Understanding of Carbon Partitioning in Tomato Fruit

Dissertation

zur Erlangung des akademischen Grades

doctor rerum agriculturarum

(Dr. rer. agr.)

Eingereicht an der

Landwirtschaftich-Gärtnerischen Fakultät

der Humboldt-Universität zu Berlin

von

Hazem Abd El-Rahman Obiadalla Ali (M.Sc.)

geb. am 14.09.1969 in Sohag, Ägypten

Präsident der
Humboldt-Universität zu Berlin
Prof. Dr. Jürgen Mlynek

Dekan der
Landwirtschaftich-Gärtnerischen Fakultät
Prof. Dr. Uwe Jens Nagel

Gutachter: 1- Prof. Dr. F. Pohlheim
2- Prof. Dr. L. Willmitzer
3- Dr. J. Kossmann

Tag der mündlichen Prüfung: 10.06.2003

The work presented in this thesis was carried out between November 1999 and December 2002 at the Max-Planck-Institute für Molekulare Pflanzenphysiologie, Golm.

This Ph.D. thesis is the account of work done between November 1999 and December 2002 in the department of Prof. L. Willmitzer in the Max-Planck Institute of Molecular Plant Physiology, Golm, Germany. It is results of my own work and has not been submitted for any degree or Ph.D. at any other university.

Eidesstattliche Erklärung

Die Dissertation ist das Ergebnis praktischer Arbeit, welche von November 1999 bis Dezember 2002 durchgeführt wurde im Department von Prof. L. Willmitzer im Max-Planck-Institut für Molekulare Pflanzenphysiologie, Golm, Germany. Ich erkläre, das ich die vorliegende Arbeit selbständig und ohne unterlaubte Hilfe angefertigt habe. Es wurden keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, und die den benutzten Quellen wörtlichen und inhaltlichen Stellen sind als solche kenntlich gemacht.

Berlin, Februar 2003

Hazem Abd El-Rahman Obiadalla Ali

TABLE OF CONTENTS

1 General Introduction 1
2 Review of literature 3
2.1 Carbon Metabolism in Photosynthetic Tissue 3
2.1.1 Feedforward control of photosynthesis 4
2.1.2 Feedback control of photosynthesis 4
2.2 Carbohydrate Allocation from photosynthetic "source" to heterotrophic "sink Tissues" 7
2.3 Mobilisation of Sucrose in sink tissues 8
2.4 Uptake of carbon into amyloplasts 10
2.5 The synthesis of starch 11
2.6 Starch degradation 13
2.7 Starch phosphorylation 13
2.8 Glycolysis 15
2.9 Fruit metabolism 16
3 Material and Methods 17
3.1 Chemicals 17
3.2 Vectors and Bacterial Strains 17
3.2.1 Vectors 17
3.2.2 Bacterial 18
3.3 Transformation and Cultivation of Bacteria 18
3.4 DNA manipulations 18
3.5 Cloning 19
3.6 Plant Material 19
3.7 Sampling of fruits 19
3.8 Transformation and Cultivation of tomato 20
3.9 Selection of plants with reduced cp-FBPase AGPase and GWD protein 20
3.9.1 Selection of plants with reduced cp-FBPase activity 20
3.9.2 Selection of plants with reduced AGPase activity 21
3.9.3 Selection of plants with reduced GWD protein levels 21
3.10 Western Blot Analysis 21
3.11 RNA (Northern) Blot Analysis 22
3.12 Determination of enzyme Maximum Catalytic Activities 23
3.12.1 Extraction Procedures and Assay Condition 23

