pathway labels the hexose monophosphate pool from C-6 over that of C-1 (thereby contributing to an overestimation of flux to pentan synthesis) should also be evaluated.

5.3.2 METABOLISM BETWEEN SUGARCANE VARIETIES

5.3.2.1 Dry mass, sucrose, protein and PFP activity

To ascertain whether the partitioning of hexose monophosphates between high and low sucrose storing *Saccharum* species followed a similar pattern to that between immature and mature internodes, three sugarcane varieties were selected for analysis. Selection of the two *Saccharum* species hybrids (NCo376 and US6656-15) and one *S. spontaneum* (Coimbatore) was based on the significant difference in sucrose content at the time of sampling (Table 5.11). Protein content in US6656-15 and Coimbatore was higher than in NCo376 (Table 5.11). As with the developing stalk, measurements of ^{14}C incorporation into the cellular fractions were expressed on a protein basis to better reflect the contribution of the cytosolic compartment to metabolism. The specific PFP activities in NCo376, US6656-15 and Coimbatore were 48.6 ± 4.6, 96.6 ± 11 and 86.3 ± 11 nmol min^{-1} mg protein^{-1}, respectively. Specific PFK activities were 31.9 ± 9.1 (NCo376), 42.2 ± 9.7 (US6656-15) and 47.2 ± 5.9 (Coimbatore) nmol min^{-1} mg protein^{-1}, respectively.

5.3.2.2 Metabolism of [U-^{14}C]glucose
Carbon partitioning

Of the [U-^{14}C]glucose supplied, 44.6 %, 63.0 % and 83.4 % was taken up by internode 7 tissue slices of NCo376 (1.50 g fresh mass), US6656-15 (1.0 g fresh mass) and Coimbatore (0.94 g fresh mass), respectively. The total ^{14}C recovered in the cellular components of extracted tissues was 91.3 %, 105.0 % and 91.1 % for NCo376, US6656-15 and Coimbatore, respectively. In all three varieties, more than 90.0 % of

Table 5.11   Dry mass, sucrose and soluble protein content in internode 7 of two interspecific hybrids (NCo376, US6656-15) and one *S. spontaneum* (Coimbatore).

Variety	Tissue dry mass (%)	Sucrose content (mmol gram dry mass^{-1})	Protein content (mg gram dry mass^{-1})
NCo376	13.70 ± 3.50	1.14 ± 0.17	3.35 ± 0.29
US6656-15	16.10 ± 0.78	0.76 ± 0.09	5.77 ± 0.80
Coimbatore	26.41 ± 4.29	0.21 ± 0.05	4.54 ± 0.75

-Each value is the mean ± SD of three separate samples.

Table 5.12 Incorporation of ^{14}C and percentage distribution (in parenthesis) into the cellular components of tissue discs (internode 7) from two interspecific hybrids (NCo376, US6656-15) and one *Saccharum spontaneum* (Coimbatore), supplied with [U-^{14}C]glucose for 5 h.

Cellular components	NCo376	US6656-15	Coimbatore
	(kBq mg protein^{-1})		
CO$_2$	7.22 ± 1.3 **(7.24)**	14.21 ± 0.6 **(13.55)**	15.41 ± 1.2 **(13.50)**
Water-soluble			
sucrose	64.04 ± 17 **(64.26)**	38.28 ± 0.90 **(36.50)**	25.02 ± 9.9 **(21.94)**
glucose	8.11 ± 1.0 **(8.13)**	6.94 ± 1.2 **(6.62)**	3.65 ± 1.3 **(3.20)**
fructose	1.56 ± 0.5 **(1.57)**	1.53 ± 0.3 **(1.46)**	2.57 ± 0.92 **(2.25)**
acidic/basic	3.98 ± 1.0 **(4.00)**	24.14 ± 2.9 **(23.03)**	46.89 ± 11.8 **(41.08)**
Water-insoluble	13.17 ± 2.8 **(13.21)**	17.30 ± 3.8 **(16.49)**	19.15 ± 2.43 **(16.77)**
Lipid-soluble	1.64 ±0.23 **(1.64)**	2.46 ± 0.16 **(2.34)**	1.42 ± 0.88 **(1.24)**
Total uptake	99.72 ± 23	104.86 ± 9.9	114.13 ± 28

-Each value is the mean ± SD of three separate plants.

the incorporated [U-^{14}C]glucose was metabolised (Table 5.12). The increased percentage of ^{14}C recovered in sucrose from internode 7 tissues of NCo376 reflected an increased partitioning of hexose monophosphates into sucrose synthesis (Table 5.12). The percentage ^{14}C allocated to sucrose synthesis in US6656-15 and Coimbatore was lower than that of NCo376 (Table 5.12). Carbon allocation to sucrose storage was inversely related to partitioning into total respiration (CO_2 production and the anionic/cationic fraction) between the three different varieties (Table 5.12). The percentage carbon distribution into the insoluble matter was marginally higher in US6656-15 and Coimbatore than in NCo376 (Table 5.12). Incorporation of ^{14}C into the lipid-soluble fraction was negligible in all three varieties investigated (Table 5.12).

Respiratory flux

To estimate carbon flux into the respiratory pathway across the varieties, the specific activity of the endogenous glucose pool (Table 5.13) was calculated from the endogenous glucose concentration (Table 5.13) and the amount of ^{14}C recovered in glucose following labelling (Table 5.12), as described in section 5.3.1.3. As with the developing stalk, the assumption made was that glucose was located exclusively in the cytoplasm and that the ^{14}C-glucose was equally distributed. Using the specific activity of the respective glucose pools, a comparative rate of carbon flux into the respiratory pathways between the varieties was estimated from the sum total of ^{14}C incorporated into CO_2, the anionic/cationic and lipid-soluble fractions. The highest flux rate into the respiratory pathway was evident in tissue slices of Coimbatore and US6656-15, and the lowest in NCo376 (Table 5.13). The respiratory flux rate calculated for internode 7 tissue slices of NCo376 was comparable to that reported previously (Table 5.7 and 5.8).

Table 5.13 Estimated carbon flux into respiration in internode 7 tissue discs of NCo376, US6656-15 and Coimbatore, labelled with [U-^{14}C]glucose. The flux was calculated using the ^{14}C incorporation values from the glucose labelling experimental data recorded in Table 5.12.

Variety	Endogenous glucose	Specific activity of the glucose	^{14}C incorporation into CO_2, acidic/ basic and lipid fraction	Estimated flux into respiration
	(μmol mg protein^{-1})	*(kBq μmol^{-1})*	*(kBq 1.0 h^{-1} mg protein^{-1})*	*(nmol min^{-1} mg protein^{-1})*
NCo376	9.0 ± 1.1	0.9 ± 0.04	2.6 ± 0.5	47.4 ± 9.1
US6656-15	4.0 ± 0.2	1.7 ± 0.3	8.2 ± 0.7	78.6 ± 7.1
Coimbatore	1.7 ± 0.1	2.1 ± 0.8	12.7 ± 2.8	100 ± 22.0

-Each value is the mean ± SD of three separate samples.

5.4 **DISCUSSION**

Three major sinks for incoming sucrose exist within storage tissues. These include storage as sucrose, conversion to oligo- or polysaccharides and respiration (ap Rees, 1995). In order to identify additional regulatory factors controlling carbon partitioning between sucrose accumulation, structural polysaccharide synthesis and respiration in sugarcane internodal tissues, the first requirement is to characterise changing patterns of carbon allocation between these major components.

Since sucrose is the *in vivo* source substrate to sugarcane stem tissue (Hatch and Glasziou, 1964), [U-^{14}C]sucrose was first supplied to excised sugarcane internodal tissue discs to investigate the metabolic fate of sucrose. Results from the present study indicate that whilst sucrose is stored in the mature internodes, a higher percentage of labelled sucrose is allocated to total respiration (CO_2 production and the acidic/basic cellular components) and insoluble matter synthesis in the younger internodes. Sucrose accumulation in the developing sugar beet root is similarly associated with a decreased allocation of incoming carbon (from ^{14}C-sucrose) into structural carbohydrates, proteins and the acidic/basic fraction (Giaquinta, 1979).

In sugarcane, the increased percentage ^{14}C recovered in sucrose from internodes 2 to 7 could be interpreted to reflect a decreased hydrolysis/cleavage of the incoming labelled sucrose, and hence a decreased supply of hexose monophosphates for utilisation by respiration and insoluble matter synthesis. Alternatively, the increased label in sucrose may be indicative of a regulated change in the partitioning of hexose monophosphates from pathways of non-sucrose related biosynthetic activity to sucrose resynthesis. Since sugarcane internodal tissues contain significant levels of acid invertase (Hatch and Glasziou, 1963; Hatch et al., 1963; Gaylor and Glasziou, 1972; Lingle and Smith, 1991; Singh and Kanwar, 1991; Venkataramana and Naidu, 1993; Dendsay et al., 1995), SuSy (Lingle and Smith, 1991; Buczynski et al., 1993; Lingle and Irvine, 1994) and neutral invertase (Hatch et al., 1963; Gaylor and Glasziou, 1972;

Lingle and Smith, 1991; Singh and Kanwar, 1991; Venkataramana and Naidu, 1993; Dendsay et al., 1995), it has been proposed that incoming sucrose is likely to be cleaved/hydrolysed and resynthesised at all stages of maturation (Komor, 1994). To address the proposal that the developmental pattern of carbon partitioning between the different cellular fractions reflects a competition for hexose monophosphates, internodal tissue slices were supplied with [U-^{14}C]glucose.

It is evident that more than 90 % of the supplied ^{14}C-glucose is metabolised, showing that glucose is phosphorylated and incorporated into cellular metabolism with equal efficiency in all internodes investigated. Additionally, an increasing percentage of the hexose monophosphate pool is partitioned towards net sucrose synthesis with maturation. Similar findings have recently been documented by Komor et al. (1996). Although ^{14}C from fructose may rapidly label sucrose via the reversible reaction catalysed by SuSy (Geigenberger and Stitt, 1993), a prerequisite for the metabolism of glucose is the irreversible phosphorylation to Glc-6-P by HK. Hence, the increased movement of label into sucrose from the source substrate, glucose, is a reflection of increased hexose monophosphate partitioning into sucrose. This result is consistent with the increased in vivo accumulation of sucrose (refer to Chapter 4).

The higher incorporation of ^{14}C into the glucosyl moiety of sucrose in the immature internodes does not negate the rapid equilibrium of hexose monophosphates. Rather, these results may reflect the removal of labelled UDPGlc by both SPS and SuSy, as opposed to the removal of labelled Fru-6-P by SPS only. Although the approach used to determine the relative contribution of SuSy to unidirectional sucrose synthesis will likely be an underestimate, results support the contribution of SuSy to the movement of carbon into sucrose in immature sugarcane internodal tissues. In internodes 2 and 3, the minimum estimated contribution of SuSy to sucrose synthesis would be 48.0 % and 38.7 %, respectively. An increased involvement of SuSy in younger internodes has also been reported by Komor (1994). In other tissue types, the movement of label into sucrose via SuSy, is indicative of the reaction operating very close to the theoretical Keq in vivo (Geigenberger and Stitt, 1993). That SuSy does not make any

appreciable contribution to the movement of label into sucrose in the more mature internodes, may be indicative of fine control mechanisms operating to stimulate cleavage activity *in vivo*, or that SuSy expression is down-regulated. However, as significant levels of extractable SuSy activity have been reported in mature tissues (Buczynski et al., 1993; Lingle and Irvine, 1994), it is feasible that fine control mechanisms operate to stimulate cleavage activity of SuSy *in vivo*. This, however, remains to be investigated. Collectively, the present study implicates a precise developmental control in the partitioning of hexose monophosphates between the routes of sucrose synthesis and non-sucrose storage related biosynthetic activity.

To investigate whether the pattern of carbon partitioning to sucrose between low and high sucrose storing varieties is analogous to that observed between immature and mature internodes, ^{14}C distribution patterns (from ^{14}C-glucose) were compared across three varieties which differed significantly in sucrose content. As with the developing internodes, more than 90 % of the supplied ^{14}C-glucose is metabolised, similarly indicating that glucose is phosphorylated and incorporated into cellular metabolism with equal efficiency across the varieties investigated. The percentage ^{14}C allocated to sucrose synthesis in US6656-15 and Coimbatore was lower than that of NCo376. Carbon allocation into sucrose is consistent with the prevailing intracellular sucrose content across the varieties. The higher proportion of hexose monophosphates allocated to non-sucrose storage related biosynthetic activity in the poorer, sucrose storing varieties indicates that metabolism is more analogous to that of the immature internodes. However, of the ^{14}C metabolised by non-sucrose related biosynthetic activity, a far higher proportion enters the respiratory pool (CO_2 production and anabolic biosynthesis) in the lower sucrose storage varieties, whereas there is a more coordinated decrease in both insoluble matter synthesis and respiration in the developing stalk.

