Analysis of intermediate carbon metabolism in strawberry plants

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Declaration

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Summary

Strawberry (*Fragaria x ananassa*) fruit quality is largely determined by the relative amounts of sugars and organic acids present, as well as soluble solid content. This study had three components: 1) Characterisation of cytosolic carbohydrate metabolism and carbon partitioning to sugars and organic acids in two commercial varieties, 2) analysis of transgenic strawberry fruit with increased pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (PFP) activity and 3) analysis of transgenic strawberry fruit with increased ß-fructosidase (invertase) activity in either cytosol or apoplast. Analyses of transgenic strawberry may inform similar attempts in grape berries.

Festival and Ventana, two popular commercial strawberry cultivars in South Africa, were fairly similar with respect to sugar and organic acid content. Twelve cytosolic enzymes were investigated. Temporal differences in maximum catalytic activity were observed for invertase, PFP, pyruvate kinase and ADP-glucose pyrophosphorylase (AGPase). Invertase, PFP and AGPase activity also differed between the cultivars. One enzyme, SuSy, could not be analysed effectively, due to the purification method employed. These analyses established methodology for the analysis of transgenic berries.

Constructs were designed to constituitively express *Giardia lamblia* PFP (GL-PFP), or to express *Saccharomyces cerevisiae* invertase (SCI) in a fruit-specific manner. A second invertase construct was designed to target SCI to the apoplast. Strawberry (cv. Selekta) was transformed and the presence of each transgene confirmed by PCR. Untransformed Selekta was used as control in both transgenic studies.

Transgenic lines were selected based on GL-PFP activity in leaves and total PFP activity in ripe fruit. Sugar and organic acid content of ripe berries with high PFP activity was determined. Although berries displayed marked changes in sugar composition, the total sugar content was similar to controls, in all except one line. Organic acid content was decreased, leading to a clear reduction in organic acid-to-sugar ratio. This points to a gluconeogenic role for PFP in strawberry fruit.

Transgenic berries were screened for SCI activity. Berries containing untargeted SCI exhibited total invertase activity similar to controls and were not analysed further. Berries with apoplast-targeted SCI displayed three-fold increases in invertase activity compared to controls. Total sugar content was reduced and exhibited reduced sucrose content relative to hexoses. Despite the effect of increased invertase activity on metabolites, maximum catalytic activity of enzymes involved in cytosolic sucrose, hexose and organic acid metabolism were unchanged. Transgenic plants selected in these studies were subsequently vegetatively replicated and future work will include immature fruit.

Opsomming

Die vruggehalte van aarbeie (*Fragaria x ananassa*) word hoofsaaklik bepaal deur die verhouding tussen suikers en organiese sure, asook totale suiker- en suur-inhoud. Hierdie studie het drie komponente behels, naamlik 1) die karakterisering van sitosoliese koolhidraatmetabolisme en koolhidraat-afbakening tussen suikers en organise sure in twee kommersiële variëteite, 2) die analise van transgeniese aarbei vrugte met hoër pirofosfaat: D-fruktose-6-fosfaat 1-fosfotransferase (PFP) aktiwiteit en 3) die analise van transgeniese aarbei vrugte met hoër invertase aktiwiteit in óf die sitosol of die apoplastiese ruimte. Inligting wat uit dié analises voortspruit mag dalk soortgelyke projekte in druiwe beïnvloed.

Twee gewilde kommersiële aarbeisoorte in Suid-Afrika, Festival en Ventana, was redelik eenders ten opsigte van suiker- en organiese-suurinhoud. Twaalf sitosoliese ensieme is ondersoek. Daar was verskille in die maksimum katalitiese aktiwiteit van invertase, PFP, pirofaat kinase en ADP-glukose pirofosforilase (AGPase) in sommige ontwikkelingstadiums. Verskille in invertase-, PFP- en AGPase-aktiwiteit is ook waargeneem tussen die twee soorte. Een ensiem kon, as gevolg van die suiweringsmetode wat gebruik is, nie effektief geanaliseer word nie. Die metodiek vir die analise van transgeniese aarbeie is ook uitgeklaar.

Konstrukte is ontwerp om *Giardia lamblia* PFP (GL-PFP) in die hele plant uit te druk, of om *Saccharomyces cerevisiae* invertase (SCI) slegs in die vrug uit te druk. 'n Tweede invertase-konstruk is ontwerp om SCI na die apoplastiese ruimte te teiken. Aarbei plante (var Selekta) is met een van die drie konstrukte getransformeer en die aanwesigheid van elke transgeen is bevestig met polimerase-kettingreaksie (PCR). Ongetransformeerde Selekta is is beide transgeniese studies as kontrole gebruik.

Transgeniese plante is gekies volgens GL-PFP-aktiwiteit in blare en totale PFP-aktiwiteit in ryp aarbeie. Totale suiker- en organiese-suurinhoud van bessies is bepaal waar hoë PFP-aktiwiteit waargeneem is. Duidelike verskille in suikersamestelling is waargeneem, maar, met die uitsondering van een lyn, was daar nie ooglopende verskille in totale suikerinhoud nie. Daarteenoor was die organiese-suurinhoud merkbaar laer as by die kontrole-bessies. Dit het daartoe gelei dat die verhouding tussen organiese sure en suikers heelwat afgeneem het. Hierdie waarneming dui op 'n glukoneogenetiese rol vir PFP in aarbeibessies.

Transgeniese bessies is gekeur volgens SCI-aktiwiteit. Ryp bessies met ongeteikende SCI het nie merkbaar meer totale invertase-aktiwiteit getoon nie en is daarom nie verder geanaliseer nie. Daarteenoor het bessies waar SCI na die apoplastiese spasie geteiken is driemaal hoër invertase aktiwiteit getoon. Dié bessies het minder suiker (totaal) bevat en het ook heelwat minder sukrose bevat in vergelyking met heksose-inhoud. Ten spyte van die duidelike gevolg van verhoogde invertase-aktiwiteit op metabolietinhoud was daar nie ooglopende verskille in die maksimum katalitiese aktiwiteit van ander ensieme wat by sukrose, heksose en organiese-suurmetabolisme betrokke is nie. Transgeniese plante wat in hierdie ondersoeke gekies is, is later vegetatief vermeerder en toekomstige analises sal ook op nie-ryp bessies uitgevoer word.

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Preface

This dissertation is presented as a compilation of 6 chapters and each chapter is introduced separately. In Chapter 1 the general aims and motivation for this study is introduced. Chapter 2 is a review of literature that is applicable to the field of study. The individual aims and outcomes of the work undertaken for this study are presented in chapters 3 to 5. Each research chapter is presented in the format of a research publication and may be submitted for publication in whole or in part. Guidelines for authors of Plant Physiology were used as a guideline. Chapter 6 concludes this thesis by discussing the outcome of the study as a whole.

Chapter 1	General introduction
Chapter 2	Literature review: Carbon Partitioning in Plants – The roles of PFP and invertase
Chapter 3	Research chapter: Carbon partitioning and enzyme activity in two commercial strawberry cultivars
Chapter 4	Research chapter: Heterologous expression of pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase activity in strawberry fruit
Chapter 5	Research chapter: Expression of yeast invertase in strawberry driven by B33 patatin promoter
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List of Abbreviations

35S	35S ribosomal subunit
ADP	Adenosine 5'-diphosphate
ARC	Agricultural Research Council (South Africa)
AsA	Ascorbic acid (vitamin C)
ATP	Adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
CoA	Coenzyme-A
CT	Cytosolic location
CW	Apoplastic location
DNA	Deoxyribonucleic acid
DTT	1,4-dithiothreitol
EDTA	ethylenedianinetetraacetic acid
FW	Fresh weight
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
kDa	kilo Dalton
K_m	Michaelis-Menten constant
mM	Millimolar (10^{-3})
mRNA	Messenger Ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PEP	Phospho*enol*pyruvate
Pi	Inorganic phosphate (PO_4^{3-})
PMSF	Phenylmethanesulphonylfluoride
PPi	Pyrophosphate ($P_2O_7^{4-}$)
PVPP	Polyvinyl polypyrrolidone
SDS	Sodium Dodecyl Sulphate
TCA-cycle	Tri-carboxylic acid cycle (also Krebs cycle)
TCEP	Tris[2-carboxyethyl]phosphine hydrochloride

Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
U	Catalytic Units (μmol product formed per min per mg protein)
UDP	Uridine 5'-diphosphate
UTP	Uridine 5'-triphosphate
V_{max}	Maximum velocity of enzyme
WT	Control (wild-type)

Enzymes with classification

AGPase	ADP-glucose pyrophosphorylase (EC 2.7.7.27)
Aldolase	Fructose-1,6-bisphosphatase aldolase (EC 4.1.2.13)
AsA oxidase	L-Ascorbate oxidase (EC 1.10.3.3)
FBPase	Fructose-1,6-bisphosphatase (EC 3.1.3.11)
GDH	Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
GPD	Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)
HK	Hexokinase (EC 2.7.1.1)
Invertase	Beta- fructosidase (EC 3.2.1.26)
LDH	Lactate dehydrogenase (EC 1.1.1.27)
MDH	Malate dehydrogenase (EC 1.1.1.37)
MEM	NAD-dependent malic enzyme (EC 1.1.1.38)
PDG	Pyruvate dehydrogenase (EC 1.2.4.1)
PEPc	Phosphoenolpyruvate carboxylase (EC 4.1.1.31)
PFK	ATP dependent phosphofructokinase (EC 2.7.1.11)
PFP	Pyrophosphate dependent phosphofructokinase (EC 2.7.1.90)
PGI	Phosphoglucose Isomerase (EC 5.3.1.9)
PGM	Phosphoglucomutase (EC 2.7.5.1)
PK	Pyruvate kinase (EC 2.7.1.40)
SuSy	Sucrose-uridine diphosphate glucosyltransferase (EC 2.4.1.13)
TPI	Triose phosphate isomerase (EC 5.3.1.1)
UGPase	UDP-glucose pyrophosphorylase (EC 2.7.7.9)

1 General introduction

1.1 INTRODUCTION

Strawberry (*Fragaria x ananassa*) metabolite composition is a significant component of fruit quality. In particular, the relative amounts of sugars and organic acids affect fruit quality, as they are the most abundant compounds. Many studies have investigated the metabolite composition of strawberry fruit. These studies aimed to improve or better understand nutritional traits (sugar and organic acid content, especially ascorbic acid content), fruit colour (due to anthocyanins), softening and post-harvest treatments.

Strawberries accumulate significant quantities of sugars and organic acids. Cultivars contain different amounts of sugar and also differ with respect to sugar composition. Most commercial strawberry cultivars contain more hexoses (glucose and fructose) than sucrose (Ogiwara *et al.*, 1998). Citric acid is by far the most abundant organic acid in ripe strawberry, and citrate content is an accurate measure of titratable acidity (Reddy *et al.,* 2000; Kafkas *et al.,* 2007; Kamperidou and Vasilakakis, 2006). Malate content is far lower, but also contributes to total organic acid content (Ménager *et al.*, 2004; Montero *et al.*, 1996).

Physiologically speaking, strawberries have false fruit. The fleshy part of the berry is not derived from ovarian tissue as most fruits are, but is a modified receptacle, while the achenes, which appear to be seeds on the outer surface of the berry, are the physiological fruit, since they are derived from ovarian tissue (Coombe, 1976). Despite the unusual morphology of strawberry, it has become of scientific interest as a model system for non-climacteric fruit metabolism and translational genomics (Folta and Dhingra, 2006). Strawberry has specifically been proposed as a model system for grape berries, where the balance between sugars and organic acids affect the quality of both table grapes and wine (Burger, 2000). In grape berries used in wine production, the relative quantities of glucose and fructose can affect alcoholic fermentation (Berthels *et al.,* 2008), while high malate content may lead to wine spoilage due to malolactic fermentation (Mazzei *et al.,* 2007).

Non-green strawberry fruits, as carbon sinks, do not synthesise carbohydrates through photosynthesis, but instead rely on photosynthate imported from photosynthesizing tissues via phloem. In most plants the major translocated carbohydrate is sucrose, which is transported via the phloem into the fruit (Taiz and Zeiger, 2002). One of the enzymes affecting the rate at which sucrose is imported into tissues is β-fructosidase (EC 3.2.1.26, Invertase). Invertase catalyses an irreversible hydrolysis of sucrose to glucose and fructose. Plant invertases exist in three subcellular compartments namely the apoplast, cytosol and vacuole. Biochemical analysis of maize mutants with significantly lower apoplastic invertase activity revealed that the kernels exhibited reduced

photosynthate import, leading to a miniature phenotype (Miller and Chourey, 1992). Increased cell-wall invertase activity, on the other hand, is responsible for higher soluble solids (sugars, organic and amino acids) content in tomato fruit (Baxter *et al.,* 2005). Cytosolic invertases have been implicated in growth and developmental regulation (Nonis *et al.,* 2007) and provide hexoses required for glycolysis and biosynthetic processes. Heterologous expression of yeast invertase in potato and tobacco, on the other hand, led to increases in the ratio of glucose to fructose (Sonnewald *et al.,* 1997; Tomlinson *et al.,* 2004).

In order to investigate the role of invertase in fruit carbon partitioning, in particular towards sugars and organic acids, a recombinant yeast-derived invertase was expressed in either the cytosol or apoplast. Compartment-specific expression will be accomplished by adding the proteinase inhibitor II signal sequence to the mature *suc2* gene. In light of the detrimental effect of constituitive invertase overexpression on normal growth and development (Sonnewald *et al.,* 1991; Von Schaewen *et al.,* 1990), we aimed to confine expression to the strawberry fruit, by expressing invertase under the control of the B33 patatin promoter. The b33 patatin promoter from potato tubers has been observed to confer fruit-specific expression in tomato (Le *et al.,* 2006). It is suggested that strawberry will exibit the same change in glucose-to-fructose ratio as was observed in previous studies (Sonnewald *et al.,* 1997; Tomlinson *et al.,* 2004), to provide a basis for similar studies in grape berries. The effect of invertase overexpression on malate content has not been described previously.

A second component of our study focussed on shifting the balance between sugars and organic acids. Pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90, PFP) catalyses one of the key reactions in glycolysis. It catalyses a freely reversible reaction, interconverting fructose-6-phosphate (and pyrophosphate) and fructose-1,6-bisphosphate (inorganic phosphate). The exact role of PFP in metabolism has not been elucidated, although transgenic approaches have shown that PFP affects carbohydrate partitioning. In tobacco seeds PFP may affect the rate of starch degradation, leading to changes in the timing and extent of lipid deposition. Transgenic tobacco with increased PFP activity exhibits changes in carbon allocation, with both sink and source tissues containing less starch (Wood *et al.,* 2002a). Heterologous expression of PFP furthermore resulted in an increase in lipid content of tobacco seeds and hastened the deposition of lipids in the seeds (Wood *et al.*, 2002b). Transgenic sugarcane with reduced PFP activity exhibited higher sucrose content in young internodes (Groenewald, 2006). In an attempt to decrease sugar content relative to organic acid content in strawberry, we constituitively expressed PFP from a non-plant source, that would not be subject to regulation by fructose-2,6-bisphosphate.

Few studies on sugar and organic acid content in strawberry also include analysis of enzyme activity. Therefore, in addition to genetic manipulation of carbon partitioning in ripe berries,

changes in carbon allocation during development were investigated to provide insight into key reactions that may influence carbon partitioning. Berries from two commercial strawberry cultivars were harvested at four developmental stages. While invertase and PFP have both been observed to affect metabolite content and composition in sink tissues, many other enzymes are involved in pathways where these metabolites function as substrates and products. Therefore, where metabolite levels are different, enzyme activities are possibly also different. In our study, possible enzymatic causes for changes in sugar and organic acid accumulation in developing strawberry fruit were investigated. Enzymes were selected based on their relation to substrates and products of invertase and PFP and the metabolites of interest (sucrose, glucose, fructose, citrate and malate). Cytosolic localization of the selected enzymes was a determining factor, particularly for enzymes involved in organic acid metabolism. This component of the study also enabled us to establish a methodology for the analysis of transgenic strawberry and also assisted with the interpretation of data from transgenic berries, especially with regard to the role of PFP in strawberry ripening.

This study aims to investigate carbon partitioning in strawberry, firstly to provide a framework for future applications of transgenic strawberry as a model system for grape berries, secondly to investigate potential targets for gnetic manipulation and thirdly to contribute data regarding the metabolite content of two commercial cultivars that have not previously been characterised.

1.2 PROJECT AIMS AND OUTCOMES

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

Chapter 2: Carbon Partitioning in Plants – The roles of PFP and invertase.

Aim: To present the background of this study, including an overview of cytosolic carbon metabolism and the roles of PFP and invertase.

Outcomes: An overview of cytosolic carbon metabolism is presented, followed by conclusions derived from recent biotechnological research using transgenic plants. Important aspects regarding PFP and invertase are reviewed, with particular emphasis on their roles in sink metabolism.

Chapter 3: Variation in carbon partitioning and enzyme activity in two commercial strawberry cultivars.

Aim: To provide a platform for further biochemical analysis of strawberry by investigating possible enzymatic causes for different carbon partitioning profiles in two commercial cultivars and establishing protocols for the evaluation of cytosolic carbon metabolism

and carbon partitioning in strawberry fruit.

Outcomes: Two field-grown commercial strawberry varieties (Festival and Ventana) were harvested at four developmental stages and several cytosolic enzymes involved in sugar and organic acid metabolism were assayed. Sugar (glucose, fructose and sucrose) and organic acid (citric, malic and ascorbic acid) contents were determined. Enzymatic bases for the changes in sugar content were investigated by assaying twelve cytosolic enzymes involved in sugar and organic acid metabolism. Three possible enzyme targets for transgenic manipulation were identified.

Chapter 4: Over-expression of pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase activity in strawberry fruit.

Aim: To determine if increased PFP activity in fruit leads to a shift in carbon allocation from sugars to organic acids.

Outcomes: Strawberry lines expressing active protein were selected and fruit were harvested. Sugar and organic acid content and PFP activity were measured and analysed. Lines expressing recombinant PFP contained less organic acids relative to sugars. Sugar composition was also altered.

Chapter 5: Over-expression of yeast invertase in the apoplast or cytosol of strawberry using the B33 patatin promoter.

Aim: Analyse the effect of increased invertase expression on sugar and organic acid content in ripe fruit.

Outcomes: Lines where yeast invertase was active in the cytosol did not possess significantly more invertase activity than the untransformed controls. Cytosolic lines were not analysed further. Invertase activity was increased up to 3 times in lines expressing the yeast invertase in the apoplast. Total sugar, fructose and malate content of apoplastic lines was significantly ($p<0.01$) lower, leading to a reduction in sucrose-to-hexose ratio.

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2 Carbon Partitioning in Plants – The roles of PFP and invertase

2.1 INTRODUCTION

The most prominent feature of plants is their ability to assimilate CO_2 and to synthesise carbohydrates and, unlike most heterotrophic organisms, plants can synthesise all essential organic compounds instead of depending on the uptake of these compounds. Respiration is the process whereby plants convert the products of photosynthesis into energy and also into precursors for other organic compounds, including proteins and lipids. A large component of respiration is glycolysis, where glucose is oxidised to produce ATP, triose-phosphates and organic acids. Gluconeogenesis and sucrose synthesis, on the other hand, synthesise glucose and sucrose using products of glycolysis or photosynthesis (Figure 2.1). These opposing processes are regulated through a variety of complex mechanisms, including the relative concentrations of intermediate metabolites common to the three pathways.