3.12.2 Phosphoglucoisomerase (EC 5.3.1.9) 23
3.12.3 Phosphoglucomutase (EC 5.4.2.2) 24
3.12.4 Hexokinase (EC 2.7.1.1) 24
3.12.5 Fructokinase (EC 2.7.1.4) 24
3.12.6 UDP-glucose Pyrophosphorylase (EC 2.7.7.9) 24
3.12.7 Sucrose Synthase (EC 2.4.1.13) 24
3.12.8 Enolase (EC 4.2.1.11) 25
3.12.9 Triose Phosphate Isomerase (EC 5.3.1.1) 25
3.12.10 ... Phosphoglycerate Kinase (EC 2.7.2.3) 25
3.12.11 ... Phosphofructokinase (EC 2.7.1.11) 25
3.12.12 ... Pyruphosphate dependent Phosphofructokinase (EC 2.7.1.90) 25
3.12.13 ... Glyceraldehyde 3-Phosphate dehydrogenase (EC 1.2.1.12) 26
3.12.14 ... Pyruvate Kinase (2.7.1.40) 26
3.12.15 ... Phosphoenolpyruvate Phosphatase (3.1.3.60) 26
3.12.16 ... Fructose-1, 6-bisphosphatase (EC 3.1.3.11) 26
3.12.17 ... ADP-glucose Pyrophosphorylase (EC 2.2.7.27) 27
3.12.18 ... Acid Invertase (EC 3.2.1.26) 27
3.13 Determination of Soluble Sugars and Starch Content 27
3.14 Determination of Metabolic Intermediates 28
3.15 Analysis of fruit yield and flowers 29
3.15.1 Analysis of fruit weight 29
3.15.2 Analysis of fruit size 29
3.15.3 Fruit setting 29
3.15.4 Date of 50% flowering 29
3.16 Statistical Analysis of Data 29
4 Analysis of Carbohydrate Metabolism in Micro-Tom Fruits 30
4.1 Introduction 30
4.2 Aim of the work 30
4.3 Results 31
4.3.1 Development of fruit of tomato cultivar Micro-Tom 31
4.3.2 Starch and soluble sugars in developing fruits of Micro-Tom 31
4.3.3 Changes in activities in enzymes involved in conversion of sucrose to starch 33
4.3.4 Changes in activities in enzymes involved in glycolysis or the Calvin cycle .. 34
4.3.5 RNA blots of plastidial transporters 37

4.4 Discussion and conclusion 38
5 Analysis of the Function of Chloroplastic Fructose 1,6-bisphosphatase in Tomato Fruit42
5.1 Introduction 42
5.2 Aim of the work 42
5.3 Results 42
5.3.1 Recovery of Plants with Reduced FBPase Activity in the Pericarp of Tomato Fruit. 42
5.3.2 Starch and soluble sugar contents in the pericarp of the WT and transgenic lines 44
5.3.3 Changes in activities in enzymes involved in conversion of sucrose to starch 44
5.3.4 Concentration of Metabolic Intermediates in the pericarp of the WT control and transgenic lines 47
5.3.5 Analysis of fruit yield........ 47
5.3.6 Number of flower, fruit per plant, fruit set and number of days to 50% flowering. 51
5.4 Discussion and conclusion 51
6 Functional Analysis of ADP-glucose Pyrophosphorylase in Tomato Fruit........ 57
6.1 Introduction 57
6.2 Aim of the work 58
6.3 Results 58
6.3.1 Recovery of plants with reduced AGPase activity in the pericarp of tomato fruit 58
6.3.2 Starch and soluble sugar contents in the pericarp of the WT and transgenic lines 58
6.3.3 Changes in activities in enzymes involved in conversion of sucrose to starch 60
6.3.4 Concentration of metabolic intermediates in the pericarp of the WT control and transgenic lines 62
6.3.5 Analysis of fruit yield........ 62
6.3.6 Number of flowers, fruits per plant, fruit set and number of days to 50% flowering 65
6.4 Discussion and conclusion 66
7 Analysis of the Function of the GWD protein in Tomato Fruit........ 69
7.1 Introduction 69
7.2 Aim of the work 69

7.3 Results ... 70
7.3.1 Recovery of Tomato Plants with Repression of the GWD Protein ... 70
7.3.2 Starch and soluble sugar contents in the pericarp of the WT and transgenic lines ... 71
7.3.3 Starch and soluble sugar contents in the leaves of the WT and transgenic lines ... 72
7.3.4 Changes in activities in enzymes involved in conversion of sucrose to starch 73
7.3.5 Analysis of fruit yield ... 75
7.3.6 Number of flower, fruit per plant, fruit set and number of days to 50% flowering ... 77
7.4 Discussion and conclusions ... 79
8 Literature Cited ... 88
Acknowledgements ... 103