From internodes 2 to 7, the carbon partitioned into total respiration is shown to decline from 28 % to 15 %, and from 32 % to 17 % in tissues labelled with [U-^{14}C]glucose and [U-^{14}C]fructose, respectively. The total carbon allocated to insoluble matter synthesis

and respiration decreases from 64 % to 28 % between internodes 2 to 7. Sacher et al. (1963) reported that only 10 % of the total radioactivity (from ^{14}C-glucose) incorporated into the sugar pool of immature sugarcane internodal tissue slices, is mobilised (cycled) to other biosynthetic activity during a 10 h incubation period. Based on the high percentage distribution of label into structural material and respiration from the present study, it is evident that remobilisation of sucrose (catalysed by soluble acid invertase) is not the sole means of regulating carbon partitioning in sugarcane internodal tissues. In sugarcane, the accumulation of sucrose in maturing sugarcane internodes has been attributed primarily to a decline in the hydrolysis of sucrose by vacuolar acid invertase (Hatch and Glasziou, 1963; Hatch et al., 1963; Slack, 1965; Gaylor and Glasziou, 1972; Glasziou and Gaylor, 1972; Zhu et al., 1996). The precise mechanism/s operating to divert carbon away from insoluble matter biosynthesis and respiration towards increased sucrose storage, both within the developing stalk and between different varieties, is not evident from the present investigation. Although decreased cycling of cytosolic sucrose in maturing internodes has been implicated (Komor et al., 1996), underlying regulatory changes in the pathways of cell wall polyssacharide synthesis, glycolysis and the OPP pathway may operate to down-regulate the entry of hexose monophosphates into structural matter synthesis and respiration.

From the present investigation, it is not possible to estimate the relative contribution of glycolysis and the OPP pathway to the decrease in CO_2 production. In sugarcane internodal tissues, it appears that pentan synthesis is an additional source of released CO_2. The following observations lend support to this proposal. Firstly, the net conversion of UDPglucuronic acid to UDPxylose is accompanied by the release of CO_2 from carbon number 6 (Davies et al., 1964). In the younger internodes, the $^{14}CO_2$ released from [6-^{14}C]glucose exceeds that from [1-^{14}C]glucose. Usually, a C-6/C-1 ratio of unity is the predicted maximum value and is indicative of the sole contribution of glycolysis, since an increasing contribution of the OPP pathway to CO_2 release merely serves to decrease the ratio below unity (Davies et al., 1964). Ratios (C-6/C-1) of CO_2 release exceeding unity are indicative of increased pentan synthesis associated

with growth (Hill and ap Rees, 1994). In this study the C-6/C-1 ratio is below unity in internode 7 (in all varieties investigated), but this does not necessarily negate the contribution of pentan synthesis to CO_2 production. Rather, the decreasing ratio may be reflected by the overall decreased ^{14}C incorporation into the insoluble matter. Thus, the present study highlights the caution which needs to taken even when interpreting the qualitative contribution of glycolysis and the OPP pathway to respiration from a C-6/C-1 ratio of less than unity. Secondly, the relative amounts of $^{14}CO_2$ evolved from C-6-labelled and C-1-labelled glucose, respectively, are comparable after both 45 min and 3 h. This suggests that time dependent recycling of carbon via the OPP pathway is not a contributing factor to the increased C-6/C-1 ratio. In instances where specifically labelled glucose has been successfully employed to estimate the contribution of glycolysis and the OPP pathway to CO_2 production, pentan synthesis has been found to be negligible (Hill and ap Rees, 1994).

It is likely that although insoluble matter biosynthesis is a contributory factor to CO_2 release, glycolysis and the OPP pathway are solely responsible for the provision of carbon from ^{14}C-sugars to the biosynthesis of amino acids, organic acids and lipids in sugarcane internodal tissues. Biosynthesis of the combined pool of amino acids, organic acids and lipids is higher in both immature sucrose storing internodes and the lower sucrose storing varieties. The largest proportion of labelled carbon entering total respiration is recovered in the cationic and anionic fractions. Similar findings are reported for a variety of other sink tissues, where the proportion of respiratory carbon metabolised by anabolism may exceed that released as CO_2 by approximately 2.5 to 50 fold (Dixon and ap Rees, 1980 b; Edwards and ap Rees; 1986 a; Hajirezaei et al., 1994; Hill and ap Rees, 1994). In the present investigation, the percentage of carbon entering anabolism is probably a slight underestimation, as recovery of labelled carbon in protein was not determined. In a variety of other tissue sources, the proportion of label recovered in protein ranges from 25 to 200 % of that recovered in the free amino acid pool (Dixon and ap Rees, 1980 b; Edwards and ap Rees, 1986 a; Hajirezaei et al., 1994; Hill and ap Rees, 1994). Assuming a similar range for sugarcane, then the allocation of total carbon into respiration in internode 2 (as an example) would range

from 27 to 30 %, as opposed to the 26 % which was measured in the present investigation.

It is evident from the fractionation of the acidic/basic component in the developing sugarcane internodes, that an increased proportion of label is consistently recovered in the organic acid pool. This observation is indicative of the increased synthesis of organic acid sources (TCA cycle intermediates), which may subsequently be stored or utilised by biosynthesis. An increased anaplerotic synthesis of organic acids has been positively linked to the stimulation of the alternative, non-phosphorylating electron transport chain where NADH, generated from the turnover of TCA intermediates, is oxidised without concomitant production of ATP (Lambers, 1985). An inverse relationship between respiration, via non-phosphorylative electron transport, and sucrose storage is shown to exist in the developing carrot root (Steingröver, 1981). The extent to which sugars may be oxidised via the alternative pathway in developing sugarcane internodes requires further investigation.

A decreased partitioning of carbon into amino acids, associated with sucrose accumulation in internodal tissue, has also been documented for sugarcane cell suspension cultures (Wendler et al., 1990; Veith and Komor, 1993). The predominant carbon sources for amino acid biosynthesis include the organic acids, pyruvate, OAA and α-ketoglutarate, which are derived from glycolysis and the TCA cycle, respectively (Ireland, 1990). Since nitrogen precursors were not available to the excised internodal tissues slices, it is possible that amino acid biosynthesis might have been underestimated in all internodes investigated. Possibly, the predominant labelling of the organic acids noted previously may be a reflection of decreased amino acid biosynthesis. However, the labelling of the combined acidic/basic fraction is considered an adequate reflection of changes associated with partitioning into anabolic respiration.

Although carbon partitioning studies were conducted in vitro, it is proposed that this approach was adequate to assess the changes in metabolism associated with whole

stem tissue. Previously, tissue slices from sugarcane stem were largely used to elucidate mechanisms associated with the uptake of sugars (Sacher et al., 1963; Bowen and Hunter, 1972; Glasziou and Gaylor, 1972; Moore, 1995). Since data from those early labelling experiments has come under criticism due to inappropriate experimental procedure (Moore, 1995), precautions taken with the use of tissue slices in the present study included: a pre-labelling wash in buffered mannitol and not water (Lingle, 1989); a short duration labelling experiment (Moore, 1995); a post-labelling tissue wash to remove radioactivity associated with the apoplastic spaces (Lingle, 1989), and the extraction of tissues to investigate the partitioning only of labelled substrates taken up by the cells. Additionally, the inclusion of acid invertase to prevent extracellular sucrose synthesis by tissue slices during labelling had no effect on carbon partitioning. Furthermore, isotope recoveries were sufficiently high to provide a reliable indication of changes in cellular metabolism

From the present investigation, it is evident that no relationship exists between specific PFP activity and carbon partitioning into the insoluble matter in either the developing stalk or across different varieties. This contrasts with the positive relationship between PFP activity and the insoluble matter content recorded between different interspecific hybrids (refer to Chapter 4). Although there is a positive relationship between PFP activity and insoluble matter content, this is not reflected by a positive relationship between PFP activity and the synthesis of insoluble matter in those internodes. Since UDPGlc is the precursor substrate for structural polysaccharide synthesis (Dennis and Greyson, 1987; Kruger, 1990), the present results may reflect a direct incorporation of UDPGlc, from sucrose cleavage, into polysaccharide synthesis in the younger internodes. For this route of carbon allocation to insoluble matter synthesis, there would be no obligatory requirement for increased PFP activity. Alternatively, if the predominant source of UDPGlc for structural polysaccharide synthesis is derived from UDPGPPase (synthetic direction), then it appears that an induction of PFP activity is not essential to facilitate the removal of the by-product PPi, as has been suggested for other tissues (Claassen et al. 1991; Nakamura et al., 1992).

The cross-varietal comparison between PFP activity and ^{14}C partitioning indicates that there is not a requirement for higher PFP activity levels during increased carbon partitioning and flux towards sucrose synthesis. These results corroborate the inverse relationship between specific PFP activity and sucrose content described previously (refer to Chapter 4).

The lack of suitable techniques to quantify the subcellular sugar (and ^{14}C-sugar) distribution precluded absolute estimations of the rate of unidirectional carbon flux into respiration. Since the estimates rely heavily on the assumption that the specific activity of the extracted sugars reflects that of the metabolically active pool, the fluxes are likely to be an overestimation. Thus, all respiratory flux calculations are comparative. Across the three sugarcane varieties sampled, increased PFP activity appears to be associated with an increased rate of respiration. Although it was not possible to determine the relative contribution of the OPP pathway and glycolysis to respiratory carbon flux, it is feasible to assume that carbon flow to respiration will proceed not only via glycolysis but also via the OPP pathway. However, the sum total of PFP and PFK activity is in excess of (or comparable to) the estimated flux rate in the varieties analysed. Similar findings, showing a positive relationship between PFP activity levels and increased carbon flux into precursors of cellular intermediates have been documented previously (Ashihara and Horikosi, 1987; Ashihara and Sato, 1993).

The decline in both partitioning and the rate of carbon flux into respiration within the developing stalk contrasts with the profile of specific PFP activity. As the prevailing PFP and PFK activity is likely to have been sufficient to sustain the observed respiratory carbon flux through glycolysis in the immature internodal tissue slices, the maintenance of PFP activity in higher sucrose storing internodes with a lower respiratory demand is not clear. The decline in carbon flux into respiration, associated with internodal maturation, contrasts with the situation in sugarcane cell suspension cultures, where the respiratory rate is maintained during periods of sucrose mobilisation and synthesis (Wendler et al., 1990). As sucrose synthesis is not driven by a decrease in respiration, PFP and PFK activity levels are relatively constant during

the cell cycle (Wendler et al., 1990).

Conclusion

The distribution of ^{14}C from uniformly labelled sugars indicates major differences in the metabolism between the immature and mature internodes (Figure 5.1). It is evident that the increased partitioning of hexose monophosphates into sucrose storage is ascribed to a two-fold reduction in the percentage carbon allocated to both insoluble matter synthesis and total (catabolic and anabolic) respiration within the developing stalk (Figures 5.1 A and B). When characterising metabolism in internode 7 across sugarcane varieties, it appears that an inverse relationship exists between carbon partitioning into sucrose storage and respiration (Figures 5.1 B, C and D). Of the carbon entering respiration in sugarcane internodal tissue, a large proportion is allocated towards amino acid and organic acid biosynthesis.

Expressed on a protein basis, changing patterns of partitioning into respiration is similarly reflected by a change in the rate of carbon flux. The apparent reduction in partitioning and carbon flux to respiration in maturing sugarcane internodes could be due to changes in the regulation of key reactions of glycolysis, inhibition of the TCA cycle, altered gene expression or substrate availability. There does not appear to be a positive relationship between specific PFP activity and the developmental pattern of carbon partitioning and flux into respiration, although a positive relationship is evident across the varieties. To provide additional insight into factors controlling the decline in carbon partitioning into respiration, fine control of glycolysis warrants further investigation. Aspects of fine control which may be investigated include the identification of key regulatory steps and the extent to which regulation is altered during internodal maturation. Furthermore, measurement of the metabolite levels is important to determine whether the *in vivo* concentration is sufficient to sustain glycolytic enzyme activity.