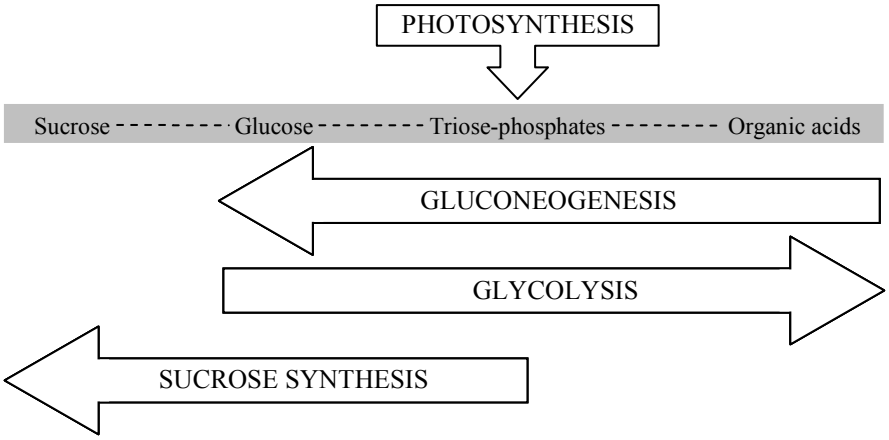

Figure 2.1. Products of photosynthesis are substrates for glycolysis, gluconeogenesis and sucrose synthesis

Plants are sessile organisms that must adapt their metabolic processes during a range of environmental factors, such as light intensity, temperature and availability of water. To provide metabolic flexibility to adapt to various conditions, plants have evolved several duplicate pathways and reactions. These duplications enable the plant to bypass key reactions that are easily affected by changing environmental conditions. Elucidating the role of specific enzymes or metabolites in plant metabolism has been complicated by this redundancy.

Sink metabolism in particular is of interest from an biotechnological perspective since the plant tissues harvested for many commercial crops such as potato, sugarcane and fruits, are considered carbon sinks. A carbon sink requires carbohydrate, usually in the form of sucrose, to be transported from the leaves via the phloem. This sucrose can then be metabolised or stored. Different plants store different compounds. For instance, seeds store either starch, proteins or lipids, while fruit can store varying amounts and types of sugars and acids.

Pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90, PFP) and

β-fructosidase (EC 3.2.1.26, invertase) are both likely candidates to modify carbon partitioning. Studies have shown that both PFP and invertase activity is correlated with sugar content in sinks. Invertase activity can determine the sugar composition and total sugar content of sinks, while PFP, catalysing a reversible reaction at a key point in hexose-phosphate metabolism, affects partitioning to many carbon pools, depending on the type of sink (lipid-storing, sucrose storing).

The purpose of this review is to give an overview of some aspects of the mechanism and regulation of cytosolic carbon metabolism and to show the possible roles of PFP and invertase in carbon partitioning in sink tissues.

2.2 CARBON PARTITIONING IN PLANT CELLS

2.2.1 Cytosolic carbon metabolism

2.2.1.1 Triose-phosphates as signal molecules in photosynthetic cells

Plant cells can either produce carbohydrates through photosynthesis or import carbohydrates from tissues where carbohydrates are synthesised (Taiz and Zeiger, 2002). The processes of photosynthesis, starch synthesis, sucrose synthesis and glycolysis are tightly regulated, and this regulation is most evident in photosynthetic tissues. Through photosynthesis, carbon, provided as CO_2, is fixed to produce triose-phosphates in the chloroplast. Triose-phosphates are substrates for sucrose synthesis and glycolysis in the cytosol, and starch synthesis in chloroplasts. The abundance of this shared substrate in each cellular compartment is affected by the activity of the pathways that use it. The interaction between the two compartments is facilitated by a concentration gradient-dependent antiport system that exchanges triose-phosphate for inorganic phosphate (Figure 2.2).

The concentration of triose-phosphate in the cytosol is dependent on the rate at which sucrose is synthesised. In light conditions, where photosynthesizing cells do not require respiration to produce ATP and reducing equivalents, sucrose synthesis is the predominant cytosolic sink for triose-phosphates. When the sucrose synthesis pathway is saturated (e.g. during high light conditions), the high triose-phosphate concentration in the cytosol will prevent the antiport system from transporting more triose-phosphates out of the chloroplast. The increased triose-phosphate concentration in the chloroplast activates ADP-glucose-pyrophosphorylase (EC 2.7.7.27, AGPase), promoting starch synthesis. Once the rate of photosynthesis decreases again, starch is degraded in the chloroplasts, producing triose-phosphate which are then transported into the cytosol, where they will be used in sucrose synthesis and respiration. Once the light-dependent reactions of photosynthesis stop, starch is degraded, producing triose-phosphates that are exported to the cytosol and used to synthesise sucrose or produce ATP and reducing equivalents through glycolysis.

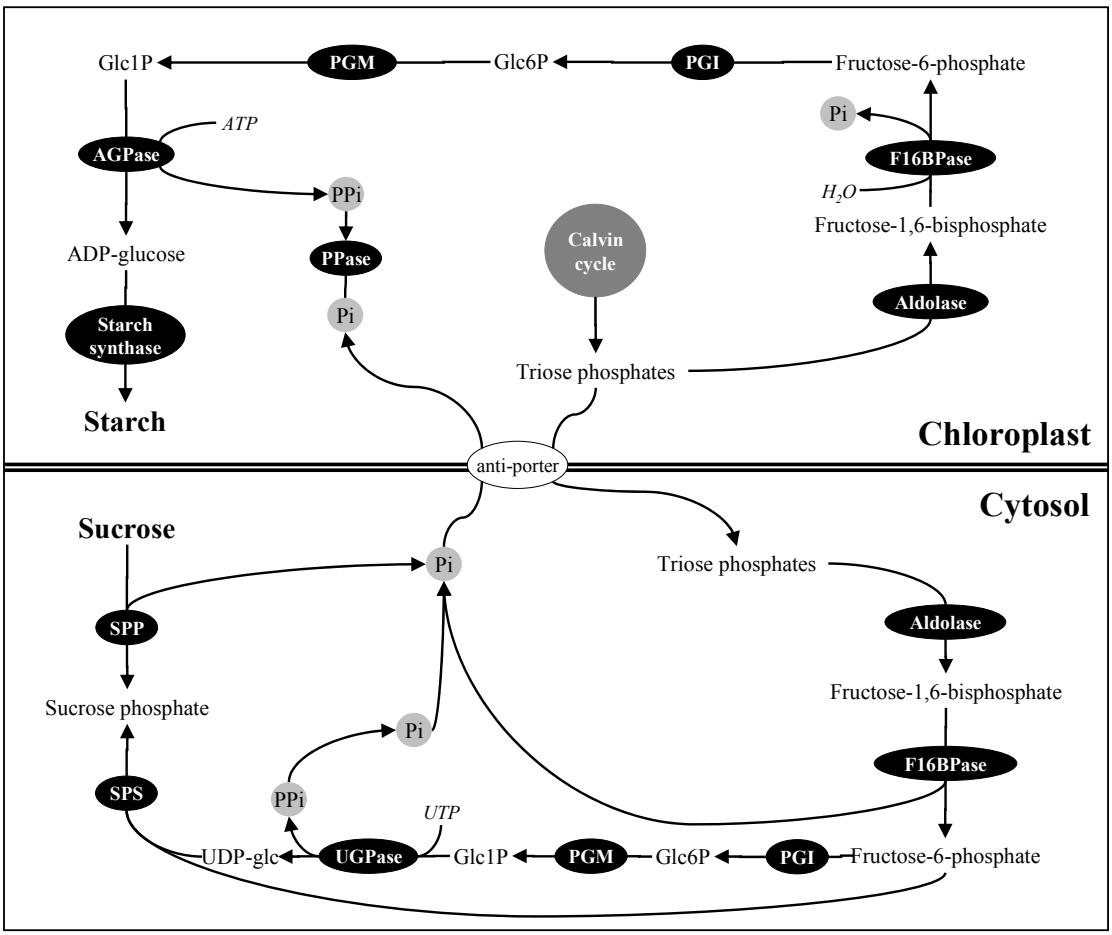

Figure 2.2. Relative rates of sucrose and starch synthesis are determined by the concentrations of inorganic phosphate (Pi) and triose phosphates in each compartment (Taiz and Zeiger, 2002)

2.2.1.2 The role of fructose-2,6-bisphosphate

The rate of sucrose synthesis in the cytosol is determined by the concentration of fructose-2,6-bisphosphate. Fructose-2,6-bisphosphate concentration is controlled by a bifuctional enzyme, containing both fructose-6-bisphosphate 2-kinase (EC 2.7.1.105) and fructose-2,6-bisphosphatase (EC 3.1.3.46) catalytic sites. These sites are regulated by the cytosolic concentrations of triose-phosphates, inorganic phosphates and fructose-6-phosphate (Cséke and Buchanan, 1983; Larondelle *et al.,* 1986; Stitt *et al.,* 1984) and have opposite effects on the phosphatase and kinase activities. The bifuctional enzyme acts as a sensor for the saturation of the sucrose synthetic pathway by increasing the concentration of fructose-2,6-bisphosphate when fructose-6-phosphate and inorganic phosphate are present in high concentrations, as is the case during sucrose synthesis. It also decreases the concentration of fructose-2,6-bisphosphate when sucrose synthesis cannot use cytosolic triose-phosphates.

Fructose-2,6-bisphosphate modulates the activity of two key cytosolic enzymes, pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90, PFP) and fructose-1,6-bisphosphatase (EC 3.1.3.11, FBPase). Both enzymes contain binding sites for fructose-2,6-bisphosphate. Fructose-2,6-bisphosphate is a competitive inhibitor for FBPase (Cséke *et al.,* 1982), while PFP is

allosterically activated by both fructose-1,6-bisphosphate (Nielsen, 1995) and fructose-2,6-bisphosphate (Theodorou and Kruger, 2001), depending on physiological conditions. An increase in fructose-2,6-bisphosphate concentration promotes increased glycolytic flux by inhibiting FBPase. A decrease in fructose-2,6-bisphosphate concentration lifts the inhibition imposed on FBPase and result in a decrease in the glycolytic activity of PFP, leading to net gluconeogenic flux (i.e. the synthesis of sucrose). The effect of fructose-2,6-bisphosphate on PFP and other factors affecting the affinity of PFP for fructose-2,6-bisphosphate will be discussed in more detail later in this chapter.

2.2.1.3 Sucrose translocation and utilization

Tissues that depend on photosynthate are called sinks and include young leaves, roots and storage organs such as developing seeds (Taiz and Zeiger, 2002). In most plants the major translocated carbohydrate is sucrose, which is transported via the phloem into the sink tissue. Phloem unloading is driven by a concentration gradient and sink cells need to maintain this gradient by removing free sucrose from the apoplast and cytosol. Sucrose can be removed by breaking it down to hexoses, or by transporting it to the vacuole, where it can be stored.

The first step in the process of cytosolic sucrose breakdown can be catalysed by either invertase or sucrose synthase (EC 2.4.1.13, SuSy). Invertase hydrolyses sucrose to form glucose and fructose (Figure 2.3), which are then phosphorylated by hexokinase (EC 2.7.1.1, HK), to produce glucose-6-phosphate and fructose-6-phosphate. SuSy transfers glucose from sucrose to UDP, producing UDP-glucose and fructose. UDP-glucose is converted to glucose-6-phosphate in a two-step process involving UDP-glucose pyrophosphorylase (EC 2.7.7.9, UGPase) and phosphoglucomutase (EC 2.7.5.1, PGM). Glucose-6-phosphate, originating from both invertase and SuSy activity, is isomerised to fructose-6-phosphate by phosphoglucose isomerase (EC 5.3.1.9, PGI). The end product of glycolysis is pyruvate, a three-carbon compound that is produced when phospho*enol*pyruvate (PEP) is dephosphorylated by pyruvate kinase (EC 2.7.1.40, PK) in a reaction that produces ATP. Plants also possess an adenylate-independent bypass where PEP carboxylase (EC 4.1.1.31, PEPc) dephosphorylates and carboxylates PEP to form oxaloacetate (four carbons), which is reduced by malate dehydrogenase (EC 1.1.1.37, MDH) to form malate. Pyruvate is imported to the mitochondria and converted to acetyl-coenzyme A. Acetyl Coenzyme-A is synthesised by pyruvate dehydrogenase (EC 1.2.4.1, PDG) and enters the tri-carboxylic acid (TCA) cycle. Malate can enter the mitochondrion and be converted to pyruvate by malic enzyme (EC 1.1.1.38), or it can be transported into the vacuole for storage.

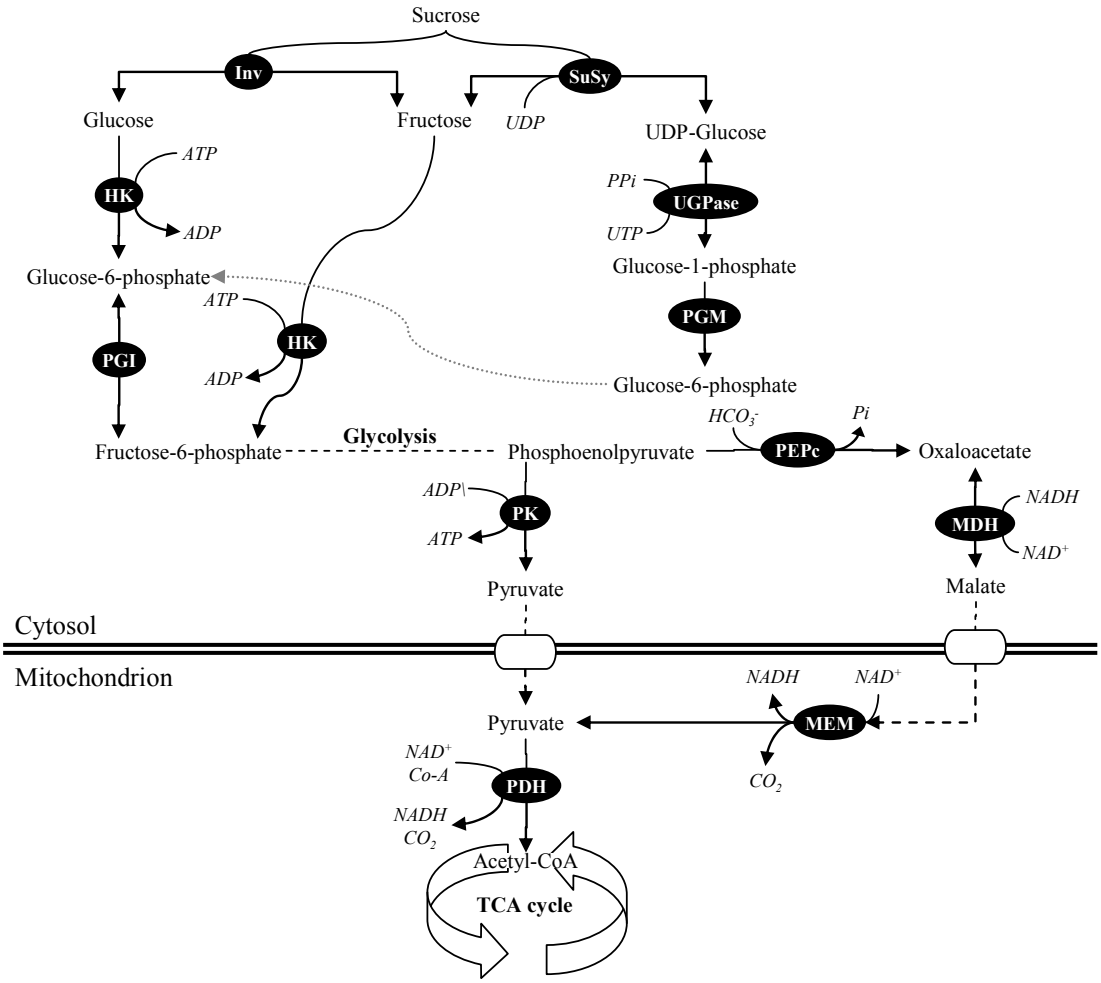

Figure 2.3. Sucrose, malate and pyruvate metabolism (Taiz and Zeiger, 2002)

Many of the intermediates of sugar metabolism and glycolysis are also substrates for the synthesis of other stored compounds. The relative proportion of these stored compounds (including sugars, starch, lipids and organic acids) are dependent on the plant species and the tissue involved. Stored compounds are made available for degradation or re-allocated to respiration, in particular when sucrose is no longer supplied to the plant tissue via the phloem.

2.2.1.4 Carbon partitioning in sink tissues

Within the cell there are several possible destinations (or sinks) for imported carbon. Carbon in the form of sucrose can be stored, degraded to hexoses (glucose and fructose), or used to synthesise starch, organic acids and cell wall components. The relative sizes of these "carbon pools" can have economic implications. In sugarcane, it would be advantageous if the maximum amount of carbon is stored as sucrose, while in potato tubers, starch is the preferred carbohydrate. In many fruits the relative amounts of sugars and organic acids are important determinants of taste and quality. Metabolite levels in sink tissues may depend on the capacity of the cells to store carbohydrates. Fruit carbohydrate accumulation patterns are furthermore dependent on enzyme activity and these can change during development and ripening. For example, starch synthesis and AGPase activity

decreased to undetectable levels as strawberry fruit ripen (Souleyre *et al.,* 2004).

2.2.1.5 Upsetting the Status Quo

Several metabolic pathways have been targeted through genetic engineering in the hope of modifying carbon allocation in plant cells. Often the targeted pathway is involved in the synthesis or degradation of an economically important compound. In potato (*Solanum tuberosum*) many attempts have been made to increase the allocation of carbon to starch at the expense of sugars (Fernie *et al.,* 2002), while in sugarcane (*Saccharum officinarum*) increased allocation of carbon to sucrose has been the target of genetic manipulation (Grof and Campbell, 2001).

Genetic manipulation of carbohydrate metabolism can produce unexpected results. Trethewey *et al.* (1998) expressed a yeast invertase and bacterial glucokinase in potato tuber, hoping that the increased capacity to hydrolyze sucrose would increase starch accumulation. Starch content was however reduced, while glycolysis was stimulated. The increase in hexose-phosphate concentration activated sucrose phosphate synthase (EC 2.4.1.14, SPS) and resulted in sucrose cycling (Trethewey *et al.,* 1999). Leggewie *et al.* (2003) found that increasing the supply of sucrose to developing tubers, by overexpression of a sucrose transporter, did not result in a significant increase in starch content, but rather increased hexoses and sucrose levels in tubers.

Excessive or insufficient utilization of sucrose in sink tissues can have dramatic effects, but the flexibility and complexity of plant metabolism often prevents researchers from effectively manipulating metabolism. Nevertheless, many valuable insights have been gained by the manipulation of carbon metabolism in plants and these insights will in turn lead to an increased understanding of the complex metabolic pathways which have provided plants with the ability to survive in many different, sometimes hostile environments.

2.2.2 Pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase

Pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90, PFP) is an enzyme occurring only in the cytosol. It catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate in the glycolytic direction, and the dephosphorylation of fructose-1,6-bisphoshate to fructose-6-phosphate in the gluconeogenic reaction (Figure 2.4). Reeves *et al.* (1974) first isolated PFP from *Entamoeba histolytica*, a lower eukaryote. The first plant isolate was from pineapples leaves (Carnal and Black, 1979) and PFP has since been isolated from a variety of plant species and tissues (Stitt, 1990).