List of Figures

Figure 1: The role of Fru-2,6-P2 in feedforward control of sucrose synthesis. 5
Figure 2: The role of Fru-2,6-P2 in feedback control of sucrose synthesis. 6
Figure 3: The predominant route of sucrose unloading and subsequent mobilization.............. 9
Figure 4: Developmental series of tomato fruits from Micro-Tom cultivar. 31
Figure 5: Starch and soluble sugar contents in pericarp and placental tissues of tomato cultivar Micro-Tom during development. (A) Starch. (B) Sucrose. (C) Fructose. (D) Glucose. Data represent the mean of five independent measurements + SE........... 32
Figure 6: Activities of enzymes involved in the conversion of sucrose to starch in the pericarp and placental tissues of fruit of the tomato cultivar Micro-Tom. (A) SuSy. (B) Acid invertase. (C) UDPase. (D) PGM. (E) AGPase. Data represent the mean of five independent measurements + SE. 35
Figure 7: Activities of some glycolytic and clavin cycle enzymes in pericarp and placental tissues of fruit from the tomato cultivar Micro-Tom during its development.(A) HK. (B) FK. (C) FGI. (D) FBPase. (E) PPi-PFK. (F) PFK. (G) TPI. (H) G3P DH. (I) PGK. (J) Enolase. (K) PK. (L) PEP phosphatase. Data represent the mean of five independent measurements+ SE. 36
Figure 8: RNA blot analysis of some plastidial transporters throughout fruit development in the tomato cultivar Micro-Tom. TPT in (A) pericarp and (B) placental tissues. Glc-6-P transporter in (C) pericarp and (D) placental tissues. ATP/ADP transporter in (E) pericarp and (F) placental tissues. 37
Figure 9: Aerial parts of plants in both WT control and α-cp-FBP-transgenic lines after 8 weeks growth in the glasshouse. From left to right: untransformed WT control, α-cp-FBP#19, α-cp-FBP#33, α-cp-FBP#34 and α-cp-FBP#34. The α-cp-FBP plants are phenotypically identical to the untransformed WT control. 43
Figure 10: FBPase activity during developmental stage (A), Western blot analysis in green (25 DAF) (B) in the pericarp of WT and α-cp-FBP-transgenic lines [total soluble fruit protein (25µg) was subjected to SDS-PAGE on a 10% (w/v) gel] and FBPase activity in the leaves of WT control and α-cp-FBP-transgenic lines (C). Data represent the mean of five independent measurements + SE. 45

Figure 11: Starch and soluble sugar contents in pericarp of WT and α-FBP-transgenic lines in tomato cultivar Moneymaker during development. (A) Starch. (B) Glucose. (C) Fructose. (D) Sucrose. Data represent the mean of five independent measurements + SE. 46

Figure 12: Activities of enzymes involved in the conversion of sucrose to starch in pericarp of the WT control and αcp-FBP-transgenic lines of fruit of the tomato cultivar Moneymaker. (A) SuSy. (B) UGPase. (C) PGM. (D) AGPase. Data represent the mean of five independent measurements + SE. 48

Figure 13: Some 65 DAF old fruits from αcp-FBP-transgenic lines (bottom) in comparison with a control fruit (above).(A) Transgenic line #19. (B) Transgenic line #33 (C) Transgenic line #34... 50

Figure 14: Aerial parts of plants in both WT control and α-AGP-transgenic lines after 13 weeks growth in the glasshouse. From left to right: WT control, transgenic line #2, transgenic line #7, transgenic line #11 and transgenic line #11. The α-AGP plants are phenotypically identical to the untransformed WT control. 59

Figure 15: AGPase activity during developmental stage (A) and Western blot analysis in green (25 DAF) (B) in the pericarp of WT control and α-AGP-transgenic lines. Total soluble fruit protein (25µg) was subjected to SDS-PAGE on a 10% (w/v) gel. 60

Figure 16: Starch and soluble sugar contents in the pericarp of the WT control and α-AGP-transgenic lines.(A) Starch. (B) Glucose. (C) Fructose. (D) Sucrose. Data represent the mean of five independent measurements + SE in both WT control and transgenic line #7, but four independent measurements + SE in transgenic line #2 and transgenic line #11. 61

Figure 17: Activities of enzymes involved in the conversion of sucrose to starch in pericarp of the WT control and α-AGP transgenic lines of fruit of the tomato cultivar Moneymaker. (A) SuSy. (B) UGPase. (C) PGM. (D) FBPase. Data represent the mean of five independent measurements + SE in both WT control and transgenic line #7 and four independent measurements + SE in transgenic line #2 and transgenic line #11. 63

Figure 18: Some 65 DAF old fruits from α-AGP-transgenic line #7 (bottom) in comparison with the WT control fruit (above). 65