A
Immature sucrose storing internode
(NCo376)

B
Mature sucrose storing internode
(NCo376)

C
Low sucrose storing interspecific hybrid
(US6656-15)

D
Low sucrose storing S. *spontaneum*
(Coimbatore)
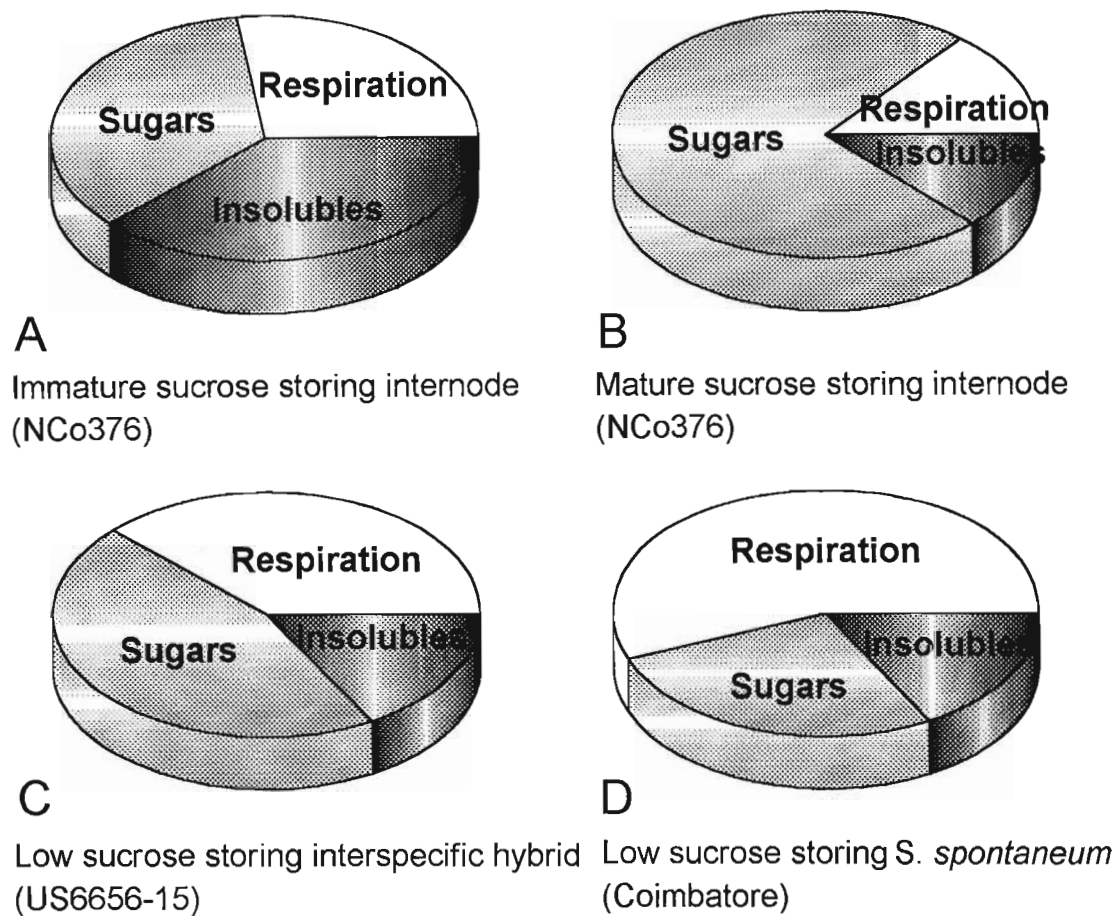
Figure 5.1 Carbon partitioning within internodal tissues of the developing sugarcane stalk of NCo376 (A and B) and between US6656-15 and Coimbatore (C and D).

CHAPTER 6

ASPECTS OF THE REGULATION OF SUCROSE METABOLISM IN SUGARCANE INTERNODAL TISSUES

6.1 INTRODUCTION

From the previous chapter it is evident that the increased partitioning of hexose monophosphates into sucrose synthesis is associated with a concomitant decrease in partitioning to total respiration (CO_2 production and biosynthesis), both within the developing stalk and between different varieties (refer to Chapter 5). Irrespective of the regulatory mechanisms which are proposed to coordinate the supply of partially oxidised TCA intermediates and reductant between various biosynthetic and electron transport pathways (Lambers, 1985; Wiskich and Dry, 1985; ap Rees, 1990), respiratory activity in sucrose importing tissues is primarily dependent on the regulation of glycolytic carbon supply to the TCA cycle (Stitt and Steup, 1985; Plaxton, 1996). Hence, the decline in carbon partitioning to respiration may reflect a down-regulation of glycolysis through an alteration in the regulation of a key reaction step, substrate limitation or a decline in gene expression.

Various interdependent mechanisms of fine control act to modulate the activity of both the irreversible and reversible enzyme catalysed reactions of glycolysis (Plaxton, 1996). Based on the analysis of metabolites levels during associated respiratory changes in many tissue types, it has been demonstrated that carbon flux is primarily regulated through fine control of enzymes catalysing the utilisation of PEP (PK and PEPCase) and the Fru-6-P/Fru-1,6-P_2 interconversion (FBPase and PFK), respectively (Adams and Rowan, 1970; Kobr and Beevers, 1971; Dixon and ap Rees, 1980 a; Turner and Turner, 1980; Day and Lambers, 1983; Beaudry et al., 1989; Turpin et al.,

1990; Geigenberger and Stitt, 1991 b; Hatzfeld and Stitt, 1991).   Coarse modulation of enzyme activity provides an alternative means of regulating the magnitude of glycolytic flux in certain tissues (Dixon and ap Rees, 1980 b; Beaudry et al., 1987; Enomoto et al., 1994; Podestá and Plaxton, 1994).   In other tissues, carbon cycling between sucrose and the hexoses/hexose monophosphates (Wendler et al., 1990; Geigenberger and Stitt, 1991 a; MacRae et al, 1992; Geigenberger and Stitt, 1993), and between the latter and the triose-P (Hatzfeld and Stitt, 1990; Hatzfeld et al., 1990; Hill and ap Rees 1994; Hill and ap Rees, 1995) has highlighted the contribution of the reaction sequence from SuSy to PFP to the turnover of sucrose based on supply and demand for carbon by growth and respiration.   Both substrate levels and effectors also act in part, to modulate the activity of those reversible (near-equilibrium) catalysed reactions (Avigad, 1982; Edwards and ap Rees, 1986 b; Morrel and ap Rees, 1986; Hatzfeld et al., 1990; Geigenberger and Stitt, 1991 a; Merlo et al., 1993).

The underlying importance of identifying physiological processes which influence partitioning resides in the development of strategies to improve productivity.   Although sucrose breakdown and glycolytic activity is at the core of carbon allocation to various biosynthetic pathways (Blakeley and Dennis, 1993), the regulation of this pathway by phosphorylated intermediates has not been examined in sugarcane internodal tissues (Moore, 1995). Furthermore, insignificant recycling of carbon between the hexose monophosphates and triose-P in elongating internodal tissues was proposed to occur (Sacher et al., 1963).   Due to the significant levels of extractable specific PFP activity measured in sugarcane (Chapter 4; Lingle and Smith, 1991), this observation by Sacher et al. (1963) is difficult to comprehend.   Since sucrose breakdown and glycolysis comprises a series of enzyme catalysed reactions, each responsive to the levels of respective substrates, and/or allosteric activators/inhibitors, the extent to which enzyme activity is regulated by these fine controls in sugarcane internodal tissues remains to be determined.

The aim of the present investigation was to examine fine control of sugarcane sucrose cleavage and glycolytic enzyme activity in relation to respiratory carbon partitioning.

In this study, several enzyme catalysed reactions from SuSy to PK were examined in maturing internodal tissue. The mechanisms of fine control selected for investigation included alteration in substrate/product concentration and allosteric regulation. Using this approach the objective was to identify the tightly controlled reactions *in vivo* and to determine the extent of regulatory change which is proposed to accompany the change in respiration. With the exception of sucrose synthase (Buczynski et al., 1993), there are no published reports on the kinetic and regulatory properties of enzymes catalysing the reaction sequence from sucrose to pyruvate in sugarcane internodal tissue. Hence, the steady state contents of key intermediates were compared with the known kinetic and regulatory properties of enzymes purified from several other plant species.

It is reported that there is no change in the regulation of PFK, FBPase and PK activity by variation in the substrate to product concentration during internodal maturation. There is also no decrease in the activity levels of PFK, PFP and PK coinciding with the decrease in respiration. A decrease in the hexose monophosphate concentration during maturation is reported.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

6.2.1.1 Biochemicals

[U-^{14}C]glycerol and the 250 mm x 4.6 mm Spherisorb SAX (5 µm) column were purchased from Amersham International and Phenonenex, respectively.

6.2.1.2 Plant Material

Details pertaining to the sampling of the two *Saccharum* species hybrids, NCo376 and US6656-15 are recorded in section 3.1.2.

6.2.2 METHODS

6.2.2.1 Preparation of plant material

The preparation of tissue samples is recorded in section 3.2.1.   For the metabolite studies, internodal tissues were rapidly excised into liquid nitrogen which had been super-cooled by degassing.

6.2.2.2 Metabolite extraction and measurement

The sugars, hexose monophosphates, Fru-1,6-P$_2$, Fru-2,6-P$_2$, triose-P, PEP, pyruvate, Pi and PPi were extracted according to Ball and ap Rees (1988), as described in section 3.2.2. UDPGlc and UTP were extracted according to Stitt et al. (1983). Samples were powdered in liquid nitrogen and extracted in 1.2 mL 20 mM Na-Hepes (pH 8.5) containing 2 mM EDTA, 8 mL methanol and 3 mL chloroform, after which 8 mL H$_2$O was added.   The extracts were incubated at 25°C for 10 min and then centrifuged at 4000g for 15 min.   The supernatant was removed, dried down in a rotary evaporator at 40 - 50°C and the pellet resuspended in 1 mL deionised, distilled H$_2$O. Samples were filtered (0.22 µm Millex-GV$_4$ filters) prior to fractionation by HPLC.   All internodal samples were extracted and measured in triplicate.

Unless otherwise indicated, all metabolites were measured enzymatically at 340 nm using a Beckman DU 7500 spectrophotometer. The amount of metabolite present was stoichiometric with change in A, produced either from the oxidation of NADH or reduction of NAD/NADP. The extraction efficiency for each intermediate was determined from the percentage recovery of exogenously supplied metabolite, which was spiked onto the frozen tissue samples during grinding. The concentration of exogenously supplied metabolite added was comparable to that obtained in the tissues. For the sugars, glycolytic intermediates, UTP and Pi, extraction efficiency exceeded 95.0 %, 80.0 %, 92.0 % and 96.0 %, respectively.

Glucose and Fructose

Glucose and fructose were determined sequentially in a 1 mL reaction volume using the Boehringer Mannheim sugar analysis kit. The principle of the analysis was based on the method of Bergmeyer and Bernt (1974) and is described in section 3.2.2. Following the determination of glucose, fructose content was measured following the addition of HPI which converted the Fru-6-P produced from HK to Glc-6-P. The change in A was measured from the reduction of NADP during the conversion of Glc-6-P to gluconate-6-phosphate.

Glc-6-P and Fru-6-P

Sequential measurements of Glc-6-P and Fru-6-P were determined according to Lang and Michal (1974). The standard 1 mL reaction contained 100 mM Tris-Cl (pH 7.6), 5 mM $MgCl_2$, 0.2 mM NADP and sample extract (50 - 150 µL). Glc-6-P was measured following the addition of 0.4 IU Glc-6-P dehydrogenase. The reaction was monitored until completion. Any change in A associated with the addition of Glc-6-P dehydrogenase alone, was determined by including an equivalent second volume of enzyme in the reaction. The change in A following the second addition of enzyme was subtracted from the initial A change. Fru-6-P was then measured by the change in A after adding 1.6 IU HPI. Similarly, any interference in A due to the addition of HPI alone was corrected for by including a second volume of enzyme after completion of the reaction.

Triose-P and Fru-1,6-P_2

Triose-P and Fru-1,6-P_2 were measured sequentially in a 1 mL volume containing 100 mM Tris-Cl (pH 7.6), 1 mM EDTA, 0.15 mM NADH and sample (50 - 150 µL), as described by Michal and Beutler (1974). The reaction was initiated with 1.36 IU TPI and 0.6 IU glycerol-3-P dehydrogenase, and the triose-P levels were measured from the change in A. Once the reaction was complete, Fru-1,6-P_2 was quantified following the addition of 0.27 IU aldolase. Any interference in A change associated with the addition of the coupling enzymes alone was corrected for, as described in the preceding paragraph.

Fru-2,6-P_2

The Fru-2,6-P_2 content was measured from the activation of partially purified potato PFP (van Schaftigen et al., 1982) in the standard PFP assay reaction (described in section 3.2.5.1). Contaminating Fru-2,6-P_2 in commercial Fru-6-P was first removed by treating the preparation with 200 mM HCl for 30 min and then neutralising with 1 M Tris (Kruger et al., 1983 b). A linear calibration curve was constructed by measuring the rate of partially purified PFP in the presence of 0 to 60 pmol ml^{-1} commercial Fru-2,6-P_2. Using the calibration curve ($r^2 = 0.986$), the concentration of Fru-2,6-P_2 in the sugarcane internodal extracts was determined from the PFP activity rates.

PPi

PPi was determined according to a modification of the method of Viola and Davies (1991). The standard 1 mL reaction contained 100 mM Tris-Cl (pH 7.6), 1 mM EDTA, 10 mM Fru-6-P, 10 µM Fru-2,6-P_2, 0.15 mM NADH, 0.27 IU aldolase, 1.36 IU TPI, 0.6 IU glycerol-3-P dehydrogenase and 50 - 150 µL sample extract. PPi was quantified following the addition of 0.10 IU PFP. On completion of the reaction, a second volume of PFP was added to determine background A change.