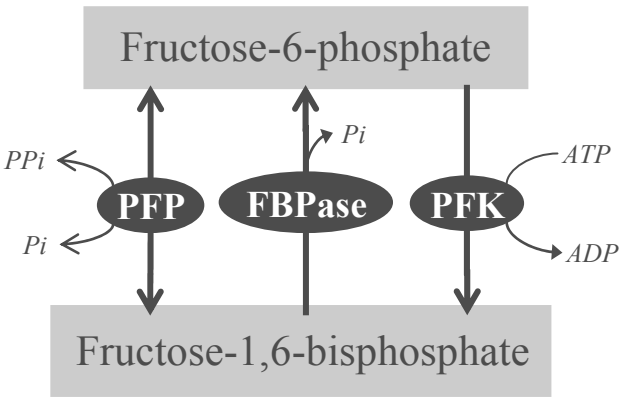

Figure 2.4. Conversion of fructose-6-phosphate to fructose-1,6-bisphosphate is catalysed by three enzymes

2.2.2.1 PFP in glycolysis/gluconeogenesis

PFP is one of three enzymes involved in the interconversion of fructose-6-phosphate and fructose-1,6-bisphosphate (Figure 2.4). ATP: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11, PFK) acts in the glycolytic direction by phosphorylating fructose-6-phosphate, using ATP as phosphate donor. Fructose-1,6-bisphosphatase (EC 3.1.3.11, FBPase) acts in the gluconeogenic direction. PFP catalyses a reversible reaction and uses pyrophosphate (PPi) instead of ATP. The readily-reversible reaction catalysed by PFP provides flexibility, as it is active in both the glycolytic and gluconeogenic pathways. PFP activity is modulated by its substrates as well as fructose-2,6-bisphoshate (Cséke *et al.,* 1982; Fernie *et al.*, 2001; Kombrink *et al.,* 1984; Montavon and Kruger, 1992; Nielsen and Wischmann, 1995; Stitt, 1989; Theodorou and Plaxton, 1996; Van Schaftingen *et al.,* 1982), and activity is dependent on the aggregation state and subunit composition of the enzyme which varies between different tissue types (sink/source), developmental stages and environmental conditions (Kruger and Dennis, 1987; Botha and Botha, 1991a; 1991b; Theodorou *et al.,* 1992; Theodorou and Plaxton, 1996).

2.2.2.2 PFP and fructose-2,6-bisphosphate

Fructose-2,6-bisphosphate activates both the glycolytic and gluconeogenic reactions of plant-derived PFP and in some cases can affect the aggregation state of the enzyme (Balogh *et al.,* 1984; Kruger and Dennis, 1987; Wu *et al.,* 1983). The glycolytic reaction is activated by decreasing the K_m for fructose-6-phosphate and increasing the V_{max} (Botha *et al.,* 1986; Sabularse and Anderson, 1981; Theodorou and Plaxton, 1996; Van Schaftingen *et al.,* 1982). It is still unclear to what extent fructose-2,6-bisphosphate increases affinity for pyrophosphate, as there is a variation of a slight decrease in K_m to a slight increase depending on the source of the enzyme that was characterised (Bertagnolli *et al.,* 1986; Botha *et al.,* 1986; Kombrink *et al.,* 1984; Van Schaftingen *et al.,* 1982).

Most plant PFP proteins are multimeric and are composted of two immunologically distinct peptides of approximately 66 kDa (α-subunit) and 60 kDa (β-subunit). These subunits can aggregate as dimers, tetramers or octamers (Botha and Botha, 1991a; Kruger and Dennis, 1987;

1991b; Theodorou *et al.,* 1992; Theodorou and Plaxton, 1996). In grapefruit, PFP exists in two isoforms, a heterotetramer ($\alpha_2\beta_2$) that is sensitive to regulation by fructose-2,6-bisphosphate and a homodimer (β_2) that is not regulated by fructose-2,6-bisphosphate (Van Praag *et al.*, 2000). In the absence of the α-subunit, PFP dephosphorylates fructose-1,6-bisphosphate, thereby promoting gluconeogenic flux.

2.2.2.3 Manipulating PFP activity

Illuminated leaves of tobacco plants with strongly decreased PFP expression had similar levels of PPi, triose- and hexose-phosphates, but 30% less UDP-glucose and significantly more fructose-2,6-bisphosphate when compared to the wild-type, while leaves harvested at the end of the dark-period, i.e. sink leaves, contained 50% more hexose-phosphates and 30-40% less 3-phosphoglycerate and PEP compared to the wild type (Paul *et al.,* 1995). Since mature photosynthesizing tobacco leaves also contain less fructose-2,6-bisphosphate than needed to activate all PFP proteins (Nielsen and Wischmann, 1995), this indicates that the plants with less PFP will simply have a greater proportion of the enzymes fully activated by fructose-2,6-bisphosphate. It has also been observed that tobacco leaves with decreased PFP activity had elevated fructose-2,6-bisphosphate levels, but no significant changes in hexose phosphates, sucrose, glucose, fructose or starch compared to the wild type (Nielsen and Stitt, 2001).

Wood *et al.* (2002a) expressed an isoform of PFP that is insensitive to fructose-2,6-bisphosphate to increase total PFP activity in tobacco. They also found few changes in leaf carbon partitioning, although a decrease in starch accumulation was observed at the start and end of the light period. Given that starch synthesis, sucrose synthesis and glycolysis all require triose-phosphates, this may indicate that the increased PFP activity increases the capacity of cytosolic carbon metabolism at times when starch synthesis would occur.

Manipulating PFP activity in other sink tissues showed similar results to Paul *et al.* (1995), where potato tubers with decreased PFP activity had slightly more hexose-phosphates and less glycerate-3-phosphate and PEP, and a four- to fivefold increase in fructose-2,6-bisphosphate (Hajirezaei *et al.,* 1994). Lipid deposition and embryo growth rate increased in tobacco seeds with increased PFP activity, while starch content decreased (Wood *et al.,* 2002a; 2002b). These results indicate that PFP activity may play a greater role in developing tissues, but that increased activation by fructose-2,6-bisphosphate may compensate for decreased PFP expression.

2.2.2.4 The role of PFP

The two distinguishing characteristics of PFP (compared to PFK and FBPase), i.e. that it catalyses a reversible reaction and uses pyrophosphate instead of ATP, contribute to the uncertainty about its role in metabolism. It has been implicated in the regulation of cytosolic pyrophosphate concentrations (Ap Rees *et al.,* 1985b; Ap Rees, 1988; Claassen *et al.,* 1991; Dancer and Ap Rees,

1989; Simcox *et al.,* 1979) and providing a bypass to PFK and FBPase when these enzymes are not able to sustain flux (Botha and Botha, 1993a; Dennis and Greyson, 1987; Focks and Benning, 1998). PFP, as an enzyme synthesising PPi, might indirectly affect sucrose degradation via SuSy by modulating the activity of UGPase (Ap Rees, 1988; Black *et al.,* 1987; Huber and Akazawa, 1986). Its independence on ATP suggests a role in phosphate-limiting conditions (Duff *et al.,* 1989; Kato-Noguchi, 2002; Mertens, 1991; Murley *et al.,* 1998; Theodorou *et al.,* 1992). The involvement of PFP in hexose-to-triosephosphate cycling (Krook *et al.,* 2000) and the inverse correlation between sucrose accumulation in sugarcane (Bindon and Botha, 2002; Groenewald, 2006; Whittaker, 1997) point to a role in maintaining high metabolic activity, e.g. during germination and rapid growth.

The two prime factors determining fruit quality are the organic acid and sugar content (Green, 1971). Sugars are the substrate for glycolysis, while organic acids are products. PFP is believed to influence the flux through glycolysis, and increased PFP activity may therefore shift the allocation of carbon. While it would be advantageous to increase sugar content in strawberry, it may be beneficial to decrease sugar content in other fruit, such as grape berries used for wine production. The reversible reaction catalysed by PFP and the ambiguity regarding its role in metabolism mean that either of these scenarios is possible.

2.2.3 β-Fructosidase

β-Fructosidase (EC 3.2.1.26, Invertase) in plant cells occurs in three sub-cellular compartments. Cell-wall (apoplastic) and vacuolar invertases are optimally active at acidic pH and the invertase active in the cytosol operates at neutral pH. Invertases catalyse the hydrolysis of sucrose and related glycosides (e.g. raffinose), by cleaving the fructose-moiety from the molecule.

2.2.3.1 Yeast invertases

Invertase was first purified from brewer's yeast by Berthelot (1860) as part of research on fermentation of sugars into ethanol. Yeast (*Saccharomyces cerevisiae*) produces two isoforms of invertase, one is secreted and the other is located in the cytosol. The extracellular version is a glycoprotein, while the cytoplasmic one is not (Perlman and Halvorson, 1981). These proteins are encoded by the same gene, but produce three mRNAs which are translated into two larger polypeptides and one smaller polypeptide. The larger polypeptides incorporate a signal peptide, which is cleaved from the mature peptide during post-translational modification of the extracellular invertase. The cytoplasmic invertase corresponds to the smaller polypeptide. The processed extracellular polypeptides and the unprocessed cytoplasmic polypeptides have a mass of 60 kDa.

2.2.3.2 Plant invertases

Insoluble plant invertase is quite similar to the extracellular, glycosylated invertase of yeast

(Sturm and Chrispeels, 1990), in the sense that it is also glycosylated and ionically bound to the cell wall (Fahrendorf and Beck, 1990). In addition, plants have two soluble forms of invertase. An acid-soluble isoform occurs in the vacuole and is closely related to the cell-wall invertase, in both amino acid sequence and enzyme characteristics, while a neutral/alkaline invertase occurs in the cytoplasm (Sturm, 1999). While acid and alkaline invertases in the same plant species are immunologically distinct, alkaline invertases between different plant species are not (Chen and Black, 1992). Plant cytoplasmic invertase is more similar to cytoplasmic cyanobacterial invertases than to plant acid invertases (Gallagher and Pollock, 1998; Vargas *et al.,* 2003). It is therefore not surprising that plant invertases are encoded by two gene families, with one family encoding the soluble and insoluble acid invertases and the other encoding the cytoplasmic invertase (Ji *et al.,* 2005). A key difference between acid and neutral plant invertases is that neutral invertases tend to be specific for sucrose, while acid invertases can utilise related glycosides such as raffinose and stachytose (Gallagher and Pollock, 1998; Sturm, 1999).

Plant invertases have been detected in plant extracts as early as 1943 (Glasziou 1962). Hatch *et al.* (1962) identified both acid and neutral invertases in sugarcane; however the majority of studies into the properties of invertases have focussed on the acid invertases. More recently neutral/alkaline invertases have been isolated from several plants and the genes coding for them sequenced (Gallagher and Pollock, 1998), allowing detailed study into the role of the cytosolic invertase. The optimal pH ranges of plant isozymes reflect the subcellular location, with the vacuolar and apoplastic invertases active at low pH (pH 4.5-5) and the cytosolic invertases active at neutral and slightly alkaline pH (pH 7-8).

2.2.3.3 Invertase in sucrose metabolism

Sucrose cleavage in plant cells is catalysed by both invertase and sucrose synthase (EC 2.4.1.13, SuSy). Invertase is a hydrolytic enzyme, requiring no additional substrates to cleave sucrose into glucose and fructose. SuSy is a glycosyl transferase that requires UDP to cleave sucrose into fructose and UDP-glucose. Unlike SuSy, invertases catalyse an essentially irreversible reaction. Since neutral/alkaline invertase and SuSy occur in the same compartment of the cell, the relationship between these two enzymes has been studied extensively.

Invertase is regulated by the products of the reaction it catalyses. Fructose is a competitive inhibitor at 15 mM (Gallagher and Pollock, 1998), while glucose is a non-competitive inhibitor (Isla *et al.,* 1999). *In vivo* an inhibitory protein is also present. This inhibitor has been shown to change the pH optima of acid invertase from a single peak at pH 4.5 to two pH optima, with a significant reduction of activity at pH 4.5.

2.2.3.4 The role of invertase

In many sink organs, including fruits, the relative activities of apoplastic invertase activity and

SuSy change during development. Apoplastic invertase activity is higher during the later stages of development, when sucrose is unloaded through an apoplastic route, while SuSy is more active when symplastic unloading dominates. This trend has been observed in papaya (Zhou *et al.,* 2003), grape berry (Zhang *et al.,* 2006) and potato tubers (Viola *et al.,* 2001). The loading of sucrose into and out of the phloem is dependent on a concentration gradient of sucrose and protons. The sink strength of an organ is partly determined by the ability of that organ to maintain that gradient (Carpaneto *et al.,* 2005). Apoplastic invertase plays a significant role in the unloading of sucrose when it occurs through a primarily apoplastic route. If this process is disrupted by increasing apoplastic invertase activity in source tissues, severe phenotypic effects can occur, such as repression of photosynthesis, bleaching and necrosis in matured leaves (Canam *et al.,* 2006; Dickenson *et al.,* 1991; Sonnewald *et al.,* 1991; Stitt *et al.,* 1991; Von Schaewen *et al.,* 1990).

Comparing mutant and transgenic plants with altered invertase activity revealed that invertase activity is important for normal growth and development. Biochemical analysis of maize mutants with significantly lower cell-wall invertase activity revealed the significance of invertase activity in determining the amount of photosynthate imported into the kernels, as these mutants have small kernels compared to the wild-type (Miller and Chourey, 1992). Antisense repression of invertase activity in tomato led to significantly increased sucrose and decreased hexose contents (Ohyama *et al.,* 1995). This trend was also evident when investigating the difference in acid invertase activity between wild, sucrose-accumulating tomato species and domesticated, hexose-accumulating tomato species. Fruit of the wild species did not express the acid invertase gene (Klann *et al.,* 1993). Invertase activity was also shown to be inversely correlated with sucrose uptake in tuberising potato stolons (Viola *et al.,* 2001).

The essential role of invertase in growth and development is evidenced by the severe phenotypic effects observed in plants with constituitively altered invertase activity; therefore sink-specific expression of invertase has been employed. Previous reports have shown that plants with high endogenous invertase activity exhibit increased soluble solids content (Baxter *et al.,* 2005), however transgenic plants expressing yeast invertase in tobacco seed exhibit increased hexose-to-sucrose ratios and decreased total sugar content (Tomlinson *et al.,* 2004). In potato tubers, lower sucrose content was observed (Sonnewald *et al.,* 1997). It is therefore likely that recombinant invertase activity in fruit would lead to decreased sugar content.

2.3 CONCLUSION

Strawberry is of scientific interest as a model system for non-climacteric fruit metabolism and translational genomics (Folta and Dhingra, 2006). Strawberry has specifically been proposed as a model system for grape berries, where the balance between sugars and organic acids, as well as

their final concentrations, affect the quality of both table grapes and wine. Cytosolic sugar and organic acid metabolism in commercial strawberry fruit was characterised to provide a starting point for the analysis of transgenic fruit. This provided data on developmental changes in sugar and organic acid content, as well as enzyme activity.

Increased PFP or invertase activity has the potential to modify carbon partitioning in strawberry fruit. To this end, *Giardia lamblia* PFP was expressed constituitively in strawberry, while *Saccharomyces cerevisiae* invertase was expressed in either the cytosol or apoplast. The pleiotropic effect of altered invertase activity necessitates a targeted approach to address the detrimental effect of altered invertase activity in photosynthesising leaves, therefore the gene was expressed under the control of a fruit-specific promoter. Several plants were selected for continued analyses based on activity of the recombinant enzyme.

In wine production, sugar content and composition of grape berries affect both the fermentation process and quality of the finished product. Grapes containing more fructose than glucose may produce stuck alcoholic fermentations, where yeasts cannot metabolise all available sugar (Berthels *et al.,* 2008). High residual sugar content, due to stuck fermentation, renders wines microbiologically unstable and unsuitable for bottling and sale. Recently, low-alcohol wines have increased in popularity (Malherbe *et al.,* 2003). Low-alcohol wines are currently mainly produced by reducing sugar content after harvest or alcohol content after fermentation, but this often negatively affects sensory properties of the wine. Should heterologous expression of invertase lead to reduced sugar or content, it may be advantageous to the South African wine industry, where the warm climate leads to high sugar and low organic acid content.

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3 Carbon partitioning and enzyme activity in two commercial strawberry cultivars

3.1 INTRODUCTION

Strawberry (*Fragaria x ananassa*) quality is largely determined by the relative amounts of sugars and organic acids present. The composition of strawberry has been the focus of many studies, in keeping with the economic importance of nutritional traits (sugar and organic acid content, especially ascorbic acid content), berry colour (due to anthocyanins), softening and post-harvest treatments. Recently however, strawberry has become of scientific interest as a model system for non-climacteric fruit metabolism and translational genomics (Folta and Dhingra, 2006). Strawberry has specifically been proposed as a model system for grape berries, where the balance between sugars and organic acids affect the quality of both table grapes and wine.

Sugar content of strawberry varies from cultivar to cultivar, with the relative quantities of sucrose, fructose and glucose also differing. Wild strawberries tend to accumulate equal amounts of sucrose, glucose and fructose, while most cultivated strawberry cultivars accumulate less sucrose (Ogiwara *et al.*, 1998). Total sugar content of ripe berries in 40 cultivated strawberry varieties varied between 32 and 80 mg per gram fresh weight, with an average sugar content of 59 mg per gram. Strawberries accumulate sugars and organic acids (especially citric and malic acid) during ripening, with the ratio of sugars to acids continuously increasing as the berries ripen (Ménager *et al.*, 2004; Montero *et al.*, 1996).

The metabolism of sucrose is the determining factor of sink strength for many sink tissues, including potato tubers and tomato fruit. In tomato fruit, invertase has been reported to affect both total sugar content and sugar composition (Klann *et al.*, 1993; Kortstee *et al.*, 2007), with higher invertase activity associated with higher soluble solids (sugars, organic- and amino acid) content (Baxter *et al.*, 2005). Another key reaction is the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. This reaction is catalysed by three enzymes, ATP: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11, PFK), fructose-1,6-bisphosphatase (EC 3.1.3.11, FBPase) and pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90, PFP). PFK phosphorylates fructose-6-phosphate using ATP, while FBPase dephosphorylates fructose-1,6-bisphosphate. PFP catalyses a reversible reaction and uses pyrophosphate (PPi) instead of ATP. PFP has been reported to influence sugar composition in sugarcane (Whittaker and Botha 1999, Groenewald 2006), and to affect the composition of lipids in tobacco seed (Wood *et al.*, 2002b). It has been suggested that PFP impacts the flux of carbon through glycolysis. The end product of glycolysis is pyruvate, a three-carbon organic acid that is produced when phospho*enol*pyruvate (PEP) is dephosphorylated by pyruvate kinase (EC 2.7.1.40, PK). Plants also possess an adenylate-

independent bypass where PEP carboxylase (EC 4.1.1.31, PEPc) dephosphorylates and carboxylates PEP to form oxaloacetate (four carbons), which is then reduced by malate dehydrogenase (EC 1.1.1.37, MDH) to form malate. Malate can be converted to pyruvate by malic enzyme (EC 1.1.1.38), or it can be transported into the vacuole for storage. Pyruvate is the substrate for the tri-carboxylic acid cycle in mitochondria and is used for the synthesis of citrate by citrate synthase (EC 4.1.3.7). Citrate is one of the principle stored carbohydrates in strawberry.

Two commercial strawberry varieties that are popular in South Africa were selected. Both varieties were cultivated in the same area, using the same method and berries were available simultaneously. The study aimed to broaden the knowledge-base with regard to strawberry fruit metabolism by investigating possible enzymatic causes for changes in sugar and organic acid accumulation. A secondary aim of this study was to establish the methodology for further analysis of strawberry, especially transgenic berries. Sugar and organic acid content of strawberry, along with the maximum catalytic activity of cytosolic enzymes involved in sugar and organic acid metabolism were determined. Possible correlations between sugar and/or organic acid content and enzyme activity are investigated.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Strawberry (*Fragaria x ananassa* cv. Ventana and Strawberry Festival) were harvested at Loewenstein, Paarl, South Africa in September 2007 from matted rows with mini-tunnels. Approximately twenty berries were harvested for each developmental stage, quartered and randomly separated into three replicates before freezing in liquid nitrogen. Tissue was stored at -80ºC until further analysis. Developmental stages were as follows: ripe (fully expanded and 100% red), turning (fully expanded and 50% red), white (fully expanded) and green (receptacle distinguishable from seeds).