Pi

Pi was determined using a colorimetric assay (Joyce and Grisolia, 1960). Extracts (50 µL) were added to 0.5 M trichloroacetic acid in a final volume of 200 µL. A colour

reagent was prepared by mixing 4 mL 16 % (m/v) ammonium molybdate in 10 N H_2SO_4 and 2 g $FeSO_4$ in 36 mL H_2O. The colour reagent (100 μL) was added to the 200 μL sample/trichloroacetic acid and the A measured at 660 nm. Concentration of Pi in sugarcane internodal extracts was calculated from a linear calibration curve, constructed by measuring A in the presence of 0 to 60 μM Pi (r^2 = 0.98).

Pyruvate and PEP

Pyruvate and PEP were measured sequentially according to Czok and Lamprecht (1974). Pyruvate was measured following the addition of 1.0 IU LDH to a 1 mL reaction volume containing 300 mM Na-Hepes (pH 7.6), 3 mM EDTA, 37 mM KCl, 1.2 mM ADP, 7.5 mM NADH and 50 - 150 μL sample extract. Following the determination of pyruvate, PEP was measured by adding a combination of PK and LDH (2.0 IU and 2.75 IU, respectively). The change in A associated with the addition of LDH and the LDH/PK combination alone was accounted for.

UDPGlc and UTP

Anion exchange chromatography was used to separate UDPGlc and UTP according to a modified method of Viola et al. (1994). Separation was performed by HPLC on a 25 cm Spherisorb SAX (5μm) column, at a flow rate of 1 mL min^{-1}, using a mobile phase of (A) 10 mM ammonium phosphate pH 3.0 and (B) 450 mM ammonium phosphate pH 4.5. The gradient employed was 100 % of (A) for 2 min, followed by a linear gradient to 20 % of (B) over 8 min, and finally to 65 % of (B) over a further 20 min. The gradient was maintained at 65 % of (B) and 35 % of (A) for 5 min, and the column washed for 10 min with 100 % of (A) prior to application of the next sample. Metabolite peaks from the injected sugarcane extracts (20 μL injection volume) were resolved at 260 nm and integrated using the computer package Chrom-A-Scope (Version 1.7, Barspec). Regression analyses of standard curves for UDPGlc and UTP showed a linear increase (r^2 = 0.99) between 0 and 50 nmol 20 μL^{-1}. Comparable levels of UDPGlc in sugarcane extracts were obtained when the enzymatic method was employed (Viola et al., 1994).

6.2.2.3 Enzyme extraction and measurement

All enzymes were extracted and measured as described in section 3.2.5.

PFP and PFK activity

The measurement of PFP and PFK activity is described in sections 3.2.5.1 and 3.2.5.2.

SuSy activity

SuSy was measured in the cleavage direction at the pH optimum (pH 6.8) for both isoforms in sugarcane (Buczynski et al., 1993). The standard reaction was based on a modification of Huber and Akazawa (1986) and contained 100 mM Na-Hepes (pH 6.8), 2 mM $MgCl_2$, 1.5 mM NAD, 25 mM sucrose, 0.15 IU UDPGlc dehydrogenase and 1 mM UDP. Activity was initiated with UDP.

UDPGPPase activity

UDPGPPase was measured in the direction of Glc-1-P formation. The standard reaction contained 100 mM Tris-Cl (pH 8.5), 5 mM $MgCl_2$, 1 mM UDPGlc, 1 IU PGM, 1 IU Glc-6-P dehydrogenase and 0.6 mM NADP. Activity was initiated by the addition of 2.5 mM PPi (Sowokinos et al., 1993).

Aldolase activity

The standard reaction for the measurement of aldolase contained 50 mM Tris-Cl (pH 7.8), 1 mM EDTA, 0.2 mM NADH, 5 mM $MgCl_2$, 10 IU TPI, 1 IU glycerol-3-P dehydrogenase and 2 mM $Fru-1,6-P_2$ (Moorhead and Plaxton, 1988). Following the addition of $Fru-1,6-P_2$, activity was measured.

PK activity

The standard reaction for the measurement of PK contained 50 mM Na-Hepes (pH 6.9), 50 mM KCl, 10 mM $MgCl_2$, 0.15 mM NADH, 2 mM PEP, 5 % (m/v) BSA, 2 mM DTT, and 20 IU LDH. PK activity was initiated following the addition of 2 mM ADP (Moorhead and Plaxton, 1988).

6.2.2.4 Protein measurement

This procedure is described in section 3.2.6.

6.2.2.5 ^{14}Carbon labelling studies

Both [U-^{14}C]glucose and [U-^{14}C]glycerol were used in the labelling experiments at a concentration of 2.0 GBq mmol^{-1}. The tissue preparation and labelling is described in section 3.2.7.1. Extraction of the labelled tissues and the fractionation of the neutral water-soluble component are also discussed in sections 3.2.7.2 and 3.2.7.3.

6.3 RESULTS

6.3.1 METABOLITE DETERMINATIONS

6.3.1.1 Metabolite levels in the developing stalk

To better represent the metabolic compartment containing the metabolites, levels were expressed on a protein basis.   Protein content decreased from 2.28 ± 0.20 mg gram fresh mass^{-1} (internode 3) to 1.22 ± 0.18 mg gram fresh mass^{-1} (internode 5), and then remained constant.

Due to limited material in internode 2, metabolites were extracted from internodes 3 to 9, and the levels compared (Figure 6.1).   Sucrose content increased from internodes 3 to 9, whilst glucose and fructose increased from internode 3 to 4 and then decreased (Figure 6.1 A).   There was an increase in the levels of UDPGlc (Figure 6.1 B), and to a lesser extent in Glc-6-P (Figure 6.1 B), Fru-6-P and Fru-1,6-P_2 (Figure 6.1 C) between internodes 3 and 5.   From internodes 5 onwards, UDPGlc declined and then remained constant (Figure 6.1 B), whilst levels of Glc-6-P, Fru-6-P and Fru-1,6-P_2 fluctuated slightly (Figures 6.1 B and 6.1 C).   Fru-2,6-P_2 levels declined from internodes 4 to 6, after which there was no further decrease (Figure 6.1 D).   There was no significant change in the levels of triose-P, PEP and pyruvate between internodes 3 and 9 (Figures 6.1 D and 6.1 E).   Pi content increased from internodes 3 to 5 and then remained constant (Figure 6.1 F).   Levels of PPi were unchanged (Figure 6.1 F). UTP decreased from internodes 3 to 4, after which levels were constant (Figure 6.1 F).

To examine potential regulatory effects on sucrose cleavage and glycolytic enzyme activity (based on reported kinetic properties for purified preparations from other tissues), metabolites levels in sugarcane were also expressed on a concentration basis (Table 6.1).   The calculation of metabolite concentrations *in vivo* are very difficult due to the heterogenous intracellular compartmentalisation of many metabolites and the
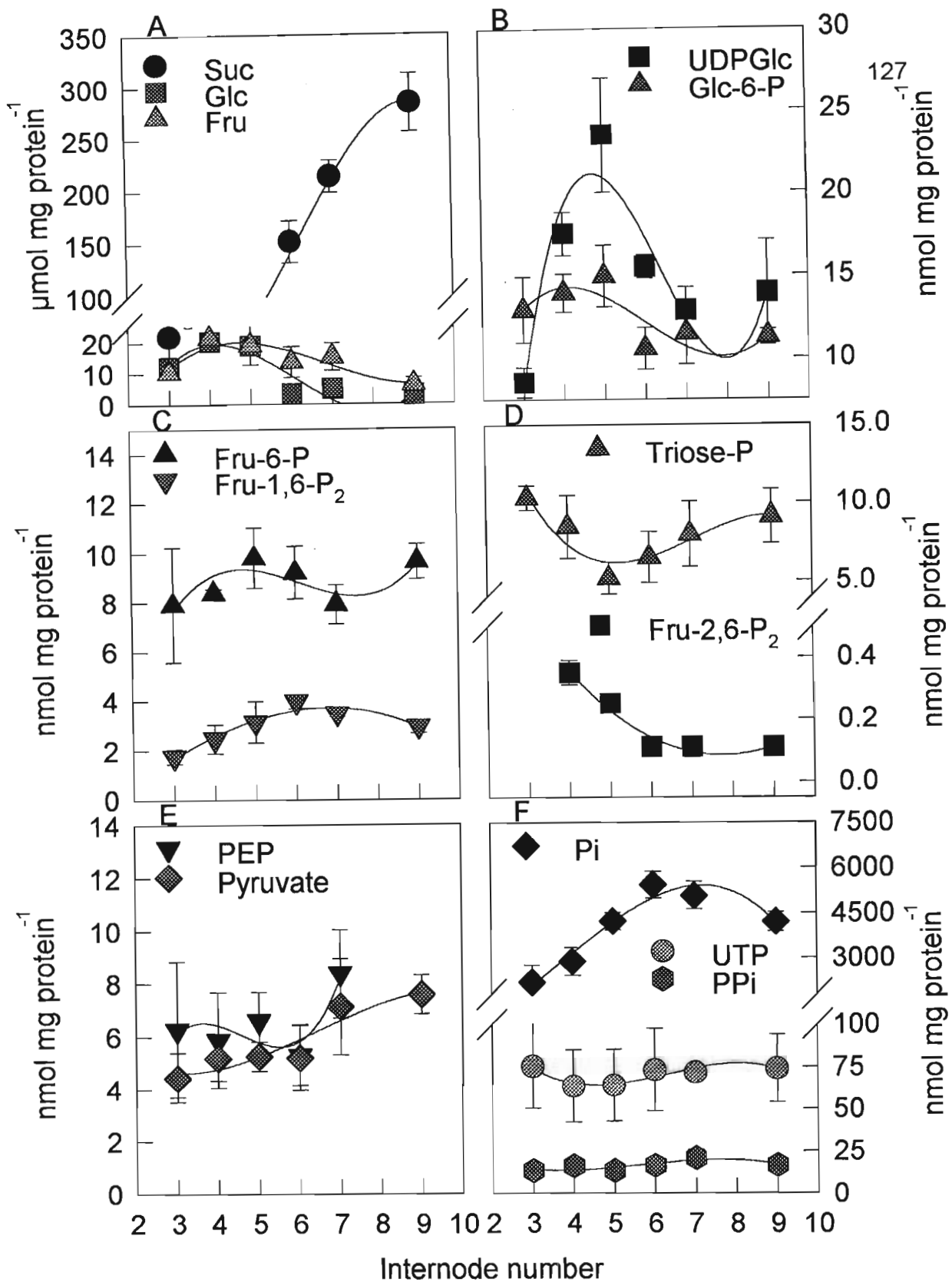
Figure 6.1 Levels of metabolites measured from internodes 3 to 9 in the developing stalk of NCo376. (A) Suc, Glc and Fru, (B) UDPGlc and Glc-6-P, (C) Fru-6-P and Fru-1,6-P_2, (D) Triose-P and Fru-2,6-P_2, (E) PEP and pyruvate, and (F) Pi, PPi and UTP. Each value is the mean ± SD of three to four separate samples.

Table 6.1 Metabolite concentration in sugarcane internodal tissue. All intermediates, with the exception of the sugars and Pi, were assumed to be confined to the cytosol. The metabolites levels presented in Figure 6.1 were used to estimate the average concentration.

	Internode 3	Internode 4	Internode 5	Internode 7	Internode 9
% cytosolic volume	10	10	10	10	10
Metabolite					
		Metabolite concentration			
		(μM)			
Suc	55×10^3	129×10^3	216×10^3	256×10^3	284×10^3
Glc	31×10^3	40×10^3	29×10^3	6×10^3	3×10^3
Fru	27×10^3	43×10^3	30×10^3	15×10^3	7×10^3
UDPGlc	187	275	302	196	158
Glc-6-P	301	263	211	130	112
Fru-6-P	178	153	123	85	104
Fru-1,6-P_2	42	46	40	37	32
Fru-2,6-P_2	ND	3.0	2.3	1.0	1.0
Triose-P	239	158	72	93	94
PEP	139	110	85	88	ND
Pyruvate	102	56 - 101	102	82	79
Pi	3.4×10^3	3.7×10^3	5.1×10^3	5.1×10^3	4.6×10^3
PPi	270	290	180	189	166
UTP	1600	999	801	909	657

inability to measure the cytosolic volume accurately. The approach taken in the present study was to assume that the storage vacuole occupied 90 % of the total cell volume in the higher sucrose-storing internodal tissues. Concentration was calculated from the metabolite levels and expressed as nmol gram fresh mass^{-1}, taking into consideration the 10 % cellular volume containing the intermediates (Table 6.1). The exception was made for the sugars and Pi, which were assumed to be uniformly distributed within the cell. The rationale behind this assumption was based on previous investigations which have shown that both Pi and sucrose phosphatase are located in the vacuole and cytosol (Hawker et al., 1987; Preisser et al., 1992), and that compartmentalisation of the sugars is not restricted to the vacuole (Hawker, 1965; Welbaum and Meinzer, 1990; Preisser et al., 1992). It is feasible that if the cytosolic volume comprises less than 10 % of the cell volume, calculations of the metabolite concentrations within the developing stalk will be an underestimation. Alternatively, if the cytosolic cellular volume is greater than 10 %, then the metabolite concentration in the cytosol will be an overestimation. Also subject to assumption is that the sugars and Pi are evenly distributed within the cell. If the distribution pattern is heterogenous, then the absolute concentration of these compounds occurring within the cytosol could be either over- or underestimated.