All enzymes, excluding ascorbate oxidase (Sigma) were purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other reagents were of analytical grade and were purchased from Roche, Merck (Whitehouse Station, New Jersey, USA) or Sigma (St. Louis, Missouri, USA).

3.2.2 Biochemical analyses

3.2.2.1 Protein extraction and enzyme activity

Berries were ground in liquid nitrogen and proteins extracted in 300 mM Hepes-KOH buffer (pH 7.2) containing 2 mM $MgCl_2$, 2 mM EDTA, 10% (v/v) glycerol, 2.5% (v/v) PEG 6000, 0.05% (v/v) Triton X-100, 5 mM DTT, 2% (m/v) PVPP and 1 mM PMSF.

Samples were desalted using Sephadex G25 spin columns (Helmerhorst and Stokes, 1980) and protein quantified using the Bio-Rad (Hercules, California, USA) Protein Assay (Bradford, 1976)

with BSA as standard.

Enzyme assays were performed as described previously with modifications as described in Table 3.1. All reactions were monitored at 340 nm on a Powerwave X Microplate Spectrophotometer with KC4 Kineticalc for Windows version 2.7 (Bio-Tek Instruments, Winooski, Vermont, USA). Catalytic activity was expressed in terms of mg protein (nmol/min/mg protein = mU).

3.2.2.2 Metabolite extraction and quantification

Soluble sugars and organic acids were extracted from ground berry tissue by overnight incubation at 70ºC in 20 volumes of 80% ethanol. Extracts were diluted 20 times before enzymatic analysis of glucose, fructose and sucrose. Sugar concentrations were measured by means of a spectrophotometric enzymatic coupling assay (Jones *et al*., 1977) with modifications as described in Stitt *et al.* (1989). Phosphorylation of glucose by 1.5U hexokinase (EC 2.7.1.1) and 1U glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and fructose by 4U phosphoglucose isomerase (EC 5.3.1.9) was quantified by following the reduction of NAD to NADH at 340 nm. Sixty units invertase (EC 3.2.1.26) was added to hydrolyse sucrose to glucose and fructose.

The enzymatic quantification of citric and malic acid was modified from Velterop and Vos (2001). Assays were carried out at 25°C in a total volume of 250 µL. Malic acid was quantified with 2.5U Glutamate-oxaloacetate transaminase (EC 2.6.1.1) and 2U L-malate dehydrogenase (EC 1.1.1.37, MDH) in the presence of 25 µL extract. Citric acid concentration was measured with 4U MDH, 4U L-lactate dehydrogenase (EC 1.1.1.27) and 0.2U Citrate Oxaloacetate-Lyase (EC 4.1.3.6) in the presence of 10 µL extract. Absorbance data (340 nm) was captured on a Powerwave X Microplate Spectrophotometer.

Ascorbic acid (AsA) was extracted and quantified with the AsA oxidase assay (Rao and Ormrod, 1995). Plant extracts were obtained from frozen tissue ground in 6% (w/v) meta-phosphoric acid. Reduced AsA was determined at pH 5.9 by measuring the decrease in A_{265} (extinction coefficient of 14.3 cm^{-1} mM^{-1}) after the addition of 2 units of AsA oxidase (EC 1.10.3.3) to 1 mL of the reaction mixture. Oxidised AsA was measured as the increase in A_{245} after the addition of a recently discovered reductant, tris[2-carboxyethyl]phosphine hydrochloride (TCEP), and an incubation step at room temperature. Total AsA is given as the sum of reduced AsA and oxidised AsA.

3.2.2.3 Statistical analyses

Microsoft Office Excel 2002 was used for all calculations. Quantitative analyses are presented as mean values and the reproducibility of the results is expressed as standard error. Statistical analysis of the experimental results was carried out based on Student's t-test. Differences reaching a minimal confidence level of 80% were considered as being statistically significant.

Table 3.1 Enzyme activity assays

Enzyme[a]	Assay components[b]	Initiation substrate	Reference
AGPase	40 mM Hepes (pH 7.5), 4 mM $MgCl_2$, 0.8 mM $NADP^+$, 0.2 mg/mL BSA, 1 mM ADP-glucose, 1U GDH, 1U PGM	1 mM PPi	Rocher *et al.*, 1989
FBPase	50 mM Hepes-KOH (pH 7.2), 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 2U PGI, 1U GDH	0.6 mM fructose-1,6-bisphosphate	Kruger and Beevers, 1984
GDH	50 mM Hepes-KOH (pH 7.2), 5 mM $MgCl_2$, 0.5 mM $NADP^+$	1 mM glucose-6-phosphate	Doehlert *et al.*, 1988
HK	50 mM Hepes-KOH (pH 8.0), 4 mM $MgCl_2$, 2 mM glucose, 0.5 mM $NADP^+$, 1U GDH.	2.5 mM ATP	Doehlert *et al.*, 1988
Invertase	100 mM Hepes-KOH (pH 7), 200 mM sucrose, 10 mM $MgCl_2$, 0.8 mM NAD, 1U GDH, 1U HK, 1U PGI.	1 mM ATP	Schaarschmidt *et al.*, 2004
PEPc	100 mM Tris-HCl (pH 8.5), 4 mM $MgCl_2$, 6 mM $NaHCO_3$, 0.3 mM NADH and 5U MDH	5 mM PEP	Ocaña *et al.*, 1996
PFK	100 mM Hepes-KOH (pH 7.2), 2 mM $MgCl_2$, 0.15 mM NADH, 1 mM ATP, 1U aldolase, 10U TPI , 1U GPD	10 mM fructose-6-phosphate	Burrell *et al.*, 1994
PFP	50 mM Hepes-KOH (pH 7.2), 2 mM $MgCl_2$, 1 mM PPi, 0.15 mM NADH, 0.01 mM fructose-2,6-bisphosphate, 1U aldolase, 10U TPI, 1U GPD	10 mM fructose-6-phosphate	Botha and Botha, 1990
PGI	100 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 10 mM fructose-6-phosphate, 1U GDH	10 mM fructose-6-phosphate	Doehlert *et al.*, 1988
PK	50 mM Tris-HCl (pH 7.0), 100 mM KCl, 15 mM $MgCl_2$, 0.15 mM NADH, 1 mM ADP, 2U LDH	5 mM PEP	Burrell *et al.*, 1994
SuSy	100 mM Hepes-KOH (pH 7.5), 15 mM $MgCl_2$, 0.2 mM NADH, 20 mM UDP-glucose, 1 mM PEP, 1U PK, 1U LDH	10 mM fructose	Schäfer *et al.*, 2004
UGPase	100 mM Tris-HCl (pH 7.0), 2 mM $MgCl_2$, 0.5 mM $NADP^+$, 12 mM UDP-glucose, 1U GDH, 1U PGM	1 mM PPi	Doehlert *et al.*, 1988

[a]AGPase, ADP-glucose pyrophosphorylase (EC 2.7.7.27); FBPase, Fructose-1,6-bisphosphatase (EC 3.1.3.11); GDH, Glucose-6-phosphate dehydrogenase (EC 1.1.1.49); HK, Hexokinase (EC 2.7.1.1); Invertase, β-fructosidase (EC 3.2.1.26); PEPc, Phosphoenolpyruvate carboxylase (EC 4.1.1.31); PFK, ATP dependent phosphofructokinase (EC 2.7.1.11); PFP; Pyrophosphate dependent phosphofructokinase (EC 2.7.1.90); PGI, Phosphoglucose Isomerase (EC 5.3.1.9); PK, Pyruvate kinase (EC 2.7.1.40); SuSy, UDP-glucose: D-fructose 2-α-D-glucosyltransferase (EC 2.4.1.13); UGPase, UDP-glucose pyrophosphorylase (EC 2.7.7.9)

[b]Aldolase, Fructose-1,6-bisphosphatase aldolase (EC 4.1.2.13); GPD, Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8); LDH, L-Lactate dehydrogenase (EC 1.1.1.27); MDH, L-Malate dehydrogenase (EC 1.1.1.37); PEP, phospho*enol*pyruvate; PGM, Phosphoglucomutase (EC 2.7.5.1); PPi, Sodium pyrophosphate ($Na_2P_4O_7$); TPI, Triose phosphate isomerase (EC 5.3.1.1)

3.3 RESULTS AND DISCUSSION

3.3.1 Sugar and organic acid accumulation

Sugars increased in concentration during berry development. Total sugar content of Ventana and Festival berries was similar at all stages of development except for green berries, being slightly but significantly ($P<0.01$) higher in Festival (Figure 3.1). Festival and Ventana had similar levels

of sucrose and fructose, while the glucose content was 1.5 to 2 times higher. Most commercial strawberry varieties have similar hexose (glucose and fructose) contents, while the sucrose content is generally lower, however Cordenunsi *et al.* (2002) observed that Oso Grande, a parent cultivar of Festival also contained more sucrose relative to other cultivars.

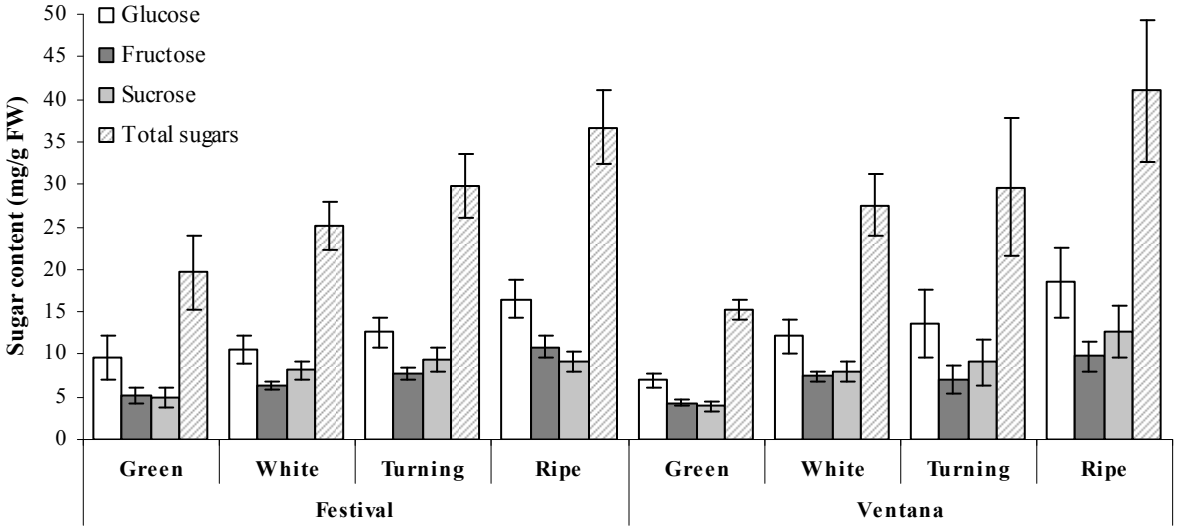

Figure 3.1 Sugar content of berries from two strawberry cultivars (n=9)

Total organic acid was expressed as the sum of the citrate and malate content, the two main organic acids present in strawberry (Cordenunsi *et al.,* 2002; Ménager *et al.*, 2004; Montero *et al.*, 1996). Values obtained in this study were comparable to those previously reported. Total organic acid content in ripe berries, expressed on a fresh weight basis, was similar for both cultivars (Figure 3.2). In terms of dry weight, citrate content was lowest in green berries and it appeared to decrease as the berries matured from white to ripe fruit. Citrate content was 2 to 3 times higher than the malate content. The malate content remained fairly constantly during berry development, however no malate was detected in green berries.

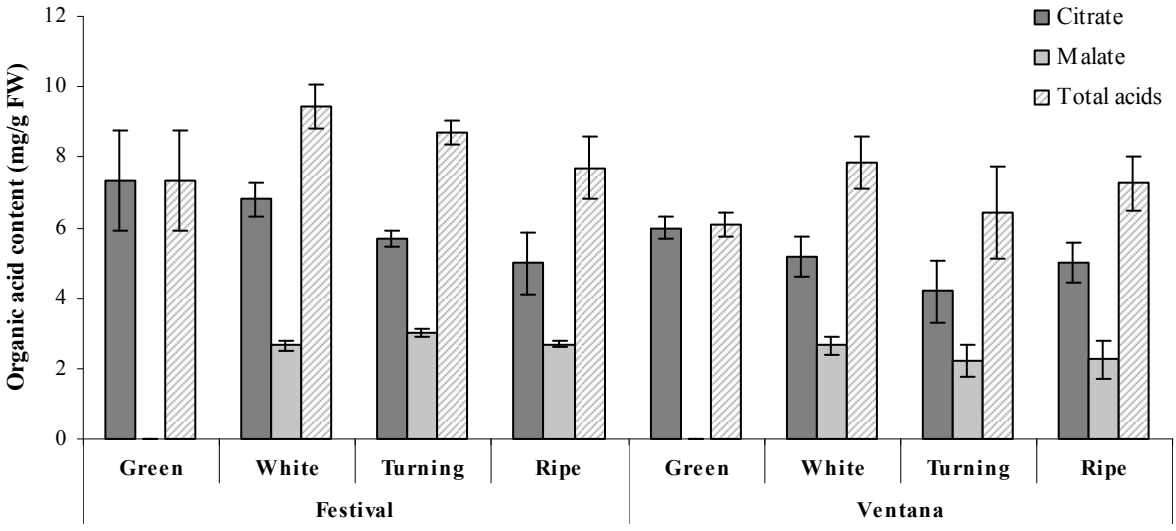

Figure 3.2 Organic acid content of berries from two strawberry cultivars (n=9)

Ascorbate content in green berries was significantly higher compared to other stages of

development (Figure 3.3), however this was shown to be due to the higher density of green berries. When expressed in terms of dry weight, ascorbic acid content was similar at all stages (data not shown). Ascorbic acid content was comparable to published data (Cordenunsi *et al.,* 2002).

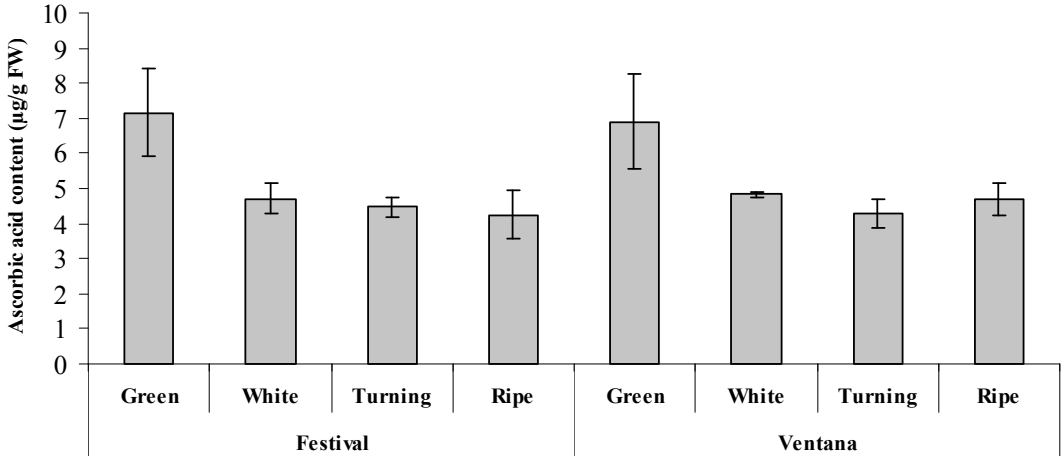

Figure 3.3 Ascorbic acid content of berries from two strawberry cultivars (n=3)

Total sugar and organic acid content, expressed as soluble solid content, increased during development (Figure 3.4), while the ratio between organic acids and sugars decreased (Figure 3.5).

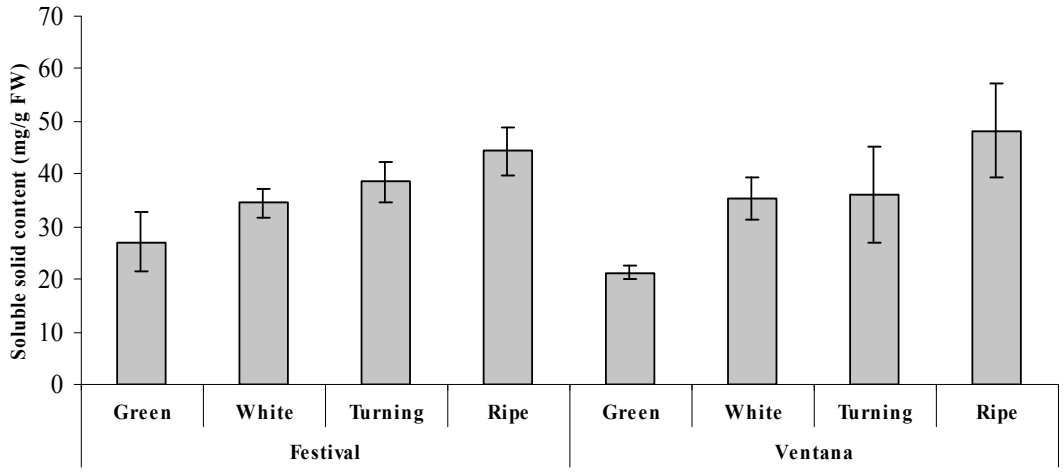

Figure 3.4 Soluble solids of strawberry. (n=9)

This is the first report on the metabolite distribution of Festival and Ventana, which are popular commercial varieties in South Africa. The similarity between the cultivars with respect to soluble solid contents and acid to sugar ratios is not surprising, as both cultivars were selected by producers based on commercial viability, which is in part determined by soluble solid content and the balance between sweetness and acidity.

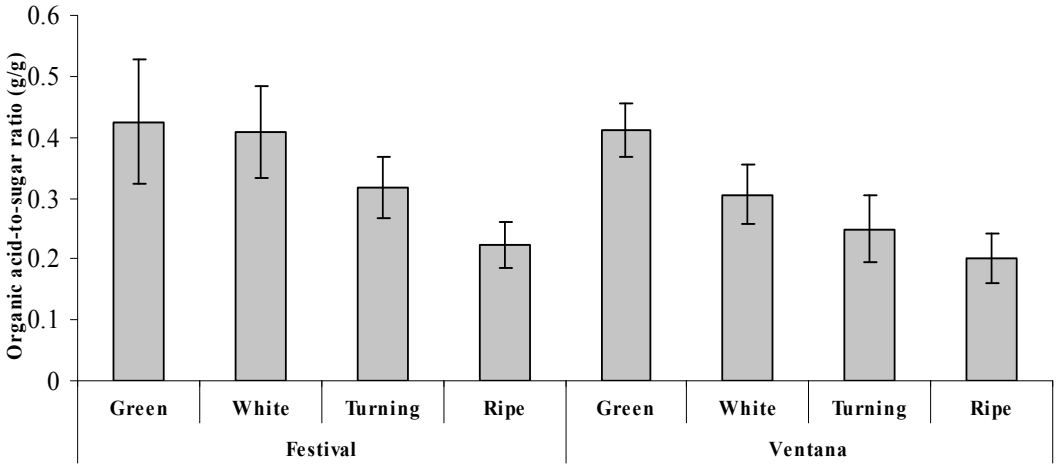

Figure 3.5 Organic acid to sugar ratio of strawberry. (n=9)

3.3.2 Enzyme activity during berry development

In order to determine the enzymatic basis for changes in sugar and organic acid accumulation during berry development, catalytic activity of a range of cytosolic enzymes involved in sugar and organic acid synthesis and degradation were measured. Low protein yield from berries (less than 0.1 mg/mL) necessitated the optimization of the extraction protocol. Both buffer composition and extraction volume were modified to provide acceptable yield. The catalytic activities for enzymes involved in sucrose synthesis and degradation, hexose metabolism and cytosolic organic acid synthesis were determined at four developmental stages for Festival (Table 3.2) and Ventana (Table 3.3).