6.3.1.2 Metabolite ratios in the developing stalk

To characterise any developmental alteration in flux and to identify the reaction/s subject to regulation, the *in vivo* product to substrate ratios for several enzymes were examined. Since fine control of plant glycolysis is primarily exerted at the reactions catalysing PEP and Fru-6-P utilisation, regulation of PK, PFK, PFP and FBPase was first examined.

The ATP and ADP contents were not measured in the present study. It was, however, possible to use the theoretical Keq and the pyruvate/PEP ratio to calculate the ATP/ADP ratio that would have to be present in the cytosol for PK to operate at equilibrium *in vivo*. A similar approach was used for the estimation of *in vivo* cytosolic ATP/ADP ratios required for PFK to catalyse an equilibrium reaction. As the apparent

Keq for PK ranges from 3.19×10^5 to 1.15×10^8 (Table 6.2), a mid-range Keq of 7.2×10^6, together with the pyruvate/PEP ratios (Table 6.2), were used to estimate the required ATP/ADP ratio. ATP to ADP ratios of 9.86×10^6 (internode 3), 5.86×10^6 (internode 5) and 7.70×10^6 (internode 7) were calculated. As these ratios are not likely to exist *in vivo,* the reaction catalysed by PK was considered to be very far removed from equilibrium in all internodes investigated. Although the reaction was considered to be highly regulated, the product to substrate ratio remained constant (Table 6.2), indicating no significant change in the regulation of PK between internodes 3 and 7.

By applying the same principle to the PFK catalysed reaction, a mid-range Keq of 1050 (Table 6.2) was used to calculate the cytosolic ATP/ADP ratio required for the enzyme to operate at equilibrium *in vivo.* This resulted in ratios ranging from 0.21×10^{-3} (internode 3) to 0.28×10^{-3} (internode 9). Since these ratios are significantly different from those required for PK to operate at equilibrium in vivo, the existence of *in vivo* ATP/ADP ratios in this range is not possible. Similarly, it was concluded that the PFK catalysed reaction was far removed from equilibrium in all internodes investigated. The ratio of Fru-1,6-P_2 to Fru-6-P was not different between internodes 3 to 9 (Table 6.2), indicating that regulation of PFK by variation in the substrate/product ratio was not altered.

Regulation of FBPase was investigated by using both the apparent Keq for the reaction and the levels of Fru-6-P and Fru-1,6-P_2 to calculate the amount of inorganic Pi required for FBPase to operate at equilibrium *in vivo.* A concentration ranging from approximately 36 (internode 3) to 53 mM (internode 9) would be required. The Pi concentration between internodes 3 and 9 ranged from 3.41 to 4.61 mM (Table 6.2). Thus, FBPase catalysed a reaction far displaced from equilibrium in sugarcane. There was no indication from the Fru-6-P/Fru-1,6-P_2 ratios that any change in the regulation of FBPase occurred between internodes 3 and 9 (Table 6.2).

Table 6.2 Comparisons of the theoretical equilibrium constants for reactions catalysed by PK, PFK, FBPase and PFP with the ratios of substrates in internodes 3 to 9 of NCo376. Ratios were calculated from the concentrations shown in Table 6.1.

Internode number	PK $\frac{[Pyruvate][ATP]}{[PEP][ADP]}$ (Keq = 3.19×10^5-1.15×10^8)[A,B]	PFK $\frac{[Fru-1,6-P_2][ADP]}{[Fru-6-P][ATP]}$ (Keq = 300 - 1800)[A,B]
	$\frac{[Pyruvate]}{[PEP]}$	$\frac{[Fru-1,6-P_2]}{[Fru-6-P]}$
3	0.73 ± 0.30	0.23 ± 0.061
4	0.92 ± 0.24	0.31 ± 0.130
5	1.24 ± 0.16	0.32 ± 0.090
6	0.95 ± 0.02	0.43 ± 0.038
7	0.93 ± 0.10	0.44 ± 0.065
9	ND	0.30 ± 0.020
	PFP $\frac{[Fru-1,6-P_2][Pi]}{[Fru-6-P][PPi]}$ (Keq = 3.3)[C,D]	FBPase $\frac{[Fru-6-P][Pi]}{[Fru-1,6-P_2]}$ (Keq = 174 M)[E]
	$\frac{[Fru-1,6-P_2]}{[Fru-6-P][PPi]}$	$\frac{[Fru-6-P]}{[Fru-1,6-P_2]}$
3	0.80×10^{-3}	4.25 ± 1.30
4	0.96×10^{-3}	3.24 ± 1.40
5	1.59×10^{-3}	3.11 ± 1.20
6	2.20×10^{-3}	2.32 ± 0.20
7	2.01×10^{-3}	3.10 ± 0.33
9	2.00×10^{-3}	3.30 ± 0.13

-Theoretical equilibrium constants are from [A]Davies et al.,1964; [B]Newsholme and Start, 1973; [C]Weiner et al., 1987; [D]Stitt, 1989; [E]Leegood and ap Rees, 1978.

Mass-action ratios for the PFP catalysed reaction (PPi hydrolysing direction) were calculated based on the assumption that total PPi, Fru-6-P, and Fru-1,6-P were equally distributed in the cytosolic compartment. Based on the product to substrate ratios and the apparent Keq for the PFP reaction (Table 6.2), the amount of Pi required for PFP to catalyse an equilibrium reaction was calculated and shown to range from 4125 (internode 3), 2075 (internode 5) to ± 1650 µM (internode 7 and 9). From the concentration of Pi (Table 6.1), the mass-action ratio for sugarcane PFP was estimated at 5.3 (internode 3), 9.18 (internode 5), and 11.71 - 8.60 (internode 7 - 9).

Variation in the substrate to product concentration of the reversible reactions catalysed by SuSy, UDPGPPase and HPI were also examined. The mass-action ratio for the reaction catalysed by SuSy (cleavage direction) was calculated from the concentrations of the substrate, sucrose and products, UDPGlc and Fru from Table 6.1. As it was not possible to effectively separate UDP from ADP by anion exchange chromatography in this study, the UDP concentration was estimated from the UTP concentration according to ap Rees et al. (1986 b). To calculate the UDP concentration, a constant ATP/ADP ratio of 2.7, and the reported theoretical equilibrium constant for adenylate kinase (0.91) was used. The mass-action ratio of the reaction catalysed by SuSy was very close to the theoretical Keq between internodes 3 and 5 (Table 6.3). From internodes 5 to 9, the estimated mass-action ratio became displaced from equilibrium (Table 6.3), likely as a consequence of the increased sucrose concentration (Figure 6.1 and Table 6.1). The mass-action ratio was displaced such that the cleavage of sucrose would be favoured to restore the equilibrium (Table 6.3).

The Glc-1-P levels were too low to be measured accurately in sugarcane. Therefore, the metabolite ratio (UTP/UDPGlc:PPi) rather than the mass-action ratio was calculated for the UDPGPPase reaction (Table 6.3). The metabolite ratio of the UDPGPPase catalysed reaction was relatively consistent between all internodes investigated (Table 6.3). The mass-action ratio for the reaction catalysed by HPI was both constant and close to the theoretical Keq in all internodes (Table 6.3).

Table 6.3 Comparison of the theoretical equilibrium constants for the reactions catalysed by SuSy, UDPGPPase, and HPI with the ratios of substrates in internodes 3 to 9 of NCo376. Data obtained to calculate the ratios are from Table 6.1.

Internode number	**SuSy** [Fru][UDPGlc] / [Sucrose][UDP] (Keq = 0.15 - 0.56)[A]	**UDPGPPase** [UTP][Glc-1-P] / [UDPGlc][PPi] (Keq = 2.9 - 3.6)[A,B]
	[Fru][UDPGlc] / *[Sucrose][UDP]*	*[UTP]* / *[UDPGlc][PPi]*
3	0.138	0.058
4	0.225	0.015
5	0.128	0.015
6	0.045	0.024
7	0.031	0.025
9	0.015	0.025

	HPI [Fru-6-P] / [Glc-6-P] (Keq = 0.36 - 0.47)[C]
	[Fru-6-P] / *[Glc-6-P]*
3	0.59 ± 0.26
4	0.57 ± 0.07
5	0.58 ± 0.03
6	0.86 ± 0.07
7	0.65 ± 0.04
9	0.92 ± 0.07

Theoretical equilibrium constants are from [A] Weiner et al., 1987, [B] Edwards and ap Rees, 1986 b, [C] ap Rees et al., 1977.

6.3.1.3 Comparison of metabolite ratios between two interspecific hybrids

To determine whether the increased partitioning and rate of carbon flux into respiration in US6656-15 (refer to Chapter 5) was reflected by a change in the control of the reactions catalysing Fru-6-P utilisation/production, regulation of PFK, PFP and FBPase (by variation in the substrate to product ratio) was compared between NCo376 and US6656-15. The differing levels of extractable PFP activity from internode 7 between the two varieties provided additional rationale for investigating fine control of the Fru-6-P/Fru-1,6-P_2 interconversion.

From the concentration of Fru-1,6-P_2, Fru-6-P and PPi (Table 6.4), metabolite ratios were constructed for the reactions catalysed by PFP, PFK and FBPase (Table 6.5). Similar to NCo376, the metabolite concentration of internode 7 from US6656-15 was estimated based on the assumption that the vacuole occupied 90.0 % of the total cell volume. As described previously (refer to section 6.3.1.2), PFK and FBPase were tightly regulated *in vivo*. However, since the Fru-6-P/Fru-1,6-P_2 ratios were comparable between NCo376 and US6656-15, there was no change in the regulation of either PFK or FBPase. The metabolite ratio of the PFP reaction was also comparable between US6656-15 and NCo376 (Table 6.5). According to calculations, a similar concentration of Pi was required for PFP to operate close to the theoretical Keq in both NCo376 and US6656-15 (refer to section 6.3.1.2).

The mass-action ratio for the reaction catalysed by HPI was also included to establish whether Fru-6-P is equilibrated with Glc-6-P in US6656-15. From Table 6.5 it is evident that the mass-action ratio was both similar and very close to the theoretical Keq in NCo376 and US6656-15, indicating that the two hexose monophosphates were equilibrated with equal efficiency in both tissue-types.

Table 6.4 Concentration of Glc-6-P, Fru-6-P, Fru-1,6-P_2 and PPi in internode 7 of NCo376 and US6656-15.

Variety	Metabolite			
	Glc-6-P	Fru-6-P	Fru-1,6-P_2	PPi
	(μM)			
NCo376	117 ± 18	90 ± 12	40 ± 3	162 ± 32
US6656-15	140 ± 20	113 ± 18	60 ± 12	211 ± 18

-Each value is the average $\pm$ SD of three to four separate samples.

Table 6.5 Comparison of the theoretical equilibrium constants for the reactions catalysed by PFP, PFK, FBPase and HPI with the ratios of substrates in internode 7 of NCo376 and US6656-15. Data obtained to calculate the ratios are from Table 6.4.

	PFK	PFP
	$\dfrac{[Fru\text{-}1,6\text{-}P_2][ADP]}{[Fru\text{-}6\text{-}P][ATP]}$	$\dfrac{[Fru\text{-}1,6\text{-}P_2][Pi]}{[Fru\text{-}6\text{-}P][PPi]}$
	(Keq = 300 - 1800)	(Keq = 3.3)
	$\dfrac{[Fru\text{-}1,6\text{-}P_2]}{[Fru\text{-}6\text{-}P]}$	$\dfrac{[Fru\text{-}1,6\text{-}P_2]}{[Fru\text{-}6\text{-}P][PPi]}$
NCo376	0.44	2.71×10^{-3}
US6656-15	0.53	2.56×10^{-3}
	FBPase	**HPI**
	$\dfrac{[Fru\text{-}6\text{-}P][Pi]}{[Fru\text{-}1,6\text{-}P_2]}$	$\dfrac{[Fru\text{-}6\text{-}P]}{[Glc\text{-}6\text{-}P]}$
	(Keq = 174 M)	(Keq = 0.36 - 0.47)
	$\dfrac{[Fru\text{-}6\text{-}P]}{[Fru\text{-}1,6\text{-}P_2]}$	$\dfrac{[Fru\text{-}6\text{-}P]}{[Glc\text{-}6\text{-}P]}$
NCo376	2.27	0.76
US6656-15	1.88	0.80

-References for the theoretical equilibrium constants are in Tables 6.2 and Table 6.3.