Table 3.2 Activity in protein extracts of Festival strawberries. Mean ±SE for n=9

	Green	*White*	*Turning*	*Ripe*
Inv	219 ± 63	248 ± 24	295 ± 34	150 ± 11
PGI	409 ± 37	505 ± 23	365 ± 17	434 ± 25
UGPase	2521 ± 237	2840 ± 219	2566 ± 191	2910 ± 167
PFP	88.5 ± 14.4	62.1 ± 13.3	20.7 ± 6.8	37.6 ± 2.6
PFK	32.1 ± 1.8	43.5 ± 2.3	27.7 ± 3.2	38.0 ± 4.0
FBPase	52.5 ± 7.7	37.7 ± 3.0	42.3 ± 4.5	68.4 ± 9.7
HK	42.9 ± 3.2	22.3 ± 5.6	26.3 ± 5.4	26.2 ± 6.0
GPD	54.6 ± 1.9	55.1 ± 4.4	62.4 ± 3.6	62.7 ± 5.1
PEPc	4.06 ± 1.19	6.49 ± 1.34	4.64 ± 0.89	7.54 ± 1.38
PK	16.9 ± 2.0	22.0 ± 1.8	9.60 ± 2.2	34.4 ± 15.4
SuSy	0.03 ± 0.05	0.12 ± 0.17	0.20 ± 0.27	[a]
AGPase	0.67 ± 0.47	1.56 ± 0.76	4.43 ± 0.83	2.88 ± 0.61

[a] Activity below detection limit

Table 3.3 Activity in protein extracts of Ventana strawberries. Mean ±SE for n=9.

	Green	*White*	*Turning*	*Ripe*
Inv	156 ± 22	198 ± 26	185 ± 30	155 ± 17
PGI	470 ± 51	501 ± 38	500 ± 62	507 ± 54
UGPase	2858 ± 240	3140 ± 212	2849 ± 124	3057 ± 104
PFP	93.3 ± 12.8	81.1 ± 13.2	60.5 ± 21.0	56.6 ± 14.6
PFK	31.2 ± 1.9	42.5 ± 1.4	39.6 ± 6.3	42.8 ± 5.3
FBPase	67.3 ± 11.3	24.3 ± 3.9	41.3 ± 7.3	65.0 ± 11.7
HK	44.8 ± 4.7	19.8 ± 4.3	22.6 ± 4.0	18.7 ± 4.8
GPD	59.3 ± 5.4	53.3 ± 3.7	57.1 ± 4.8	55.8 ± 3.3
PEPc	6.23 ± 1.29	5.33 ± 1.03	5.62 ± 1.46	10.10± 3.97
PK	22.2 ± 3.0	28.9 ± 1.8	14.2 ± 1.7	55.1 ± 10.6
SuSy	[a]	[a]	[a]	[a]
AGPase	[a]	[a]	[a]	[a]

[a] Activity below detection limit

No correlations were observed between the activity of most of the enzymes measured and metabolite concentrations during ripening. Only invertase, AGPase and SuSy activity differed between the two cultivars (Table 3.2).

Invertase activity in white and turning Festival berries was significantly ($P<0.01$) higher than Festival, while activity in green and ripe berries did not differ significantly ($P>0.01$) between cultivars. The sugar content of white and turning berries was however similar.

AGPase activity in Festival berries was very low compared to other enzymes assayed and was not detected in Ventana berries. In Festival, AGPase activity increased up to the turning stage after which activity decreased. AGPase activity modulates starch synthesis and degradation, a process which occurs mainly in plastids. Strawberry (cv Elsanta) does not accumulate starch after the green berry stage (Souleyre *et al.*, 2004) and starch-containing plastids are not present in ripe berries (Schwab *et al.,* 2001).

SuSy activity was very low and only detected in unripe Festival berries. SuSy has been reported to show maximal activity in green fruit (Souleyre *et al.*, 2004). In the same study it was reported that desalting of protein extracts from strawberry using Sephadex G-25 lead to low recovery of some enzyme activities. As this was the only method employed to desalt extracts, it may have effectively eliminated SuSy activity, while more active enzymes were not as adversely affected.

PFP and FBPase activity exhibited similar trends during development (Figure 3.6). PFP activity was highest in green berries and decreased up to the turning stage after which activity increased. FBPase activity was highest in green and ripe berries.

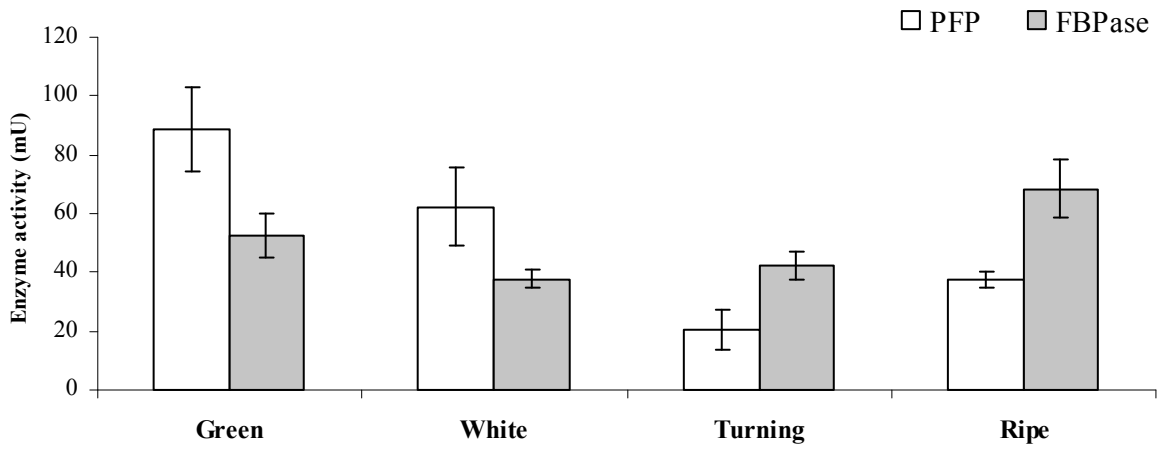

Figure 3.6 Changes in PFP and FBPase activity during development as measured in Festival berries.

Green strawberries may still possess chloroplasts and the ability to photosynthesise, therefore higher FBPase activity may indicate that sucrose synthesis is taking place. On the other hand, ripe berries no longer photosynthesise, but contain accumulated organic acids. Organic acid content decreases during ripening, while sugar content increases. FBPase and PFP may therefore be involved in the conversion of organic acid to sugars in ripening berries.

Lack of significant changes in enzyme activity during ripening is not surprising. Even large perturbations of carbohydrate metabolism do not necessarily lead to changes in the maximum catalytic activity of enzymes. For instance, an introgression line of tomato with both increased soluble solid content and invertase activity did not exhibit changes in other sugar- and starch metabolising enzymes (Baxter *et al.,* 2005). The temporal changes in metabolite content of Festival and Ventana berries may be explained by changing *in vivo* enzyme activity. Substrate and product regulation of enzyme activity may affect the *in vivo* activity while relative quantities of enzyme are not affected. This study measured maximum catalytic activity which gives an indication of the amount of enzyme present rather than *in vivo* activity. The advantage of measuring maximum catalytic activity rather than *in vivo* activity is that potential targets for transgenic manipulation are more clearly visible. In this study FBPase, PFP and invertase were highlighted as likely candidates. Overexpression of any of these enzymes, especially isoforms that are not subject to the same regulation as the endogenous isoforms are likely to have a clear effect on metabolite content.

3.4 CONCLUSION

This is the first report on the metabolite composition of Festival and Ventana, popular commercial varieties in South Africa. Since Festival and Ventana were quite similar with respect to metabolite content, enzymatic differences in cultivar metabolism could not be pinpointed, however potential targets (invertase, PFP and FBPase) for transgenic manipulation of metabolite accumulation were identified. For future studies, the selection and analysis of varieties with

varying carbohydrate composition may allow for a better correlation between accumulation of soluble solids and enzyme activity. This study also established methodology to analyse transgenic strawberry. To further investigate the potential of strawberry as model system, this approach could be repeated in grape berries.

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4 Heterologous expression of pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase activity in strawberry fruit

4.1 INTRODUCTION

Cytosolic carbon metabolism is tightly regulated in all metabolically active plant cells (Taiz and Zeiger, 2002). One of the enzymes that is central to this regulation is pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90, PFP). PFP links triose-phosphate and hexose-phosphate metabolism by catalysing a reversible reaction where fructose-6-phosphate is phosphorylated to fructose-1,6-bisphosphate, using pyrophosphate as donor (Figure 4.1). Fructose-2,6-bisphosphate activates plant-derived PFP (Balogh *et al.,* 1984; Kruger and Dennis, 1987; Wu *et al.,* 1983) in the glycolytic reaction by decreasing the K_m for fructose-6-phosphate and increasing the V_{max} (Botha *et al.,* 1986; Sabularse and Anderson, 1981; Theodorou and Plaxton, 1996; Van Schaftingen *et al.,* 1982). PFP can exist in several combinations of its subunits. In grapefruit, PFP exists in two isoforms, a heterotetramer ($\alpha_2\beta_2$) that is sensitive to regulation by fructose-2,6-bisphosphate and a homodimer (β_2) that is not regulated by fructose-2,6-bisphosphate (Van Praag *et al.*, 2000), but it can also form other combinations with varying proportions of the α- and β-subunits. In the absence of the α-subunit, PFP becomes less sensitive to regulation by fructose-2,6-bisphosphate (Yan and Tao, 1984).

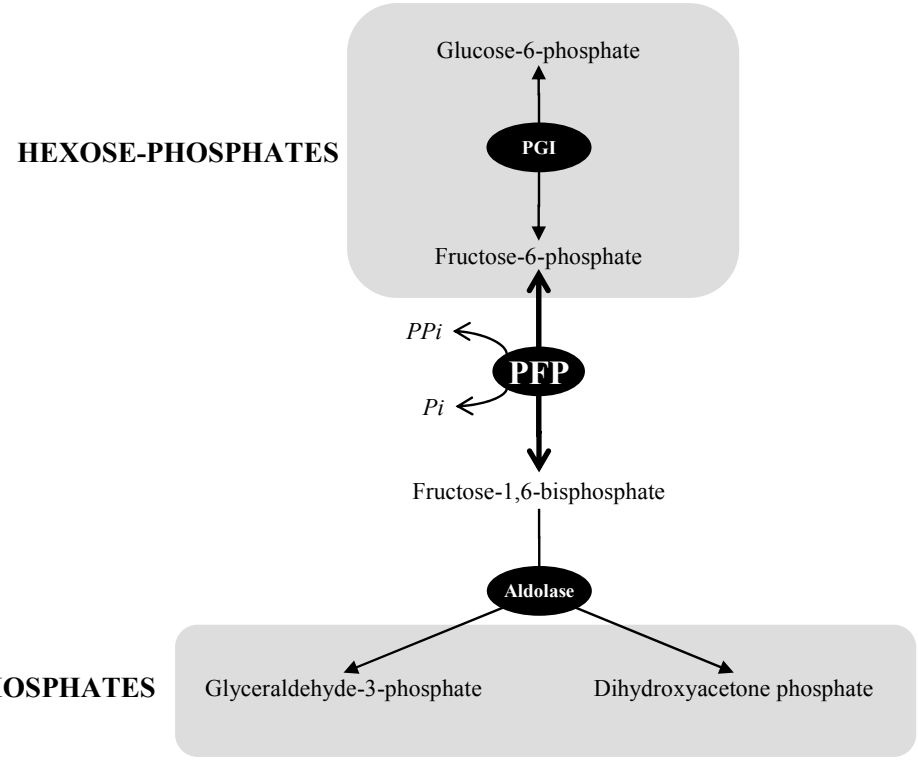

Figure 4.1 PFP links hexose-phosphate and triose-phosphate metabolism

Fructose-2,6-bisphosphate concentration is controlled by a bifuctional enzyme, containing catalytic sites for fructose-6-bisphosphate 2-kinase (EC 2.7.1.105) and fructose-2,6-bisphosphatase

(EC 3.1.3.46), that acts as a sensor for the levels of triose-phosphates and hexose-phosphates in the cytosol. Both sites are regulated by the relative concentrations of triose-phosphates, inorganic phosphates and fructose-6-phosphate (Cséke and Buchanan, 1983; Larondelle *et al.,* 1986; Stitt *et al.,* 1984). High cytosolic hexose-phosphate concentrations lead to an increase in fructose-2,6-bisphosphate content, which in turn activates PFP and leads to an increase in glycolytic flux.

In sink tissue such as fruit, hexose- and triose-phosphates are products of sucrose degradation and are substrates for the synthesis of metabolites, structural carbohydrates, proteins and lipids. Different sink tissues store different types of compounds. The effect of PFP on carbon partitioning therefore depends on the major carbon sinks present in the cells and, since PFP catalyses a reversible reaction, direction of flux through PFP. Transgenic tobacco and potato plants with reduced PFP activity (up to 97%) had more fructose-2,6-bisphosphate and less triose-phosphate in the form of 3-phosphoglycerate, but there were no significant changes in metabolic flux and plant morphology (Hajirezaei *et al.,* 1994; Nielsen and Stitt, 2001; Paul *et al.,* 1995). These findings suggest that plants may have an excess of PFP or complementary activity, or that PFP does not play an important role in the metabolism of the tissues that were investigated. Transgenic tobacco with increased PFP activity, on the other hand, exhibit changes in carbon allocation, with both sink and source tissues containing less starch (Wood *et al.,* 2002a). Heterologous expression of PFP furthermore resulted in modified lipid composition of tobacco seeds and hastened the deposition of lipids in the seeds (Wood *et al.*, 2002b). In tobacco seeds PFP may therefore affect the rate of starch degradation, leading to changes in the timing of lipid deposition. In sugarcane, PFP activity was lower in mature, sucrose-storing internodes than in young, metabolically active internodes. Maturing internodes become less metabolically active, with biosynthetic processes slowing down and sucrose accumulation increasing, suggesting that PFP activity is inversely correlated with sucrose content (Groenewald, 2006; Whittaker and Botha, 1999).

This study is not only aiming to manipulate carbon allocation to sugars and organic acids in strawberry, but is also attempting to provide insight into the possible outcome of a similar approach in grape berries. PFP clearly affects carbon allocation in sink tissues, however its role in fruit has not been established. The influence of modified PFP activity on carbon partitioning within sink tissues hinges on its central role in glycolytic and gluconeogenic metabolism. In order to further our understanding on the role of PFP in fruit metabolism we generated and characterised transgenic strawberry lines with elevated expression of the enzyme. In many heterotrophic tissues PFP has been observed to contribute to glycolytic flux. If this is the case in strawberry fruit, a decrease in the proportion of sugars relative to organic acids is expected when PFP activity is increased.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Strawberry (*Fragaria x ananassa* cv. Selekta) was cultivated under greenhouse conditions at the Biotechnology division of the ARC Infruitec-Nietvoorbij, Stellenbosch. Three vegetative clones of each plant were treated as biological replicates. Leaf and berry tissue was frozen on site in liquid nitrogen and stored at -80ºC until analysis. Berries were harvested over a period of six weeks at four stages of development as determined by maturity and external berry colour. Developmental stages were as follows: ripe (fully expanded and 100% red), turning (fully expanded and 50% red), white (fully expanded) and green (receptacle tissue distinguishable from seeds). Optimizations with regard to biochemical analyses were carried out on ripe berries purchased from local retailers.

All enzymes were purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other reagents were of analytical grade and were purchased from Roche Diagnostics, Merck (Whitehouse Station, New Jersey, USA) or Sigma (St. Louis, Missouri, USA).

4.2.2 Vector construction and plant transformation

A construct (p95.624) carrying the *Giardia lamblia* PFP gene (Rozario *et al*., 1995, GENBANK U12337) was kindly provided by Dr. D. Dennis (Performance Plants, Queens University, Kingston, Canada). The coding sequence (1727 bp) was excised with *Eco*RI and *Xba*I and cloned into pART7 between the 35S promoter from cauliflower mosaic virus and the transcriptional termination sequence from the octopine synthase gene. The expression cartridge was removed from pART7 as a *Not*I fragment and introduced into the binary vector pART27 (Gleave, 1992). The resulting construct (Figure 4.2) was mobilised into the *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method. Strawberry leaf disks were infiltrated via *A. tumefaciens* in 2% (w/v) sucrose, as described in Burger *et al.* (manuscript in preparation). Transformed plants were selected on Kanamycin-containing medium.

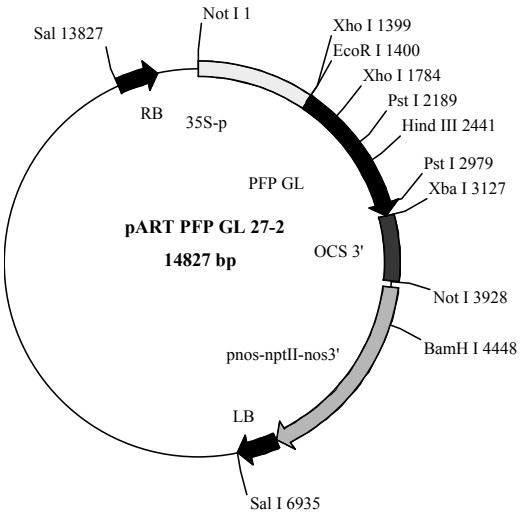

Figure 4.2 Plasmid pART PFP GL27-2 used in *Agrobacterium*-mediated transformation

4.2.3 PCR analysis

Genomic DNA was extracted from leaf material using a Nucleon PhytoPure DNA extraction kit (Tepnel Life Sciences, Manchester, United Kingdom). DNA concentration was determined at 260 nm on a Powerwave X Microplate Spectrophotometer with KC4 Kineticalc for Windows version 2.7 (Bio-Tek Instruments, Winooski, Vermont, USA) and standardised to 40 ng DNA per gram fresh weight.

Polymerase chain reaction (PCR) was used to select lines containing the *Giardia lamblia* PFP gene with the following primers: forward: 5'-ATG TCT GCT TTC GAG GTT TAC-3' and reverse: 5'-TCC GTC AGC TCA ACA TAC TTG TTA-3' (Integrated DNA Technologies, Coralville, Iowa, USA). Untransformed control (WT) plants were used as negative control and the plasmid containing the full-length DNA sequence for *Giardia lamblia* PFP was used as positive control. PCR products (344 bp) were visualised in a 0.8% agarose gel with ethidium bromide.

4.2.4 Biochemical analyses

4.2.4.1 Protein extraction

Leaf tissue was ground in liquid nitrogen and proteins extracted in 10 volumes of 100 mM Tris-HCl buffer (pH 7.2) containing 2 mM $MgCl_2$, 2 mM EDTA, 10% (v/v) glycerol, 5 mM DTT, 2% PVPP and Complete™ Protease Inhibitor.

Berry tissue was ground in liquid nitrogen and proteins extracted in 300 mM Hepes-KOH buffer (pH 7.2) containing 2 mM $MgCl_2$, 2 mM EDTA, 10% (v/v) glycerol, 2.5% (v/v) PEG 6000, 0.05% (v/v) Triton X-100, 5 mM DTT, 2% (m/v) PVPP. Complete™ Protease Inhibitor was replaced with 1 mM PMSF.

Samples were desalted using Sephadex G25 spin columns (Helmerhorst and Stokes, 1980) and protein quantified using Bio-Rad (Hercules, California, USA) Protein Assay (Bradford, 1976) with BSA as standard.

4.2.4.2 PFP activity assay

All assays were carried out at 25°C in a total volume of 250 µL. NADH oxidation was recorded at 340 nm with a Powerwave X Microplate Spectrophotometer.

Total PFP activity in berries was determined as described in Botha and Botha (1990), with modifications. Desalted protein extracts (10 µL) were incubated in 50 mM Hepes-KOH (pH 7.2) buffer containing 2 mM $MgCl_2$, 1 mM sodium pyrophosphate, 0.15 mM NADH, 0.01 mM fructose-2,6-bisphosphate, 1U aldolase (EC 4.1.2.13), 1U glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) and 10U triosephosphate isomerase (EC 5.3.1.1). The reaction was initiated by the addition of 10 mM fructose-6-phosphate.

Leaf protein extracts were used for the selection of plants expressing active recombinant

protein. Fructose-2,6-bisphosphate was omitted from the assay, rendering the endogenous enzyme inactive.

4.2.4.3 Metabolite extraction and quantification

Soluble sugars and organic acids were extracted from ground berry tissue by overnight incubation at 70ºC in 20 volumes of 80% ethanol. Extracts were diluted 20 times before enzymatic analysis of glucose, fructose and sucrose. Sugar concentrations were measured by means of a spectrophotometric enzymatic coupling assay (Jones *et al*., 1977) with modifications as described in Stitt *et al.* (1989). Phosphorylation of glucose by 1.5U hexokinase (EC 2.7.1.1) and 1U glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and fructose by 4U phosphoglucose isomerase (EC 5.3.1.9) was quantified by following the reduction of NAD to NADH at 340 nm. Sixty units invertase (EC 3.2.1.26) was added to hydrolyse sucrose to glucose and fructose.

The enzymatic quantification of citric and malic acid was modified from Velterop and Vos (2001). Assays were carried out at 25°C in a total volume of 250 µL. Malic acid was quantified with 2.5U Glutamate-oxaloacetate transaminase (EC 2.6.1.1) and 2U L-malate dehydrogenase (EC 1.1.1.37, MDH) in the presence of 25 µL extract. Citric acid concentration was measured with 4U MDH, 4U L-lactate dehydrogenase (EC 1.1.1.27) and 0.2U Citrate Oxaloacetate-Lyase (EC 4.1.3.6) in the presence of 10 µL extract. Absorbance data (340 nm) was captured on a Powerwave X Microplate Spectrophotometer.

4.2.4.4 Statistical analyses

Quantitative analyses are presented as mean values and the reproducibility of the results is expressed as standard error. Statistical analysis of the experimental results was carried out based on Student's t-test. Differences reaching a minimal confidence level of 90% were considered as being statistically significant. Probability values were calculated from T-scores using the Online T Distribution Calculator from Stat Trek (http://stattrek.com/Tables/T.aspx).

4.3 RESULTS AND DISCUSSION

4.3.1 Selection based on DNA amplification and PFP activity

Strawberry plants were transformed with a vector containing the full length sequence of *Giardia lamblia* PFP gene under control of the constituitive 35S promoter. Fifty putative transformants were hardened off and grown in a greenhouse. Total leaf DNA was isolated and was screened for the presence of the *Giardia lamblia* PFP gene using PCR with primers specific for the transgene. Of the 50 putative transformants, 30 lines contained the transgene. These lines were screened for active recombinant protein with PFP activity assays in the absence of fructose-2,6-bisphosphate, rendering the endogenous enzyme inactive (Figure 4.3). Four lines (PFP-09, -16, -44 and -45) with variable amounts of PFP activity were chosen for further analysis.

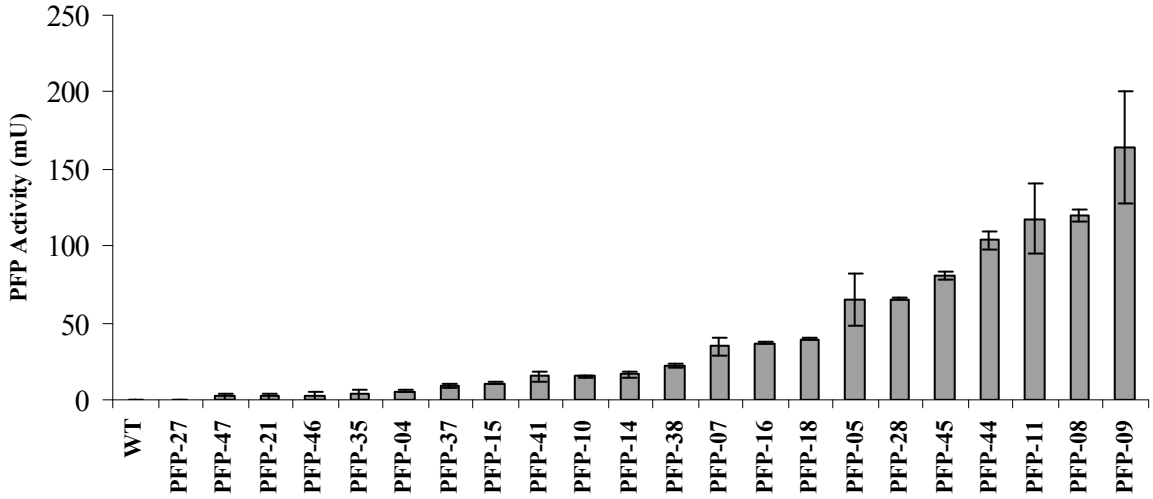

Figure 4.3 *Giardia lamblia* PFP activity in crude leaf protein extracts. Mean ±SE (n=3)

Figure 4.4 depicts total PFP activity of selected lines as measured in berry tissue and in the presence of fructose-2,6-bisphosphate. The protein extraction procedure employed for leaf tissue yielded low amounts of protein (less than 0.1 mg/mL). Both buffer composition and extraction volume were modified to provide acceptable protein yield for berry extracts (see Materials and Methods). Only ripe berries were characterised with regard to PFP activity and metabolite content, as the harvest yield of immature berries was very low and did not provide representative samples. Total PFP activity of transgenic lines was up to eight times higher than activity measured in WT plants (Figure 4.4).

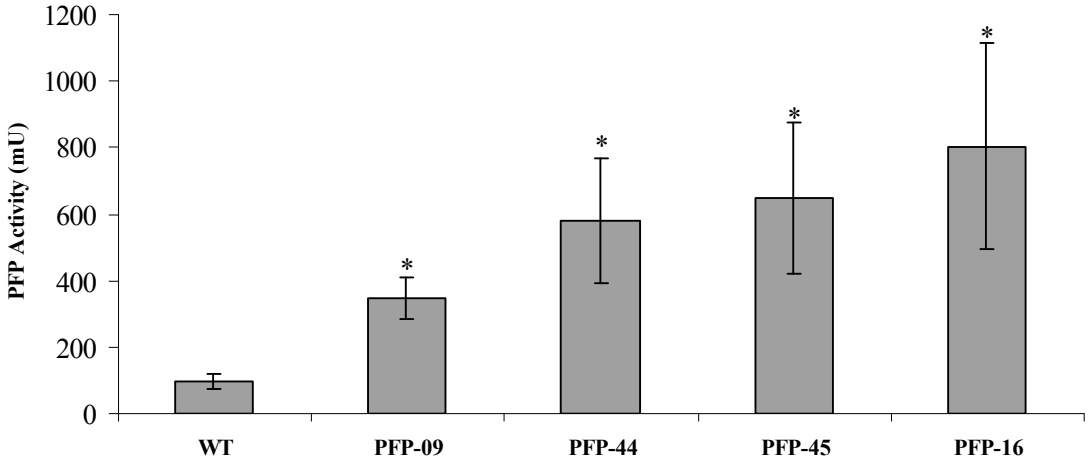

Figure 4.4 PFP activity in ripe berries. n=2 with the exception of WT (n=10). * indicates values statistically different from WT (P<0.1) at 90% confidence level.

4.3.2 Metabolite content of ripe berries

Heterologous expression of PFP did not affect total sugar content (Figure 4.5A), with the exception of line PFP-09. Sugar composition, however was different in transgenic lines with fructose content increasing up to two times (Figure 4.5B). This increase seems to have occurred at the expense of sucrose (Figure 4.5C). Glucose content did not change significantly (P>0.01) with an increase in PFP activity (Figure 4.5D). Variation between biological repeats rendered most of the

changes statistically insignificant (P>0.1). This variation most likely stems from the sampling method. Since the plants were five years old they no longer yielded many fruit, therefore berries were harvested weeks apart and treated as replicates. It has been reported that harvest date affects metabolite content (Watson *et al.,* 2002), therefore much of the observed variation is due to external factors, rather than internal biological variation.

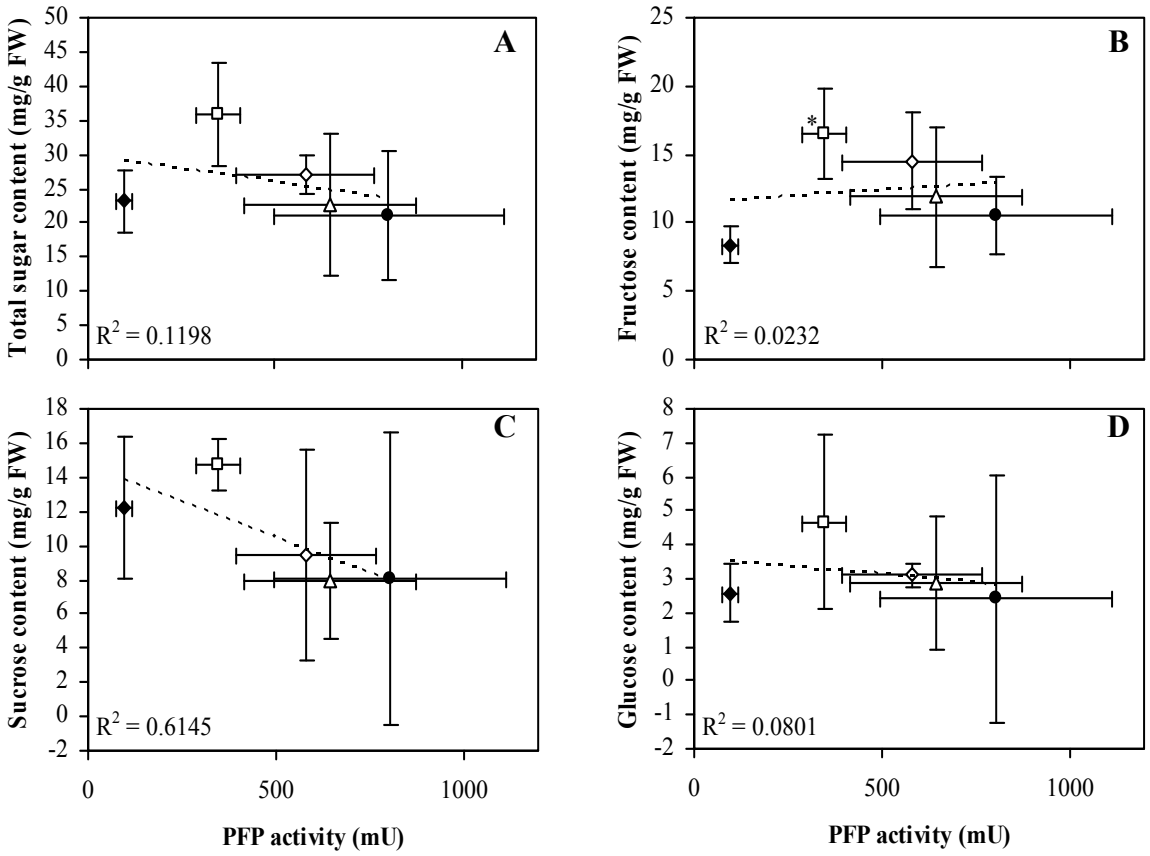

Figure 4.5 Total sugar content (A), fructose (B), sucrose (C) and glucose content (D) of ripe berries. Points represent berries from ◆ WT, □ PFP-09, ◇ PFP-44, △ PFP-45 and ● PFP-16. - - - - linear regression. * indicates values statistically different from WT (P<0.1) at 90% confidence level.

Total organic acid content of transformed lines decreased by up to 30% compared to WT (Figure 4.6). This reduction was due to decreases in both citric and malic acid content. Again PFP-09 exhibited higher organic acid content than other transgenics. Not surprisingly the soluble solids content of PFP-09 was higher than that of both control and other transgenic fruit (data not shown). Linear regressions of individual metabolites against PFP activity did not provide strong correlations, but the strongest correlation was between PFP and sucrose content (correlation coefficient 0.8).

The change in net carbon allocation to sugars and organic acids was compared between transgenic and wild-type lines (Figure 4.7). Lines expressing recombinant PFP displayed a marked reduced ratio of organic acids to sugars (up to 2.5 times). Although the sugar and organic acid content of PFP-09 were higher than other transgenics, the organic acids-to-sugar ratio was still significantly (p<0.1) decreased. Differences between the ratios of two lines (PFP-45 and -16) and

WT was statistically insignificant due to high variation between biological repeats.

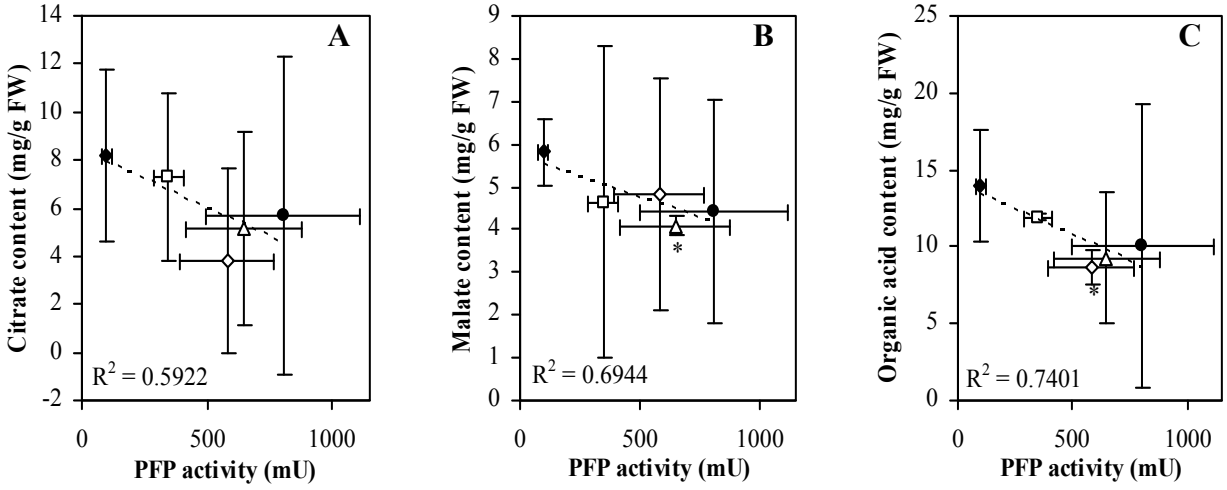

Figure 4.6 Citrate (A), malate (B) and total organic acid content (C) of ripe berries. Each point represents berries from ◆ WT, □ PFP-09, ◇ PFP-44, △ PFP-45 and ● PFP-16. - - - - linear regression. * indicates values statistically different from WT (P<0.1) at 90% confidence level.

Sugars make up the bulk of soluble solids in strawberry (Cordenunsi *et al*., 2002). In the event that heterologous expression of PFP increases the net glycolytic flux, an increase in the ratio of organic acids to sugars was expected. Although preliminary, this study suggests that the over-expression of PFP seems to positively stimulate gluconeogenesis and to negatively impact the production of organic acids. We have previously shown that PFP activity in a commercially cultivated variety (Festival) follows an activity pattern during development that is similar to that of fructose-1,6-bisphosphatase (EC 3.1.3.11, FBPase) (Chapter 3, Figure 3.6).

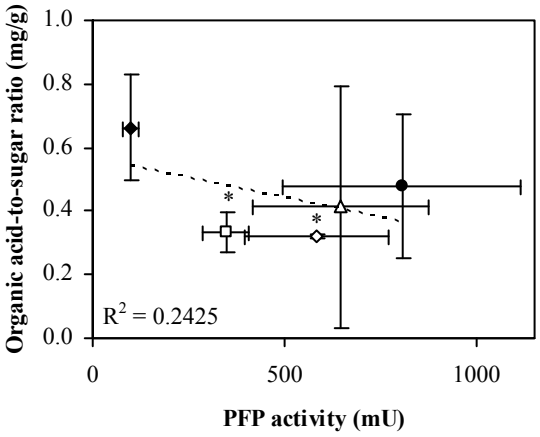

Figure 4.7 Organic acid-to-sugar ratio of ripe berries. Each point represents berries from ◆ WT, □ PFP-09, ◇ PFP-44, △ PFP-45 and ● PFP-16. - - - - linear regression. * indicates values statistically different from WT (P<0.1) at 90% confidence level.

In Chapter 3 we also observed that sugars accumulate during ripening while organic acid content decreases. It is likely that, as in grape berries, ripening coincides with increasing sugar content at the expense of organic acids (Coombe, 1976). During this time gluconeogenesis would

be the predominant pathway and increased PFP activity may contribute to flux through it. A similar effect has also been observed in watermelon (*Citrullus lanatus*) cotyledons, where PFP supplements FBPase activity when gluconeogenic flux is high (Botha and Botha, 1993).

4.4 CONCLUSION AND FUTURE WORK

This study aimed to determine whether increased PFP activity would cause a change in the allocation of carbon to sugars and organic acids. Due to low harvest yields only ripe berries were characterised. While the data has shown that increased PFP activity does affect the ratio of organic acids to sugars, there was a great deal of variation between the vegetative clones. Should this variation be minimised it will be possible to better judge the effect of modified PFP activity on carbon allocation and might provide more convincing data with respect to sugar and organic acid content.

This study allowed for the selection of lines possessing significantly increased PFP activity, accomplished through the expression of a recombinant protein that does not require activation by fructose-2,6-bisphosphate. These lines have subsequently been replicated, mostly through runners. Due to the age of the plants, some lines no longer formed runners and *in vitro* propagation was required. Resources can now be devoted towards improved experimental design, which will improve yield in the newly replicated plants. With more tissue available for analysis, the study will not only be expanded to include the immature berries, but may also allow extensive biochemical analyses, especially with regard to the role of PFP in cytosolic metabolism in strawberry.

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5 Heterologous expression of yeast invertase in strawberry driven by the B33 patatin promoter

5.1 INTRODUCTION

Strawberry (*Fragaria x ananassa*) quality is partly determined by the soluble solids content of ripe berries. Soluble solids are mainly composed of sugars, organic acids and free amino acids. The relative amounts of these compounds evidently directly affect quality. Strawberry is of scientific interest as a model system for non-climacteric fruit metabolism and translational genomics (Folta and Dhingra, 2006). Strawberry has specifically been proposed as a model system for grape berries, where the balance between sugars and organic acids, as well as their final concentrations, affect the quality of both table grapes and wine.