6.3.2 SUCROSE CLEAVAGE AND GLYCOLYTIC ENZYME ACTIVITY

6.3.2.1 Enzyme activity in the developing stalk

A subset of enzymes were measured to obtain additional insight into factors which may regulate both the partitioning and rate of carbon flux into respiration. The presence of possible enzyme activators and inhibitors was determined by preparing a series of extracts, each containing at least two different internodal tissues (Botha and Small, 1987). The measured activity in these combined tissue extracts was 108.0 ± 14 % (PFP), 109.0 ± 18 % (PFK), 97.5 ± 7.7 % (aldolase), 98.7 ± 25 % (PK), 91.8 ± 1.8 % (UDPGPPase) and 108.0 ± 10 % (SuSy) of that when the tissues were extracted separately. There was no indication of the effect of either activators or inhibitors on enzyme activity in the internodal tissues from NCo376.

The specific activity of UDPGPPase (Figure 6.2 C), PFP (Figure 6.2 A) and PK (Figure 6.2 B) increased from internodes 3 to 6, and then remained constant. PFK activity remained unchanged (Figure 6.2 A). Measurements of PFP and PFK activity in developing internodes (Figure 6.2) are the same as those presented in Table 4.1 (season 3) and Figure 4.1. The specific activity of aldolase (Figure 6.2 B) and SuSy (Figure 6.2 D) was consistent between internodes 3 and 9. The reason for the decrease in SuSy activity in internode 8 is not evident.

6.3.2.2 Enzyme activity between two interspecific hybrids

Similar to the developing stalk, the extractable activities of PFP, PFK, aldolase, PK, UDPGPPase and SuSy in internode 7 were compared between NCo376 and US6656-15. The presence of possible activators and inhibitors in NCo376 and US6656-15 was tested by measuring extracts containing a combination of tissues from both varieties. Enzyme activity in the combined tissue extracts was 94.5 ± 0.76 % (PFP), 102.0 ± 10 % (PFK), 113.0 ± 9.4 % (aldolase), 99.5 ± 25 % (PK), 105.0 ± 7.0 % (UDPGPPase) and 90.9 ± 4.4 % (SuSy) of that when the tissues were extracted separately.

Comparable levels of SuSy, UDPGPPase and aldolase were present in both NCo376
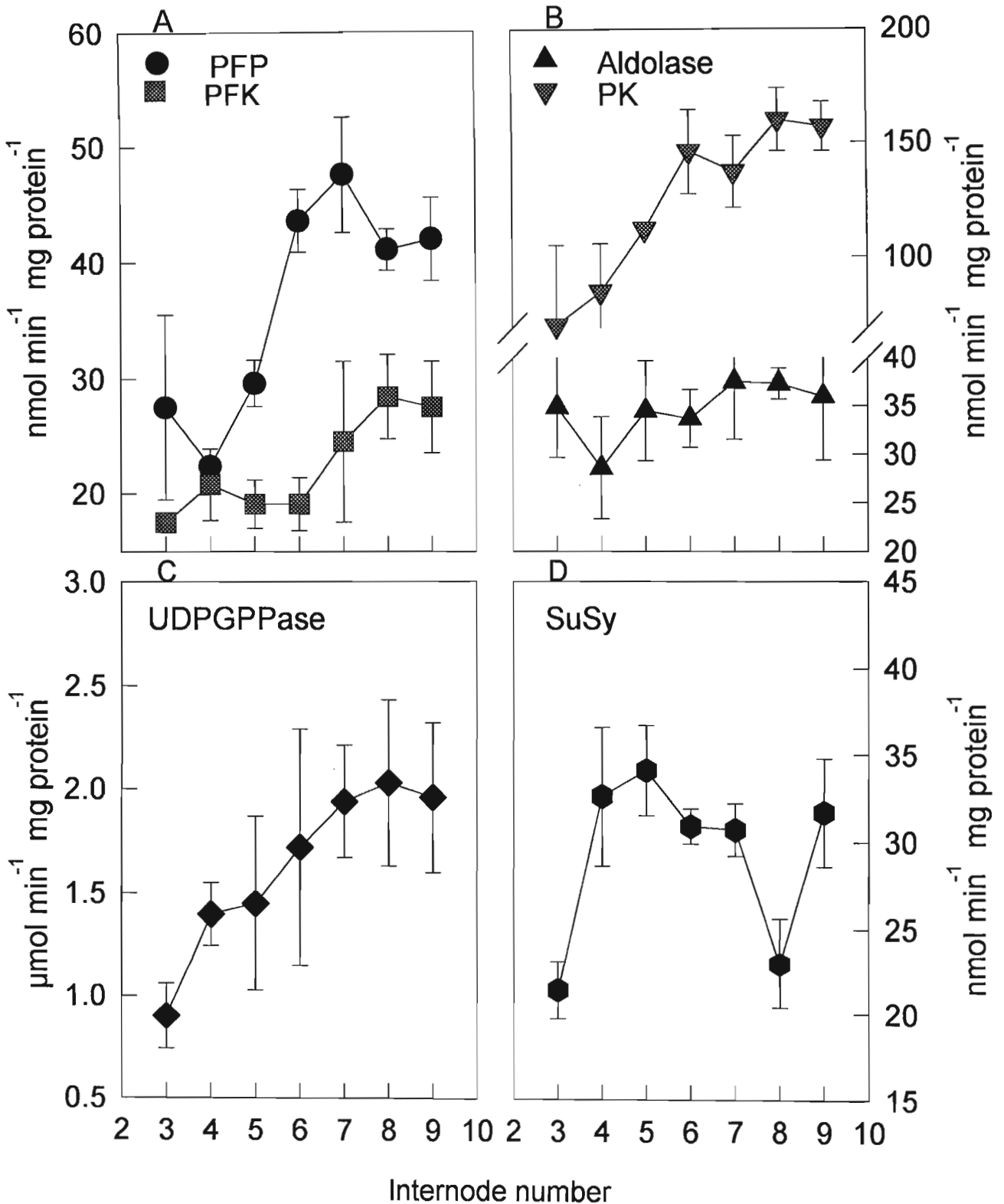
Figure 6.2 Changes in the extractable activity of (A) PFP and PFK, (B) aldolase and PK, (C) UDPGPPase and (D) SuSy between internodes 3 and 9 in NCo376. Each value is the mean ± SD of three to four separate extractions.

Table 6.6 Sucrose cleavage and glycolytic enzyme activity in internode 7 of US6656-15 and NCo376.

Enzyme	US6656-15	NCo376
	$(nmol\ min^{-1}\ mg\ protein^{-1})$	
PFP	77.8 ± 6.2	40.6 ± 7.8
PFK	32.0 ± 9.1	42.2 ± 9.7
Aldolase	47.3 ± 0.8	53.7 ± 9.4
PK	130 ± 10.0	178 ± 40.0
SuSy	35.5 ± 3.6	41.1 ± 3.6
UDPGPPase	1480.0 ± 77.0	1330.0 ± 100.0

-Each value is the mean ± SD from three separate plants.

and US6656-15 (Table 6.6). PFK and PK activity were slightly higher in US6656-15, but not significantly different from NCo376 (Table 6.6). Specific extractable PFP activity in US6656-15 was almost double the extractable activity in NCo376 (Table 6.6).

6.3.3 CARBON LABELLING OF SUCROSE FROM GLUCOSE AND GLYCEROL

To investigate whether carbon can be cycled between the triose-P and hexose monophosphate pools, internode 7 tissue slices from NCo376 and US6656-15 were labelled with [U-^{14}C]glucose and [U-^{14}C]glycerol, respectively. The choice of internode 7 is based on the observation that SuSy activity catalyses sucrose cleavage and that the highest developmental levels of PFP are recorded. The varieties NCo376 and US6656-15 were selected as PFP activity differed significantly between them. Labelling of the sugars with glycerol would necessitate the conversion to dihydroxyacetone phosphate and subsequent flux into the hexose monophosphate pool.

Partitioning of ^{14}C from glucose into the sugars (sucrose and fructose) was higher in NCo376 than in US6656-15 (Table 6.7). When the tissues were labelled with ^{14}C-glycerol, an equivalent proportion of the carbon metabolised away from the glycerol was recovered in the sugars (mostly sucrose) (Table 6.7). The percentage of carbon (from glycerol) that would have entered the hexose monophosphate pool between the varieties was calculated according to Hill and ap Rees (1994) as follows:

$$\frac{\% \ ^{14}C \ \text{partitioned into sugars from labelled glycerol} \ X \ 100}{\% \ ^{14}C \ \text{partitioned into sugars from} \ ^{14}C\text{-glucose}}$$

From the data in Table 6.7, the percentage calculated was 20.8 ± 0.19 and 43.2 ± 9.35 for NCo376 and US6656-15, respectively. Results, therefore, showed that a higher percentage of carbon entered the hexose monophosphate pool from the triose-P pool in US6656-15 than in NCo376. Additionally, there was a positive relationship between extractable specific PFP activity and the percentage labelling of the hexose

monophosphate pool between the two varieties.

Table 6.7 Comparison between the PFP activity and the percentage carbon entering the sugar pool in internode 7 tissue slices of NCo376 and US6656-15, supplied with uniformly labelled glucose and glycerol for 5 h.

	NCo376	US6656-15
-% ^{14}C partitioned into the sugars (Suc, Fru) from metabolised ^{14}C-glucose	63.89	33.90
-% ^{14}C partitioned into the sugars (Suc, Glc, Fru) from metabolised ^{14}C-glycerol	13.29 ± 0.12	14.63 ± 3.17
-PFP activity (nmol min^{-1} mg protein^{-1})	42.10 ± 4.15	74.56 ± 3.50

-For the ^{14}C-glycerol labelling the % value is the mean ± SD of three separate samples.

-For the ^{14}C-glucose labelling the % value is representative of a single labelling experiment using a bulked tissue samples from three separate plants.

-Each PFP activity value is the mean ± SD of three separate samples.

6.4 DISCUSSION

Characterising the regulation of respiratory flux is a prerequisite for elucidating the control of carbon partitioning between major biosynthetic pathways (Blakeley and Dennis, 1993; Plaxton, 1996). The present work initiates this important area of research in sugarcane internodal tissues by examining various control mechanisms acting on *in vivo* sucrose cleavage and glycolytic enzyme activity. The qualitative, traditional approach of control analysis was employed to investigate metabolic regulation in this study. This approach is directed towards investigating the key regulatory enzymes in the pathway, which in plant glycolysis are shown to exist at the PEP to pyruvate conversion and the Fru-6-P and Fru-1,6-P_2 interconversion (Adams and Rowan, 1970; Kobr and Beevers, 1971; Dixon and ap Rees, 1980 a; Turner and Turner, 1980; Day and Lambers, 1983; Beaudry et al., 1989; Turpin et al., 1990; Geigenberger and Stitt, 1991 b; Hatzfeld and Stitt, 1991). The mechanisms of fine control which were investigated in this study included variation in substrate/product concentration, substrate limitation and variation in metabolite effectors (allosteric regulation).

The metabolite ratios of the PK catalysed reaction in sugarcane are consistent with that published for other tissues (Leegood and ap Rees, 1978; Turner and Turner, 1980, Day and Lambers, 1983), and show that PK is tightly regulated *in vivo*. Stimulation of carbon flux into the TCA cycle through activation of PK is traditionally depicted by a decrease in PEP and attendant increase in pyruvate levels (Adams and Rowan, 1970; Kobr and Beevers, 1971; Turner and Turner, 1980; Beaudry et al., 1989; Stitt et al., 1990; Turpin et al., 1990). In sugarcane, the ratio of pyruvate to PEP remained unchanged, indicating that the decrease in respiration is not associated with an alteration in the fine regulation of PK activity by changing substrate/product concentration. Although the activation of a regulatory enzyme may not necessarily result in increased product levels (especially if the subsequent step in the pathway is also activated), a decrease in the precursor substrate is a prerequisite indicator of a

change in regulation (Dixon and ap Rees, 1980 a; Geigenberger and Stitt, 1991 b). As PEP concentration is not significantly altered between internodes 3 and 7 in sugarcane, it is also unlikely that an alteration in the regulation of cytosolic PEPCase by changing substrate concentration occurred. Since PEPCase, in conjunction with malate dehydrogenase, functions to replenish TCA cycle intermediates consumed by biosynthetic activity (Wiskich and Dry, 1985; ap Rees, 1990; Schuller et al., 1990; Sangwan et al., 1992), regulation of this anaplerotic by-pass may be more important in the younger sugarcane internodes where anabolic respiration is shown to be higher (refer to Chapter 5).

The overall concentration of sugarcane PEP is within the Km range of PK for PEP (30 to 200 µM) as recorded for several purified PK preparations from other plant tissues (Ireland et al., 1980; Baysdorfer and Bassham, 1984; Lin et al., 1989; Podestá and Plaxton, 1991). Hence, it is unlikely that substrate limitation of PK may have been the cause for the decline in respiration observed during internodal maturation. Similarly, sugarcane PEP concentrations are also sufficient to stimulate PEP specific phosphatase (Km = 50 µM: Duff et al., 1989 a), an alternative by-pass to PK under adenylate starvation (Duff et al., 1989 b; Theodorou and Plaxton, 1993).

From the ratios of Fru-1,6-P$_2$ and Fru-6-P, both within the developing stalk and across the varieties, it is also apparent that no change in the regulation of PFK and FBPase occurred in response to the change in carbon partitioning and flux into respiration. Previous investigations have indicated that stimulation of PFK follows the decline PEP (Adams and Rowan, 1971; Turpin et al., 1990; Geigenberger and Stitt, 1991 b; Hatzfeld and Stitt, 1991), which is consistent with reports showing allosteric regulation of purified PFK by PEP (Garland and Dennis, 1980 a; Garland and Dennis, 1980 b; Cawood et al., 1988). Sensitivity of PFK to allosteric regulation by PEP has been shown to differ between the cytosolic and plastid isoenzymes in a strictly pH dependent manner (Dennis and Greyson, 1987; Botha et al., 1988 b; Cawood et al., 1988). The levels of PEP in sugarcane are within the I$_{0.5}$ range of several purified PFK preparations for PEP (0.10 to 130 µM: Garland and Dennis, 1980 a; Isaac and Rhodes, 1986; Dennis and

Greyson, 1987; Cawood et al., 1988). Although it is reported that Pi alleviates allosteric inactivation of PFK by PEP (Cawood et al., 1988), it is possible that PFK may have been inhibited to some extent by PEP in sugarcane.

The levels of sugarcane PPi are within the range (10 to 300 μM) obtained for other tissues (Edwards et al., 1984; Botha and Small, 1987; Kesy and Kowakczyk, 1987; Weiner et al., 1987; Mohanty et al., 1993) and in excess of the Km of PFP for PPi (10 to 70 μM), characterised previously (van Schaftigen et al., 1982; Kombrink et al., 1984; Botha et al., 1986; Botha and Small., 1987; Mahajan and Singh, 1989; Stitt, 1989; Wong et al., 1990). Assuming that the Fru-2,6-P_2 extracted from sugarcane is not allosterically bound to enzymes (Stitt, 1987), then the concentration is well in excess of the Ka (2.5 to 150 nM) required to fully activate PFP (Sabularse and Anderson, 1981; van Schaftigen et al., 1982; Botha et al., 1986; Ashihara and Horikosi, 1987).

The estimated *in vivo* concentration of Fru-6-P in sugarcane is fairly low (178 to 104 μM in internodes 3 to 9) and lies within the lower limit of the concentration range (30 to 5000 μM) reported for other tissues (Botha and Small, 1987; Weiner et al., 1987; Turpin et al., 1990; Wendler et al., 1990; Geigenberger and Stitt, 1991 b; Nakamura et al., 1992; Mohanty et al., 1993; Hill and ap Rees, 1994). Furthermore, the levels of sugarcane Fru-6-P are within the lower limit required for half maximal activity of both PFK$_c$ (S$_{0.5}$ range = 25 to 700 μM) and PFP (Km range = 50 to 1200 μM) characterised from a range of other tissues (Garland and Dennis, 1980; Sabularse and Anderson, 1981; Cséke et al., 1982; van Schaftigen et al., 1982; Kombrink et al., 1984; Bertagnolli et al., 1986; Botha et al., 1986; Botha and Small, 1987; Botha et al., 1988 b; Stitt, 1989; Nakamura et al., 1992; Nielson, 1994). As neither PFP or PFK$_c$ is likely to be saturated with Fru-6-P, this has very interesting implications for the distribution of available Fru-6-P between the two enzymes and hence the regulation of Fru-6-P utilisation. If it is assumed that the cytosol comprises 10 % of the cell volume in all internodes, then the two-fold decline in Fru-6-P observed from internodes 3 to 9 may result in the down-regulation of PFP (forward direction) and PFK activity *in vivo*. Thus, substrate limitation might have been a cause for the decline in carbon partitioning to

respiration associated with internodal maturation.

No activators or inhibitors of PFK, PFP, aldolase and PFK were evident in internodal extracts from the developing stalk. As specific PFK, PFP, aldolase and PK activity did not decline from internodes 3 to 9, it is unlikely that coarse regulation of these enzymes may have been a contributory factor to the decrease in carbon partitioning to respiration. Similar observations have previously been documented for other plant species (Beaudry et al, 1987; MacRae et al., 1992).

Results from the present study suggest that PFP could also contribute to triose-P recycling in sugarcane internodal tissue. A number of observations lend support to this proposal. Firstly, the mass-action ratio of the PFP catalysed reaction in sugarcane is close to equilibrium *in vivo* as reported for other plant tissues (Cséke et al., 1982; Kruger et al., 1983 a; Edwards and ap Rees, 1986 b; Weiner et al., 1987; Hatzfeld et al., 1990; Geigenberger et al., 1993). Hence, PFP could conceivably catalyse flux in either the glycolytic or gluconeogenic direction (Stitt, 1989). As the enzyme catalyses an equilibrium reaction, an imbalance in the $Fru-6-P/PPi$ to $Fru-1,6-P_2/Pi$ ratio would have the resultant effect of stimulating PFP activity to restore the equilibrium (Stitt, 1989). The slight displacement of the mass-action ratio towards preferential catalysis in the reverse direction from internodes 3 to 5 - 9 may simply be a consequence of a change in the heterogenous distribution of intracellular Pi levels.

Furthermore, the *in vivo* $Fru-1,6-P_2$ concentration is both within the range of other tissues (20 to 450 μM) and the Km ($Fru-1,6-P_2$) of PFP (10 to 140 μM: Bertagnolli et al., 1986; Botha et al., 1986; Mahajan and Singh, 1989; Stitt, 1989; Hatzfeld et al., 1990; Turpin et al., 1990; Wendler et al., 1990; Wong et al., 1990; Nakamura et al., 1992; Nielson, 1994). Assuming that the Pi concentration in sugarcane is evenly distributed in the cell, then Pi in the cytosol is estimated to be well in excess of the required Km (129 to 750 μM) of PFP (Botha et al., 1986; Stitt, 1989; Nielson, 1994) for catalysis in the reverse direction. However, the Pi concentration in sugarcane increases two-fold from internodes 3 to 9. Increasing Pi levels become increasingly inhibitory to PFP

activity in the forward direction (Botha et al., 1986; Stitt, 1989). It is therefore possible that PFP activity (catalysing the conversion of Fru-6-P to Fru-1,6-P_2) in the more mature sugarcane internodes may have been down-regulated by Pi. Down regulation of PFP (forward direction) by Pi, combined with the decline in Fru-6-P concentration and the possible allosteric effects of PEP on PFK activity, may have contributed to the decline in respiratory carbon flow associated with maturation.

Fru-1,6-P_2 content is also well in excess of that required to stimulate FBPase by 50 % (Km = ± 5.0 µM: Herzog et al., 1984; Kruger and Beevers, 1984). However, Fru-2,6-P_2 is a potent allosteric inhibitor of FBPase$_c$ (Herzog et al., 1984; Kruger and Beevers, 1984). From the present study, the concentration of Fru-2,6-P_2 in sugarcane is probably sufficient to effect significant inhibition of FBPase$_c$ by increasing the $S_{0.5}$ for Fru-1,6-P_2 (Kruger and Beevers, 1984). Although the AMP content was not determined from the present study, increasing concentrations of the latter would serve to magnify the inhibition of FBPase$_c$ by Fru-2,6-P_2 (Herzog et al., 1984). It is thus possible that PFP may also have contributed increasingly to carbon flux from the triose-P to hexose monophosphates, especially in the younger internodes where Fru-2,6-P_2 might lead to a greater inhibition of FBPase.

Additionally, preliminary results involving the labelling of sugars from ^{14}C-glycerol show that flux from the triose-P to the hexose monophosphates may occur in sucrose storage tissues. Cycling of carbon between the hexose monophosphate and triose-P pools has previously been recorded for tissues engaged in starch biosynthesis (Keeling et al., 1988; Dancer et al., 1990 a; Hatzfeld et al., 1990; Hill and ap Rees, 1994; Hill and ap Rees, 1995). The results from the present study contrast with that of Sacher et al. (1963), who claimed negligible randomisation of carbon between the hexose monophosphates and triose-P in internodal tissue slices during sucrose accumulation in sugarcane. As both PFP and FBPase could catalyse flux from the triose-P pool to the hexose monophosphates, the extent to which PFP alone contributes to this flux in sugarcane remains to be investigated. Only in cell suspension cultures of *Chenopodium rubrum* (Hatzfeld et al., 1990) and climacteric banana (Hill and ap Rees,

1995) has PFP been shown to be solely responsible for catalysing flux from Fru-1,6-P$_2$ to Fru-6-P, since FBPase is either insufficient or absent (Hatzfeld et al., 1990; Ball et al., 1991). In sugarcane, accurate measurements of FBPase were not possible due to interference by contaminating PFP activity. The higher rate of recycling from the triose-P to hexose monophosphates, evident in *Chenopodium rubrum* cells is attributed to a requirement for PPi by SuSy and UDPGPPase (Hatzfeld et al.,1990). The significance of such a cycle in sugarcane internodal tissue will be reviewed in conjunction with the reactions occurring upstream of Fru-6-P.

The mass-action ratio of the reaction catalysed by SuSy in the younger sugarcane internodal tissues is close to equilibrium *in vivo*. Similar results have been documented for SuSy in other plant species (Geigenberger and Stitt, 1993; Merlo et al., 1993). However, if the assumptions made for the calculation of the mass-action ratio for the SuSy reaction are correct, then the enzyme is significantly displaced from equilibrium in the more mature internodes and sucrose cleavage would be favoured. This is primarily attributed to the increase in sucrose concentration. Available evidence suggests that a higher proportion of imported sucrose in maturing internodes occurs symplastically due to increased lignification and suberisation (Jacobsen et al., 1992) and that a large proportion of stored sucrose is not compartmentalised in the vacuole (Hawker, 1965; Welbaum and Meinzer, 1990; Preisser et al., 1992). These findings collectively suggest that at any point in time, the prevailing sucrose concentration in the cytosol of the mature internodes (irrespective of the variety), is likely to be higher than that of the immature internodes. Since SuSy is controlled by substrate availability *in vivo* (Avigad, 1982), catalysis is likely to proceed towards cleavage of cytosolic sucrose to restore the equilibrium. In previous reports, where the mass-action ratio of SuSy has been shown to be close to the theoretical Keq *in vivo*, the sucrose content in the tissues investigated is reported to range from approximately 10 to 100 µmol gram fresh mass^{-1} (Edwards and ap Rees 1986 b; Morrel and ap Rees, 1986; Geigenberger and Stitt, 1993). Those sucrose contents cited are comparable to the sucrose levels in the younger internodal tissues where the mass-action ratio of SuSy is close to the theoretical Keq *in vivo*. Although SuSy is capable of catalysing the synthesis of

sucrose *in vitro* (Buczynski et al., 1993), this does not necessarily imply that the *in vivo* conditions are conducive to sucrose synthesis.

The involvement of SuSy in sucrose cleavage in maturing internodes is consistent with the possible role for SuSy in sink strength in sugarcane as proposed by Lingle (1996). Similar to starch storing sinks, the continued cleavage activity by SuSy would be dependent on the effective removal of the products, UDPGlc and Fru (Stitt and Steup, 1985; Edwards and ap Rees, 1986 b). From the low concentration of UDPGlc and fructose measured between internodes 6 and 9, production inhibition of SuSy is not likely to have occurred in sugarcane (Avigad, 1982; Copeland, 1990). Hence, it is probable that the effective removal of both UDPGlc and fructose occurs. Since the reaction catalysed by fructokinase is reported to be highly regulated and far removed from equilibrium *in vivo* (Avigad, 1982; Copeland, 1990), it is feasible that fructose would have been immediately phosphorylated to Fru-6-P in sugarcane. This, however, does remain to be investigated. The consistent Glc-6-P to Fru-6-P ratio in all sugarcane internodes is very close to the theoretical Keq and provides evidence for the rapid maintenance of equilibrium of these two intermediates by PGM, similar to that reported for other sink tissues (Turner and Turner, 1980; Day and Lambers, 1983; Edwards ap Rees et al., 1986 b; Beaudry et al., 1989; Geigenberger et al., 1993). Removal of UDPGlc and Fru-6-P from cleavage activity may occur via SPS, which is primarily responsible for the synthesis of sucrose from internode 5 onwards (refer to Chapter 5). Resynthesised sucrose not deposited in the vacuole would probably serve as a substrate for subsequent cleavage by SuSy, thereby contributing to the simultaneous cycle of sucrose synthesis and cleavage in sugarcane.