β-Fructosidase (EC 3.2.1.26, Invertase) is a hydrolytic enzyme, requiring no additional substrates to cleave sucrose into glucose and fructose. Plant invertases exist in three subcellular compartments namely the apoplast, cytosol and vacuole. The optimal pH ranges of the isozymes reflect the subcellular location, with the vacuolar and apoplastic invertases active at low pH (pH 4.5 to 5) and the cytosolic invertases active at neutral and slightly alkaline pH (pH 7 to 8). Insoluble plant invertase (located in the apoplastic space) is quite similar to the extracellular, glycosylated invertase of yeast (Sturm and Chrispeels, 1990), in the sense that it is also glycosylated and ionically bound to the cell wall (Fahrendorf and Beck, 1990). Plant cytoplasmic invertase is more similar to cytoplasmic cyanobacterial invertases than to plant acid invertases (Gallagher and Pollock, 1998, Vargas *et al.,* 2003).

In tomato fruit, invertase has been reported to affect both total sugar content and sugar composition (Klann *et al.*, 1993; Kortstee *et al.,* 2007), with higher invertase activity associated with higher soluble solids content (Baxter *et al.,* 2005). Biochemical analysis of maize mutants with significantly lower cell-wall invertase activity revealed the significance of invertase activity in determining the amount of photosynthate imported into the kernels, as these mutants have smaller kernels compared to the wild-type (Miller and Chourey, 1992). Antisense repression of vacuolar acid invertase activity in tomato led to significantly increased sucrose and decreased hexose contents (Ohyama *et al.,* 1995). This trend was also evident when investigating the difference in acid invertase activity between wild, sucrose-accumulating, and tomato species and domesticated, hexose-accumulating, tomato species. Fruit of the wild species did not express the acid invertase gene (Klann *et al.,* 1993). Invertase activity was also shown to be inversely correlated with sucrose uptake in tuberising potato stolons (Viola *et al.,* 2001).

Sucrose loading from source tissues into the phloem can be severely impacted if invertases are ectopically expressed. If this process is disrupted by increasing apoplastic invertase activity in

source tissues, severe phenotypic effects can occur, such as accumulation of sugars which ultimately leads to the repression of photosynthesis, and finally to bleaching and necrosis in mature leaves (Canam *et al.,* 2006; Dickenson *et al.,* 1991; Sonnewald *et al.,* 1991; Stitt *et al.,* 1991; Von Schaewen *et al.,* 1990).

To circumvent the detrimental effect of constitutive heterologous expression a fruit-specific promoter needs to be used for invertase expression. The B33 patatin promoter from potato tubers has been observed to confer fruit-specific expression in tomato (Le *et al.,* 2006). There is however an added complication in fruit-specific expression in strawberry, since strawberry is an accessory fruit (Coombe, 1976). Strawberry achenes (seeds) are derived from ovarian tissue, making them the true fruit, while the fleshy part of strawberry is derived from tissue supporting the ovary, termed the receptacle (Figure 5.1). It is not sure whether a fruit-specific promoter will be active in the receptacle. A fruit-specific promoter from grape berries (AC1) confers achene-specific expressing in strawberry (Personal communication).

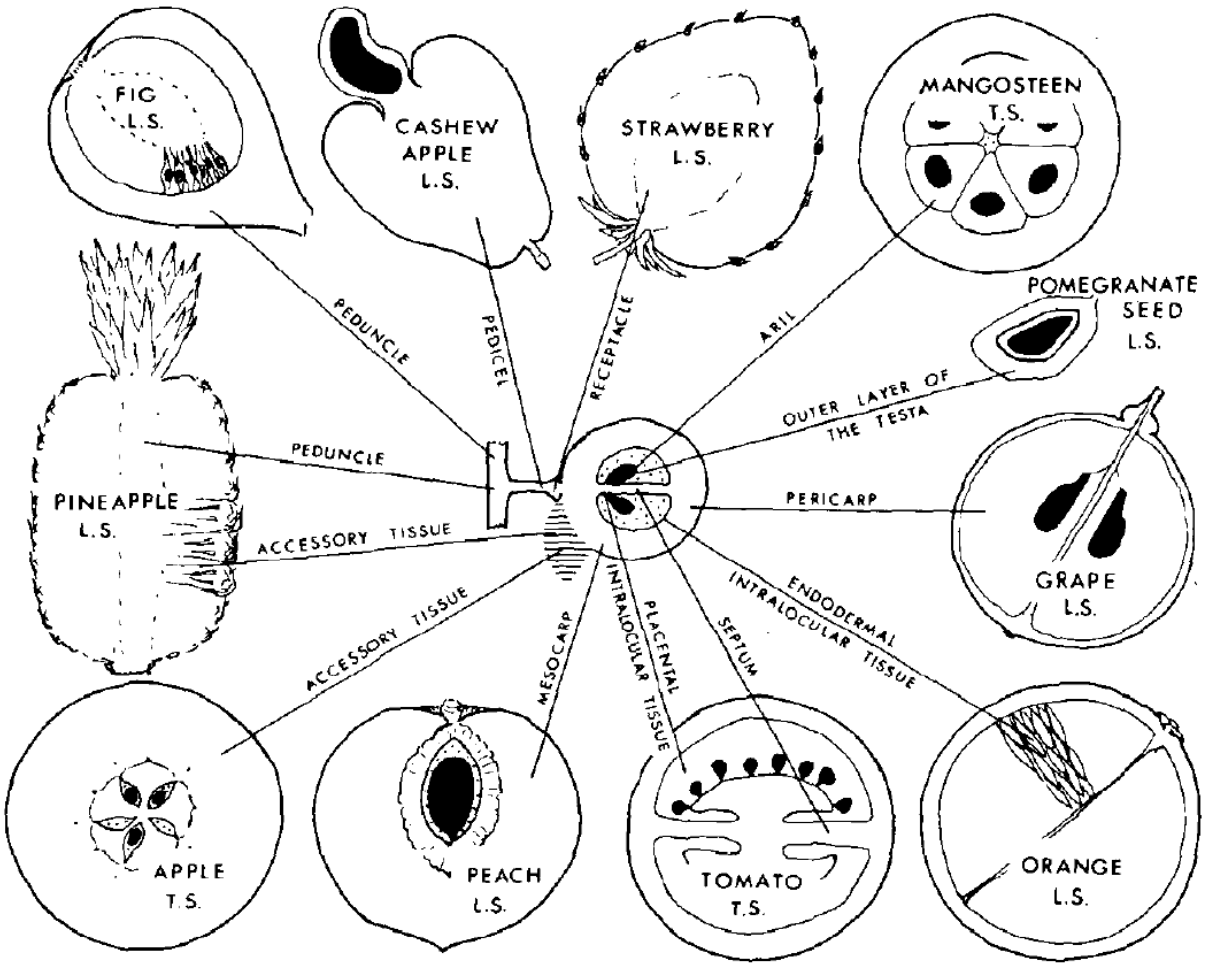

Figure 5.1 Outlines of sections of the fruits of 11 species showing the diversity of tissues which develop into fruit flesh (Coombe, 1976)

Invertase affects both sugar composition and total sugar content. Heterologous expression of apoplast-targeted invertase in other sink tissues led to an increase in the hexose-to-sucrose ratio

(Heineke *et al.,* 1992; Schaarschmidt *et al.,* 2007; Sonnewald *et al.,* 1997; Tauberger *et al.,* 1999; Tomlinson *et al.,* 2004). Furthermore, overexpression of yeast invertase in potato leads to a high glucose-to-fructose ratio (Sonnewald *et al.,* 1997). This study is specifically aimed at investigating viable targets of transgenic manipulation in grape berries, specifically those used in wine production. Past overexpression of invertase has been in tissues where sugars and organic acids are not the main stored carbohydrate, therefore we wish to elucidate the effect of fruit-specific overexpression on both sugars and organic acids.

5.2 MATERIALS AND METHODS

5.2.1 Plant tissue and materials

Strawberry (*Fragaria x ananassa* cv. Selekta) was cultivated under greenhouse conditions at the Biotechnology division of the ARC Infruitec-Nietvoorbij, Stellenbosch. Three vegetative clones of each plant were treated as biological replicates. Leaf and berry tissue were frozen on site in liquid nitrogen and stored at -80ºC until analysis. Berries were harvested over a period of six weeks at four stages of development as determined by maturity and external berry colour. Developmental stages were as follows: ripe (fully expanded and 100% red), turning (fully expanded and 50% red), white (fully expanded) and green (receptacle tissue distinguishable from seeds). Optimizations with regard to biochemical analyses were carried out on ripe berries purchased from local retailers.

All enzymes were purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other reagents were of analytical grade and were purchased from Roche Diagnostics, Merck (Whitehouse Station, New Jersey, USA) or Sigma (St. Louis, Missouri, USA).

Two constructs (pBin19 derived) were a kind gift from Prof Uwe Sonnewald (Department of Biochemistry, Friedrich-Alexander-Universität, Germany). The constructs (Sonnewald *et al.,* 1997) contained the sequence of the yeast *suc2* gene encoding the mature invertase with or without the proteinase inhibitor II signal sequence for apoplastic targeting or cytosolic localization respectively, cloned 3' of the sequence for the B33 promoter. The vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 by tri-parental mating for transformation of strawberry.

Strawberry leaf disks were infiltrated via *A. tumefaciens* in 2% (w/v) sucrose, as described in Burger *et al.* (manuscript in preparation). Transformed plants were selected on Kanamycin-containing medium.

5.2.2 Selection of lines expressing yeast invertase in berries

5.2.2.1 PCR analysis

Genomic DNA was extracted from leaf material using a Nucleon PhytoPure DNA extraction kit (Tepnel Life Sciences, Manchester, United Kingdom). DNA concentration was determined at 260 nm on a Powerwave X Microplate Spectrophotometer with KC4 Kineticalc for Windows

version 2.7 (Bio-Tek Instruments, Winooski, Vermont, USA) and standardised to 40 ng DNA per gram fresh weight.

Polymerase chain reaction (PCR) was used to select lines containing the B33 promoter and yeast *suc2* gene with the following primers: forward: 5'- GCGGTGCAAACTGAGTGAGGT A-3' and reverse: 5'-GGACTTCAAGTCATCAGAGGA G-3' (Integrated DNA Technologies, Coralville, Iowa, USA). Untransformed control (WT) plants were used as negative control and both plasmids containing the full-length DNA sequence for the B33 promoter and *suc2* gene, with or without the signal sequence, were used as positive control. PCR products were visualised in a 0.8% agarose gel with ethidium bromide.

5.2.2.2 Protein extraction

Fruit tissue was ground in liquid nitrogen and proteins extracted in 300 mM Hepes-KOH buffer (pH 7.2) containing 2 mM $MgCl_2$, 2 mM EDTA, 10% (v/v) glycerol, 2.5% (v/v) PEG 6000, 0.05% (v/v) Triton X-100, 5 mM DTT, 2% (m/v) PVPP and 1 mM PMSF.

5.2.2.3 Invertase activity assay

Both apoplastic and cytosolic localised invertase was assayed at neutral pH. Invertase activity assay was modified from Schaarschmidt *et al.* (2004). Invertase activity was coupled to NAD reduction with 1U glucose-6-phosphate dehydrogenase (EC 1.1.1.49, GDH), 1U hexokinase (EC 2.7.1.1, HK) and 1U phosphoglucose isomerase (EC 5.3.1.9, PGI) in 100 mM Hepes-KOH (pH 7) containing 0.2 M sucrose and 10 mM $MgCl_2$. Activity was initiated with 1 mM ATP. The reaction was monitored at 340 nm on a Powerwave X Microplate Spectrophotometer with KC4 Kineticalc for Windows version 2.7 (Bio-Tek Instruments, Winooski, Vermont, USA). One unit of invertase activity is defined as the amount of sucrose hydrolysed per minute per milligram protein.

5.2.3 Biochemical analyses

5.2.3.1 Metabolite extraction and quantification

Soluble sugars and organic acids were extracted from ground berry tissue by overnight incubation at 70ºC in 20 volumes of 80% ethanol. Extracts were diluted 20 times before enzymatic analysis of glucose, fructose and sucrose. Sugar concentrations were measured by means of a spectrophotometric enzymatic coupling assay (Jones *et al*., 1977) with modifications as described in Stitt *et al.* (1989). Phosphorylation of glucose by 1.5U hexokinase (EC 2.7.1.1) and 1U glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and fructose by 4U phosphoglucose isomerase (EC 5.3.1.9) was quantified by following the reduction of NAD to NADH at 340 nm. Sixty units invertase (EC 3.2.1.26) was added to hydrolyse sucrose to glucose and fructose.

The enzymatic quantification of citric and malic acid was modified from Velterop and Vos (2001). Assays were carried out at 25°C in a total volume of 250 µL. Malic acid was quantified

with 2.5U Glutamate-oxaloacetate transaminase (EC 2.6.1.1) and 2U L-malate dehydrogenase (EC 1.1.1.37, MDH) in the presence of 25 µL extract. Citric acid concentration was measured with 4U MDH, 4U L-lactate dehydrogenase (EC 1.1.1.27) and 0.2U Citrate Oxaloacetate-Lyase (EC 4.1.3.6) in the presence of 10 µL extract. Absorbance data (340 nm) was captured on a Powerwave X Microplate Spectrophotometer.

5.2.3.2 Enzyme activity assays

Enzyme assays were performed as described previously with modifications as described in Table 5.1. All reactions were monitored at 340 nm.

5.2.4 Statistical analyses

Quantitative analyses are presented as mean values and the reproducibility of the results is expressed as standard error. Statistical analysis of the experimental results was carried out based on Student's t-test. Differences reaching a minimal confidence level of 95% were considered as being statistically significant. Probability values were calculated from T-scores using the Online T Distribution Calculator from Stat Trek (http://stattrek.com/Tables/T.aspx).

Table 5.1 Enzyme activity assays

Enzyme[a]	Assay components[b]	Initiation substrate	Reference
HK	50 mM Hepes-KOH (pH 8.0), 4 mM $MgCl_2$, 2 mM glucose, 0.5 mM $NADP^+$, 1U GDH.	2.5 mM ATP	Doehlert *et al.*, 1988
PEPc	100 mM Tris-HCl (pH 8.5), 4 mM $MgCl_2$, 6 mM $NaHCO_3$, 0.3 mM NADH and 5U MDH	5 mM PEP	Ocaña *et al.,* 1996
PFP	50 mM Hepes-KOH (pH 7.2), 2 mM $MgCl_2$, 1 mM PPi, 0.15 mM NADH, 0.01 mM fructose-2,6-bisphosphate, 1U aldolase, 10U TPI, 1U GPD	10 mM fructose-6-phosphate	Botha and Botha, 1990
PGI	100 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 10 mM fructose-6-phosphate, 1U GDH	10 mM fructose-6-phosphate	Doehlert *et al.,* 1988
PK	50 mM Tris-HCl (pH 7.0), 100 mM KCl, 15 mM $MgCl_2$, 0.15 mM NADH, 1 mM ADP, 2U LDH	5 mM PEP	Burrell *et al.,* 1994

[a] HK, Hexokinase (EC 2.7.1.1); Invertase, β-fructosidase (EC 3.2.1.26); PEPc, Phosphoenolpyruvate carboxylase (EC 4.1.1.31); PFP; Pyrophosphate dependent phosphofructokinase (EC 2.7.1.90); PGI, Phosphoglucose Isomerase (EC 5.3.1.9); PK, Pyruvate kinase (EC 2.7.1.40)

[b] Aldolase, Fructose-1,6-bisphosphatase aldolase (EC 4.1.2.13); GDH, Glucose-6-phosphate dehydrogenase (EC 1.1.1.49); GPD, Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8); LDH, L-Lactate dehydrogenase (EC 1.1.1.27); MDH, L-Malate dehydrogenase (EC 1.1.1.37); PEP, phospho*enol*pyruvate; PGM, Phosphoglucomutase (EC 2.7.5.1); PPi, Sodium pyrophosphate ($Na_2P_4O_7$); TPI, Triose phosphate isomerase (EC 5.3.1.1)

5.3 RESULTS

Strawberry (cv. Selekta) was transformed with constructs containing a sequence coding for yeast invertase with or without a signal sequence. Constructs without a signal sequence (CT lines) targeted recombinant invertase to the cytosol, while constructs with the proteinase inhibitor II signal sequence (CW lines) should target the recombinant invertase to the apoplastic space (Sonnewald *et al.*, 1991). Presence of the transgenes was confirmed with PCR (data not shown).

5.3.1 Invertase activity of ripe berries

Yeast invertase is optimally active at pH 3-5 (Goldstein and Lampen, 1975), however it is also active at cytosolic pH (Sonnewald *et al.,* 1991). Assays were performed at pH 7 since coupling enzymes (GDH, HK and PGI) require neutral to slightly alkaline pH. Both recombinant and endogenous cytosolic invertase are active at neutral pH. Coupled assays of all putative CW-lines revealed significantly ($p<0.01$) higher total invertase activity in ripe berries, up to three times higher in CW-01, -02, -12 and -13 (Figure 5.2).

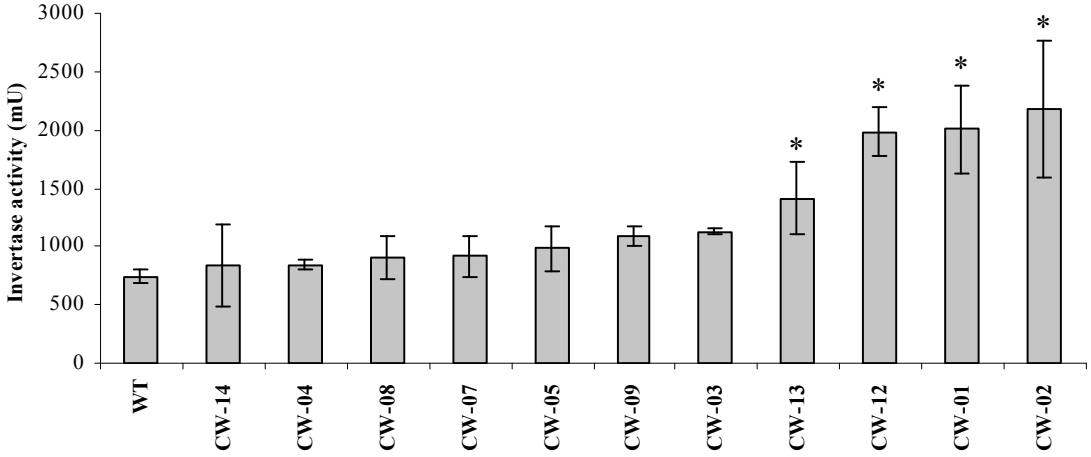

Figure 5.2 Total invertase activity at pH 7 of WT and transgenic CW berries. * indicates values statistically different from WT (P<0.01).

Based on total invertase activity at neutral pH, as well as tissue availability, three lines (CW-01, -02 and -13) were selected for continued analyses. Lines containing the untargeted gene did not exhibit significant ($P>0.05$) increases in total invertase activity (data not shown).

5.3.2 Biochemical analyses of transgenic strawberry fruit overexpressing apoplastic invertase

5.3.2.1 Metabolites

Cytosolic invertase lines were not subjected to further analyses, since there were no significant differences between their total neutral invertase activity and that of the WT. Unlike the cytosolic lines, total invertase activity in some transgenic lines expressing an apoplast targeted invertase were significantly increased (Figure 5.2). Sugar and organic acid content and composition were therefore determined in ripe berries of these lines, i.e. CW 01, CW 02, and CW 13 in comparison to the WT.

A significant (P<0.01) change was observed in the sugar composition of the CW-01 and CW-02 lines (Figure 5.3). Glucose content was increased significantly (P<0.01) in CW-01 and -02. Sucrose content in transgenic berries was two to five times reduced. The fructose content of transgenic lines was also reduced, up to two times in CW-01 and -02. A four-fold reduction in sucrose-to-hexose ratio was observed between WT and lines expressing recombinant invertase (Figure 5.3D).