Equilibrium of the UDPGlc, produced from cleavage activity, with hexose monophosphates downstream may also occur via the reversible reaction catalysed by UDPGPPase. Although Glc-1-P could not be detected in sugarcane, it is evident from the metabolite ratio of UDPGlc:PPi /UTP that the UDPGPPase catalysed reaction is not significantly displaced in sugarcane internodes. Moreover, the concentration of UDPGlc and PPi in sugarcane is within the range of the Km of purified UDPGPPase for

the pyrophosphorolytic substrates, PPi (110 to 140 µM) and UDPGlc (120 to 140 µM), respectively (Nakano et al., 1989; Sowokinos et al., 1993). Since specific UDPGPPase activity increases from internodes 3, there does not appear to be a relationship between the provision of UDPGlc for structural polysaccharides and UDPGPPase activity. This is also evident for PFP activity (refer to Chapter 5). Specific UDPGPPase activity is comparable to that of other species actively engaged in sucrose breakdown (Edwards and ap Rees, 1986 a; Morrel and ap Rees, 1986; Botha et al., 1992). Neither the requirement for such exceedingly high levels of UDPGPPase, nor the requirement to induce a further developmental increase in activity is clear for either sugarcane or other species (Edwards and ap Rees, 1986 a; Morrel and ap Rees, 1986).

The provision of PPi for UDPGPPase activity (cleavage direction) could be attained through a coupling of the reaction catalysed by PFP, as has been reported for other tissues (Edwards and ap Rees 1986 b; Huber and Akazawa, 1986; Dancer and ap Rees, 1989; Hatzfeld et al., 1990). Provision of the substrate Fru-1,6-P_2 for catalysis in the reverse direction could be mediated by PFK activity. The increase in PFK activity, similar to PFP, may be indicative of the involvement of PFK in this manner. Although PFP could supply PPi to stimulate entry of cleaved sucrose into the hexose monophosphate pool, the relative contribution of SuSy and the invertases to sucrose cleavage/hydrolysis in sugarcane is not known (Moore, 1995).

Conclusion

Mass action ratios indicated that the reactions catalysed by PFK, FBPase and PK are tightly regulated *in vivo*. There is, however, no change in the regulation of PFK, FBPase and PK activity (via an alteration in the product to substrate ratio) associated with the decrease in carbon allocation to respiration. The *in vivo* substrate concentrations are within the Km or $S_{0.5}$ range of PFK, PFP, FBPase and PK. However, there is a decrease in the Fru-6-P concentration associated with internodal maturation.

This suggests that substrate limitation may have been a cause for the decline in carbon partitioning to the respiratory pathway. Furthermore, the prevailing PEP levels and the increasing Pi concentration during internodal maturation may also have contributed to the down-regulation of PFK and PFP (forward direction), respectively. It is, however, notable that the mechanisms of fine control investigated in the present study are not the only means of control by which glycolytic enzyme activity can be modulated. Additional mechanisms of fine control such as variation in pH, subunit association-dissociation and reversible covalent modification may also have contributed to the decline in respiration through effect on glycolytic enzyme activity.

The mass-action ratio of the PFP catalysed reaction was close to the theoretical Keq, demonstrating that PFP may operate in either the glycolytic or gluconeogenic direction according to demand and supply of cellular substrate. From aspects of fine control, it is probable that the PPi-dependent sucrose cleavage pathway operates at all stages of internodal maturation and that this pathway would likely contribute to cycling within the cytosol.

CHAPTER 7

GENERAL CONCLUSIONS

The characterisation of carbon partitioning into the three major carbohydrate sinks (sucrose storage, respiration and insoluble matter synthesis) is a requirement to identify regulatory mechanisms controlling sucrose accumulation in sugarcane. Since significant differences in sucrose content are evident between the *Saccharum* species and within the developing stalk, it was hypothesised that changing patterns of carbon allocation between sucrose storage and non-sucrose storage related biosynthetic activity must exist.

Results from the present study indicate an inverse relationship in carbon partitioning between sucrose storage and respiration (CO_2 production and biosynthesis) in sugarcane internodal tissue. This relationship is consistent between different varieties and within the developing stalk. In internode 7 of two interspecific hybrids (NCo376 and US6656-15) and one *Saccharum spontaneum* (Coimbatore), carbon allocation to sucrose ranges from 64 % (NCo376) to 37 % (US6656-15) and 22 % (Coimbatore). The percentage carbon allocation to sucrose is inversely proportional to the percentage allocated to respiration, which ranges from approximately 12 % (NCo376) to 55 % (Coimbatore). Since more than 90 % of the glucose supplied is metabolised by the internodal tissues of all three varieties, these findings indicate that respiratory activity is a major sink for hexose monophosphates in the poorer sucrose storing varieties.

Within the stalk of NCo376, carbon partitioning to respiration (from uniformly labelled glucose) decreases more than 50 % from internodes 2 to 7. The reduction in carbon partitioning to respiration is evident irrespective of whether sucrose, glucose or fructose is used as the labelled substrate. Of the carbon partitioned into respiration, a larger proportion is recovered in the anabolic component (amino and organic acids). From

internodes 2 to 7 there is also a three-fold decrease in the percentage carbon partitioned to the insoluble matter. Hence, internodal maturation in the developing sugarcane stalk coincides with a redirection of carbon flux from insoluble matter biosynthesis and respiration to sucrose. The percentage carbon distribution between sucrose, insoluble matter and respiration in internodes 7 and 18 is comparable, suggesting that once the sucrose content (as a percentage of the dry mass) reaches maximum concentration, no further change in partitioning is apparent.

Collectively, the present results indicate distinct developmental changes and cross-varietal differences in carbon partitioning between sucrose storage and non-sucrose storage related biosynthetic activity. The decline in carbon allocation to the insoluble matter component and respiration, potentially suggest a change in the coarse and/or fine regulation of cell wall polysaccharide synthesis and glycolysis, respectively. Changes in carbon partitioning to respiration may also reflect a changing carbon flux through the OPP pathway. However, the relative contribution of glycolysis and the OPP pathway to glucose catabolism could not be determined due to interference by CO_2 released during pentan synthesis.

To establish whether the decline in carbon flux to respiration is associated with an alteration in the regulation of the key reaction steps of glycolysis, mass-action ratios were calculated from the measured metabolites extracted from internodes 3 to 9 of NCo376. The reactions catalysed by PK, PFK and FBPase in sugarcane are tightly regulated *in vivo*. However, there is no alteration in the regulation of those enzymes by changing substrate to product ratios coinciding with the decline in respiratory carbon flux. Similarly, in internode 7 of two interspecific hybrids (NCo376 and US6656-15), no alteration in the regulation of PFK and FBPase is evident from the metabolite ratios.

The metabolite concentrations between internodes 3 to 9 of the developing stalk were also analysed to determine whether substrate limitation of PK, PFP or PFK may have contributed to the decline in carbon allocation to respiration. In sugarcane, the PEP concentration is within the Km range of PK. Hence, it is unlikely that PEP limitation

may have been a contributory factor to the decline in respiration. However, the Fru-6-P concentration is at the lower limit of the Km and $S_{0.5}$ requirement of PFP and PFK, respectively (as determined from the kinetic properties of several purified enzyme preparations from other tissues). If it is assumed that the cytosol comprises 10 % of the cell volume in all internodes, then the concentration of Fru-6-P decreases two-fold from internodes 3 to 7. It is thus feasible that Fru-6-P limitation might have contributed to the decreased carbon partitioning to respiratory flux.

Furthermore, the PEP concentration in sugarcane internodal tissue is sufficient to have contributed to the allosteric inhibition of PFK even at the prevailing Pi concentration. There is a two-fold increase in the concentration of Pi from internodes 3 to 7. Although Pi levels are in excess of the requirement to fully activate PFP in the reverse direction, the levels measured are within the range to down-regulate activity in the glycolytic (forward) direction in the more mature internodes. Inhibition of PFP activity by Pi is also likely to be magnified by the three-fold decline in the concentration of the allosteric activator, Fru-2,6-P_2 between internodes 4 and 7. The Fru-2,6-P concentration in sugarcane is in excess of the Ka required to fully activate PFP *in vivo*, and within the range to have inhibited cytosolic FBPase activity (especially in the younger internodes). The decrease in Fru-6-P combined with the possible allosteric inhibition of PFK and PFP (in the mature internodes) may have contributed to the decline in carbon flux towards respiration. It is notable that although use was made of a range of Km and $S_{0.5}$ values recorded in the literature for purified PK, PFK and FBPase, variation in the kinetic properties is evident between those enzymes in different plant species. Thus, only from a study of the kinetic and regulatory properties of the key regulatory enzymes in sugarcane, can the extent of substrate limitation or allosteric inactivation be more accurately estimated.

The developmental profile of specific PFK, PK and PFP activity is inversely related to the proportion of carbon allocated to respiration. PFK activity remains constant, whilst PK activity increases three-fold from internodes 3 to 7. In the stalk, specific PFP activity increases approximately two-fold from internodes 3 and 7, after which levels

decline slightly and remain constant. Although the specific activities of PK, PFK and PFP extracted from the internodes were measured *in vitro*, it would appear that coarse control of these enzymes is not a contributory factor to the decline in respiration. Similarly, in internode 7 of NCo376 and US6656-15, there is no significant change in the specific PK and PFK activity, although carbon partitioning to respiration varied between these two varieties. However, it can be noted that the combined activity of both the plastid and cytosolic isoforms of the enzymes was assayed in the crude tissue extracts. Any alteration in the contribution of cytosolic isoenzyme activity to total activity was not determined in relation to respiration. It is also possible that the specific activity of the glycolytic enzymes are maintained to sustain carbon flow to the TCA cycle during internodal maturation.

PFP activity is positively correlated to respiratory flux in internode 7 of NCo376 and US6656-15. Additionally, an inverse relationship between specific PFP activity and sucrose content in internode 7 is evident across seven interspecific hybrid varieties. This negative correlation between PFP activity and sucrose is also evident in 70 clones of a F1 segregating population. These results are the first to indicate an inverse relationship between PFP activity and sucrose levels in a sucrose storage tissue.

From the present study, specific PFP activity in sugarcane appears to be controlled in part by PFP protein expression. The cross-reactivity between the anti-potato PFP$_{(total)}$ serum and the sugarcane PFP protein further indicates that there is antigenic similarity in the primary structure of PFP. The anti-potato PFP$_{(total)}$ serum is also effective at immuno-inactivating sugarcane PFP, thereby providing authenticity of the cross-reacting protein as PFP. Additionally, the immunoprecipitation studies indicate that sugarcane PFP is immunologically similar to potato and that of other plant species with respect to catalysis. Despite the immunological similarities between PFP from sugarcane and that of other plant species, substantial differences in the primary structure are shown to exist. In sugarcane, a single cross-reacting protein band is resolved. This contrasts with the presence of two (α- and β subunit) polypeptides reported for other plant species. The presence of a single cross-reacting protein band

does not negate the presence of both α- and β subunits, which in sugarcane, may be of a similar size. Additionally, the molecular mass of the sugarcane polypeptide(s) is substantially larger (approximately 72 kD) than that documented previously. The larger polypeptide(s) mass suggests that either the length of coding sequence of the PFP gene differs or that transcription or translation processing factors differ between sugarcane and other plant species. The unusual primary structure of the protein may reflect distinct differences in the regulatory and kinetic properties between sugarcane PFP and other plants. Therefore, to better understand the physiological role of PFP in sugarcane, a study of the purified protein is warranted.

In sugarcane, PFP catalyses a reaction close to equilibrium and could therefore conceivably operate in both the glycolytic and gluconeogenic direction. Since Fru-6-P forms the substrate for both PFP and PFK, and concentration declines with maturation, it is considered unlikely that *in vivo* PFP activity is primarily involved in catalysing Fru-6-P utilisation for respiratory activity. Preliminary findings have indicated that carbon flux from the triose-P to the hexose monophosphates is present in sugarcane. Although the relative contribution of FBPase and PFP activity to this reverse flux has not been investigated, the results suggest that PFP activity is functional *in vivo* and that hexose monophosphate/triose-P substrate cycling may occur in sugarcane. An involvement of PFP in the PPi-dependent sucrose cleavage pathway is also feasible. From the radiolabelling data and the estimated mass-action ratio for the reaction catalysed by SuSy, it appears that SuSy catalyses sucrose cleavage *in vivo*. The UDPGPPase and PFP reactions are sufficiently close to equilibrium *in vivo* to catalyse the formation/utilisation of PPi according to supply and demand of cellular substrate.

Although there appears to be an inverse relationship in coarse regulation of PFP and sucrose storage in sugarcane, the precise physiological role of PFP activity in internodal tissues can only be clarified from a direct approach using molecular technology to down-regulate enzyme activity. Successes will largely be based upon the complete elimination of the enzyme. As the effective manipulation in sugarcane is more likely to be attainable through use of sugarcane stem-specific promoter and a

PFP gene sequence, molecular studies should be included as a long-term research goal.

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