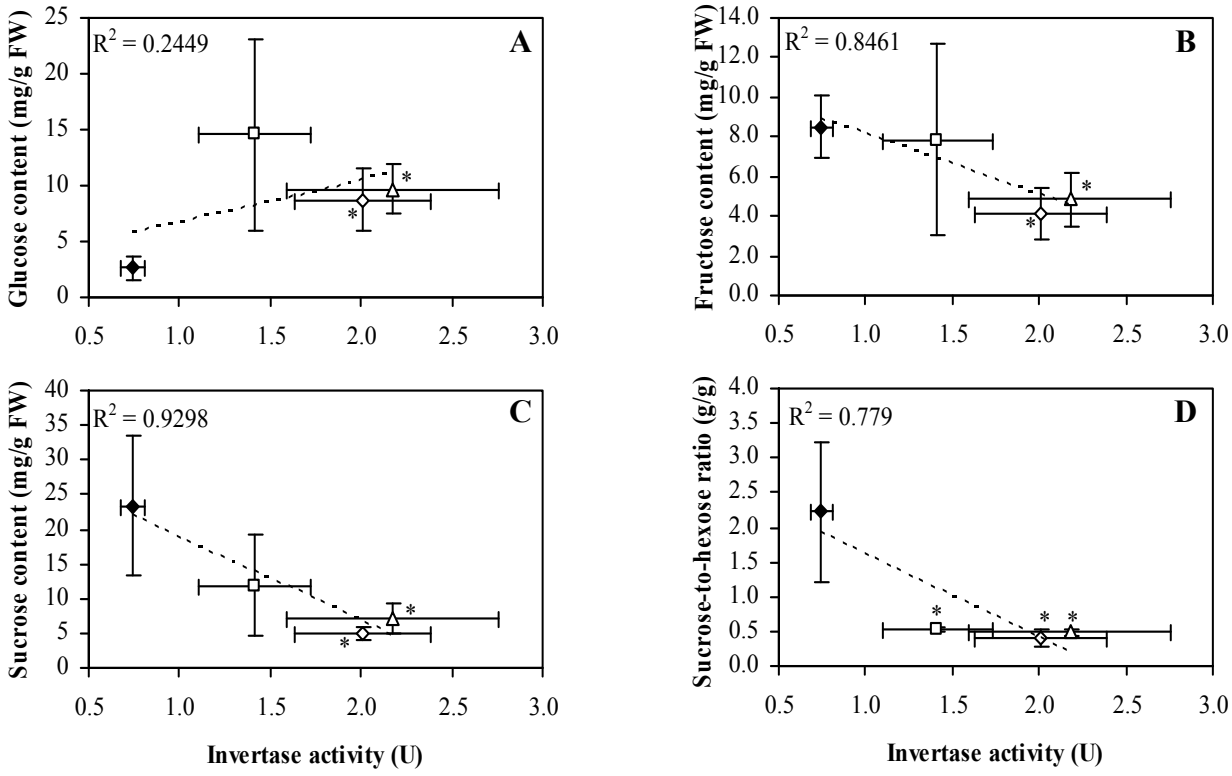

Figure 5.3 Glucose (A), fructose (B), sucrose (C) and total sugar content (D) of ripe CW berries. Points represent berries from ◆ WT, □ CW-13, ◇ CW-01 and △ CW-02. - - - - linear regression. * indicates values statistically different from WT (P<0.01) at 95% confidence level.

Total organic acid content in transgenic berries was not significantly (P>0.01) from WT (Figure 5.4A), however malate content in transgenic berries was significantly (P<0.01) reduced to up to 25% of WT (Figure 5.4B). Any potential effect of increased invertase activity on citrate content is not clear, as there was considerable variation in citrate content of control berries (Figure 5.4C). The ratio of organic acid-to-sugar was not significantly (P>0.01) reduced (Figure 5.4D).

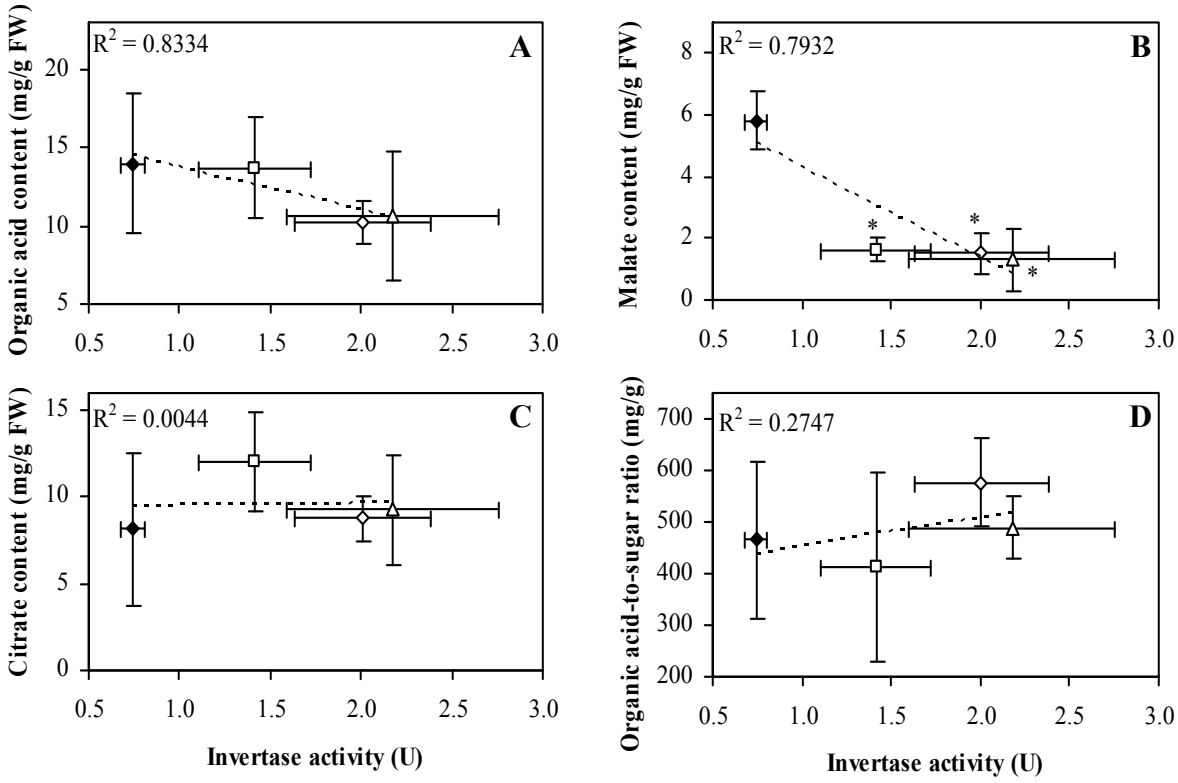

Figure 5.4 Total organic acid content (A), malate (B) citrate (C) content of ripe CW berries. Organic acid-to-sugar ratio (D) of ripe berries. Points represent berries from ◆ WT, □ CW-13, ◇ CW-01 and △ CW-02. - - - - linear regression. * indicates values statistically different from WT (P<0.01) at 95% confidence level.

5.3.2.2 Enzyme activity

Invertase catalyses the hydrolysis of sucrose to glucose and fructose. The maximum catalytic activities of several enzymes involved in the entry of hexoses into glycolysis, as well as the glycolytic enzymes responsible for the citrate and malate synthesis were determined on ripe berries (Table 5.2). No significant (P>0.01) differences were observed for maximum catalytic activities of the selected enzymes.

Table 5.2 Maximum catalytic activities of enzymes involved in cytosolic hexose and organic acid metabolism

	WT	CW-13	CW-01	CW-02
Invertase	743 ± 63	1414 ± 313	2006 ± 378	2177 ± 581
HK	76 ± 4	87 ± 10	70 ± 6	84 ± 10
PGI	1153 ± 69	1208 ± 54	1119 ± 61	1228 ± 57
PFP	66 ± 4	69 ± 3	82 ± 6	63 ± 4
PK	314 ± 19	276 ± 13	309 ± 29	315 ± 11
PEPc	55 ± 7	58 ± 5	62 ± 6	53 ± 5

5.4 DISCUSSION

5.4.1 Invertase activity of CT and CW berries

While up to three-fold increases were observed in four lines expressing cell-wall targeted yeast invertase, total invertase activity in lines expressing untargeted yeast invertase was not significantly ($p>0.05$) increased.

Differences in recombinant invertase activity have been observed depending on the subcellular location, whether expression was driven by the B33 patatin promoter (Hajirezaei *et al.,* 2000) or other promoters (Canam *et al.,* 2006). Expression of cytosolic yeast invertase in tobacco was also less consistent over time than expression of vacuole- or apoplast-targeted yeast invertase (Sonnewald *et al.,* 1991). The lack of activity increase in CT lines, while clear increases were observed in CW lines may also stem from the greater pleiotropic effects when invertase is expressed in the cytosol, as was observed by Sonnewald *et al.* (1991) in tobacco.

The B33 promoter was shown to be activated by light in stems of *Arabidopsis* seedlings (Naumkina *et al.,* 2007) and may therefore have led to increased invertase activity during the initial tissue culture selection phase. It is therefore possible that transformants with increased levels of cytosolic invertase activity suffered from stunted growth, as was observed in tobacco and potato (Canam *et al.,* 2006; Dickenson *et al.,* 1991; Sonnewald *et al.,* 1991; Stitt *et al.,* 1991; Von Schaewen *et al.,* 1990), while transformants expressing apoplastic yeast invertase were not as severely affected. Therefore, lines with significantly higher levels of cytosolic invertase activity may not have survived tissue culture, or may not have been propagated further.

5.4.2 Effect of increased invertase activity on carbohydrate metabolism

Despite a three-fold increase in invertase activity, maximum catalytic activities of cytosolic enzymes involved in sugar and organic acid metabolism were not affected significantly. Other studies have shown that increased invertase activity does not necessarily affect maximum catalytic activity of cytosolic carbon-metabolising enzymes (Baxter *et al.,* 2005; Hajirezaei *et al.,* 2000). This is likely due to substrate and product regulation of enzyme activity affecting the *in vivo* activity while relative quantities of enzyme are not affected.

Heterologous expression of invertase did not clearly affect total sugar content. Fructose content was significantly reduced and glucose content was increased, therefore hexose content was not significantly ($P>0.01$) different from WT. The increased availability of hexoses through hydrolysis via the yeast invertase may have lead to preferential phosphorylation of fructose by a hexokinase with higher affinity for fructose than glucose. Potato tubers with elevated invertase activity contained far more glucose than fructose (Sonnewald *et al.,* 1997) and it was proposed that potato tubers do not posses sufficient capacity for glucose phosphorylation. They subsequently expressed a glucokinase along with the invertase, and these tubers contained little or no glucose (Trethewey *et*

al., 1998).

In some cases there were fairly close linear relationships between metabolites and invertase activity, while in others, the linear relationship did not include all data points. Most notably glucose and citrate contents of CW-13 led to low correlation coefficients for those metabolites (0.5 and 0.07 respectively). The greatest effect of invertase activity was observed for sucrose-to-hexose ratio and malate content. In both cases a polynomial regression provided the best fit, increasing R^2 to around 0.99. This was also observed by Tomlinson *et al.* (2004), where the effect of linear increases in invertase activity led to hyperbolic changes in sucrose and hexose content.

The decrease in total sugar content is in contradiction to findings in tomato fruit. Increased activity of endogenous apoplastic invertase activity in tomato fruit correlated with higher soluble solids content (Baxter *et al.,* 2005). Direct comparison between other sink tissues is complicated by the different storage types. For instance, untransformed potato tuber contains 20 to 30 times less sugar than the untransformed berries analysed in this study (Sonnewald *et al.,* 1997). However, tobacco seed expressing yeast invertase also contained less sugar and exhibited increased hexose-to-sucrose ratios (Tomlinson *et al.,* 2004). Increases in hexose (glucose and/or fructose) content relative to sucrose content were also reported in potato tuber (Heineke *et al.,* 1992; Sonnewald *et al.,* 1997; Tauberger *et al.,* 1999) and tobacco roots (Schaarschmidt *et al.,* 2007). In this study increases in hexose content was not sufficient to compensate for decreases in sucrose content, therefore total sugar content was not affected.

Due to limited productivity, very few berries were harvested from the lines expressing high CW targeted invertase. To confirm and elaborate on these results, analyses will be repeated.

As a model system for grape berries, this preliminary study in strawberries yielded promising results. The generally warm climate in South Africa leads to grape berries with high levels of sugars yielding highly alcoholic wines. In wine production, sugar and organic acid content of grape berries affect both the fermentation process and the quality of the finished product. Grapes containing more fructose than glucose often result in stuck alcoholic fermentations, where yeasts cannot metabolise all available sugar (Berthels *et al.,* 2008). High residual sugar content as a result of stuck fermentation renders wines microbiologically unstable. Due to increasing consumer demands low-alcohol wines are gaining in popularity (Malherbe *et al.,* 2003). Low-alcohol wines are mainly produced by reducing sugar content after harvest or by reducing alcohol content after fermentation with reverse osmosis. This process often negatively affects sensory properties of the wine. Should heterologous expression of invertase lead to reduced sugar or fructose content in grapevine, as has been observed in this study for strawberry, it may be advantageous to the South African wine industry. A reduction in malate content my also be beneficial, particularly in wines where malolactic fermentation is undesirable (Bauer *et al*., 2004; Mazzei *et al.,* 2007).

5.5 CONCLUSION AND FUTURE WORK

This study aimed to determine whether increased invertase activity in either cytosol or apoplast would cause a change in the allocation of carbon to sugars and organic acids. While initial selection showed that the gene for yeast invertase was present in cytosolic lines, subsequent analysis of total invertase activity did not show clear differences between ripe transgenic berries and controls. This may be due to the more severe effect that increased cytosolic invertase activity has on plants, compared to the effect of cell-wall targeted invertase. Lines containing yeast invertase targeted to the apoplastic space did however exhibit up to three fold increases in invertase activity, leading to a significant ($p<0.01$) increase in hexose content relative to sucrose as well as a reduction in total sugar content and fructose in ripe berries. The B33 patatin promoter therefore appears to be suitable for expression of transgenes in strawberry receptacle.

This study allowed for the selection of lines possessing apoplastic invertase activity three times higher than WT. These lines have subsequently been replicated, mostly through runners that form once flowering is complete. Where runners did not produce viable plants *in vitro* propagation was required. With more tissue available for analysis, the study will not only be expanded to include the immature berries, but may also allow more extensive biochemical analyses, as well as further investigation into the tissue-specificity of the B33 patatin promoter in strawberry. In order to determine if yeast invertase was successfully expressed in the cytosolic lines, more genetic studies (e.g reverse transcriptase PCR) may be employed, as well as immunological studies.

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6 General conclusion

6.1 CONCLUSION

This study had three main components: 1) Characterisation of cytosolic carbohydrate metabolism in developing strawberries and manipulation of carbohydrate partitioning through the heterologous expression of 2) pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90, PFP) and 3) β-fructosidase (EC 3.2.1.26, invertase).

This study has yielded promising results and also highlighted aspects of the experimental design which can be further improved. Heterologous expression of PFP and apoplastic invertase clearly affected carbohydrate partitioning in ripe berries. Their effect in unripe fruit could not be established, as unripe berries that were harvested were not representative samples.

Total invertase activity of ripe berries expressing cytosolic yeast invertase was not markedly higher, therefore these were not analysed further. Ripe berries expressing apoplastic yeast invertase (CW lines) or cytosolic *Giardia lamblia* PFP (PFP lines) exhibited marked changes in carbon partitioning. Selected CW lines displayed decreased sucrose, fructose and malate content, however the ratio of organic acids to sugars was not affected to any great extent. Total sugar content in PFP lines was mostly similar to controls, while the organic acid content was reduced. The ratio of organic acids to sugars was reduced by up to 50%. The sugar composition of both CW and PFP berries were markedly different from controls. CW berries contained less sucrose and more glucose relative to total sugar content. On the other hand PFP berries contained more fructose. Analysis of two commercial varieties, both popular in South Africa, also highlighted the importance of PFP in developing strawberry. Although there were no great differences in sugar and organic acid content between the two cultivars, there were temporal differences in sugar and organic acid content. In both Festival and Ventana, sucrose and fructose content in ripening berries appeared to increase as PFP activity decreased. This suggests that PFP may fulfil a gluconeogenic role in strawberry. Invertase activity was also markedly higher in white and turning berries of Festival.

The apparent lack of correlation between enzyme activity and metabolite accumulation stems from the technique used to estimate enzyme activity. We employed coupled *in vitro* assays that are optimised to measure maximum catalytic activity. Enzymes do not necessarily operate at maximum rate of activity *in vivo*, since activity is influenced by substrate, product, activator and inhibitor concentrations. Recombinant enzymes, on the other hand, are usually selected to minimise *in vivo* regulation, and therefore lead to more pronounced changes in metabolism.

As a model system for non-climacteric fruit, and grape berries in particular, strawberry provides the opportunity to evaluate the effect of transgenic manipulation within a relatively short time. The effect of increased apoplastic invertase activity is of particular significance for the wine industry.

Grape berries with less sugar yield wines with a lower alcoholic content. In warm wine producing regions, like South Africa, the sugar content in grapes is often too high resulting in highly alcoholic wine, while health-conscious consumers increase the demand for low-alcohol wines (Malherbe *et al.,* 2003). The reduction in fructose and malate content would both be advantageous for fermentation. Stuck alcoholic fermentations, where yeasts cannot utilise excess fructose, lead to undesirable high residual sugar content in wine (Berthels *et al.,* 2008). The availability of malate, on the other hand, stimulates bacterial malolactic fermentation, a process that results in a reduction in acidity which is generally undesirable in warm wine producing regions where the organic acid content of grapes is often too low (Mazzei *et al.,* 2007).

6.2 FUTURE WORK

An aim of this project was to establish a methodology for the analysis of transgenic strawberry. A key factor that determined the extent of analyses was tissue availability. Festival and Ventana berries were harvested from a commercial farm and field-grown berries were much larger than greenhouse-grown berries. Therefore, far more berries would need to be harvested from greenhouse-grown plants to enable the same amount of analyses. This was unfortunately not possible, as yield from transgenic plants in particular was much lower than required. Due to the number of control plants, yield was sufficient to conduct most of the required analyses. There were also clear differences between control and transgenic PFP lines in terms of size and vigour. PFP plants were five years old and actively growing parts were much smaller than controls.

Some of the factors that have, in the past, been shown to affect metabolite levels in plants are pot size (Townend and Dickenson, 1995), environmental factors and harvesting date (Watson *et al.,* 2002). While control over some of these factors is fairly simple, others will be influenced by practical considerations. In order to minimise the variation to the greatest practically feasible degree, all plants should be planted in large pots of the same size with fixed amount of soil. Randomised blocks will also give a more uniform indication of biological variation. Since these factors are controlled right at the beginning of the project, they will not require a high degree of maintenance. Other factors, which would require regular input, are the irrigation of the plants, climate-control of the greenhouse, regular plant maintenance (trimming, pest-control, repotting when needed) and ensuring all plants are equally vigorous. A large component of disparity in metabolite content between genetically identical plants was irregular harvesting, where some berries were harvested at the beginning of the season and compared with those harvested later. An effort should be made to harvest samples simultaneously, which should be possible with improved yield. This may minimise biological variation, since the effect of environmental changes will similar for all replicates. Harvesting over a second season and comparing data between the two harvests would

also minimise outside effects on metabolite content. With improved yield analysis of PFP and invertase lines can be extended to unripe fruit.

This study has showed the effect of heterologous expression of PFP or invertase in strawberry. Similar studies in grape berries will be required to confirm the suitability of strawberry as a model system and if similar changes in metabolite content will occur in grape berries.

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