Figure 19: Aerial parts of plants in both WT control and α-GWD-transgenic lines after 8 weeks growth in the glasshouse. From left to right: WT control, transgenic line #16, transgenic line #17, transgenic line #20 and transgenic line #20. 70
Figure 20: Immunoblot analysis of the GWD protein in (A) leaves of untransformed WT control and three selected transgenic lines [Total soluble leaf protein (15µg) was subjected to SDS-PAGE on an 8% (w/v) gel], (B) in the pericarp of the WT control and transgenic lines (25 DAF) [Total soluble fruit (pericarp) protein (30µg) was subjected to SDS-PAGE on an 8% (w/v) gel] and (C) in the pericarp of the WT control fruits between 25-70 DAF [Total soluble fruit protein (20µg) was subjected to SDS-PAGE on an 8% (w/v) gel]. 71
Figure 21: Starch and soluble sugar contents in the pericarp of the WT control and α-GWD-transgenic lines in tomato cultivar Moneymaker during development. (A) Starch. (B) Glucose. (C) Fructose. (D) Sucrose. Data represent the mean of five independent measurements + SE in the WT control and transgenic line #17, but four independent measurements + SE in transgenic lines #16 and #20 74
Figure 22: Starch and soluble sugar contents in the leaves of the WT control and transgenic tomato lines lacking the GWD protein. (A) Starch. (B) Soluble sugars. Data represent the mean of five independent measurements + SE in the WT control and transgenic line #17, but four independent measurements + SE in transgenic lines #16 and #20.. 75
Figure 23: Activities of enzymes involved in the conversion of sucrose to starch in the pericarp of the WT control and α-GWD-transgenic lines of tomato cultivar Moneymaker.(A) SuSy. (B) UGPase. (C) PGM. (D) AGPase. (E) FBPase. Data represent the mean of five independent measurements + SE in WT control and transgenic line #17, but four independent measurements + SE in transgenic lines #16 and #20.. 76
Figure 24: Some 65 DAF old fruits from α-GWD-transgenic lines (bottom) in comparison with the WT control fruit (above). (A) Transgenic line #16. (B) Transgenic line #17. (C) Transgenic line #20. 78

List of Tables

Table 1: Metabolite concentrations in the pericarp of 30 DAF old WT control and αcp-FBP-transgenic fruits. 49
Table 2: Weights and sizes of ripe tomato fruits in the WT control and αcp-FBP-transgenic lines. 49
Table 3: Number of flowers, fruits, fruit set and number of days to 50% flowering in the WT control and αcp-FBP-transgenic lines. 51
Table 4: Metabolite concentrations in the pericarp of 30 DAF old WT control and α-AGP-transgenic lines. 64
Table 5: Weights and sizes of ripe tomato fruits in the WT control and α-AGP-transgenic lines. 64
Table 6: Number of flowers, fruits, fruit set and number of days to 50% flowering in the WT control and the transgenic lines. 65
Table 7: Weights and sizes of ripe tomato fruits in WT control and α-GWD-transgenic lines. 77
Table. 8: Number of flowers, fruit set and number of days to 50% flowering in the WT control and α-GWD-transgenic lines. 77

List of Abbreviations

1,3-BPGA	1,3-bisphosphoglyceric acid
2-PGA	2-phosphoglyceric acid
3-PGA	3-phosphoglyceric acid
ADP	adenosine diphosphate
ADPglc	ADP-glucose
AGPase	ADP glucose pyrophosphorylase
ATP	adenosine triphosphate
ATP-PFK	ATP-dependent phosphofructokinase
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CoA	coenzyme A
Conc.	concentration
cp-FBPase	chloroplastic fructose-1,6-bisphosphatase
cv	cultivar
cy-FBPase	cytosolic fructose-1,6-bisphosphatase
DAF	days after flowering
D-enzyme	disproportionating enzyme
DEPC	diethyl pyrocarbonate
DHAP	dihydroxyacetone phosphate
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	(ethylene-bis[oxyethylenenitrilo]tetaacetic acid
EMS	ethyl methanesulfonate
EST	expressed sequence tag
FBPase	fructose-1,6-bisphosphatase
FK	fructokinase
Fru-1,6-P_2	fructose-1,6-bisphosphate
Fru-1,6-P_2 aldolase	fructose-1,6-bisphosphatase aldolase
Fru-2,6-P_2	fructose-2,6-bisphosphate
Fru-6-P	fructose 6-phosphate

FW	fresh weight
G3P	glyceraldehyde 3-phosphate
G3P DH	glyceraldehyde 3-phosphate dehydrogenase
G6P DH	glucose 6-phosphate dehydrogenase
GBSS	granule-bound starch synthase
Glc-1-P	glucose 1-phosphate
Glc-6-P	glucose 6-phosphate
GWD	glucan water dikinase
Hepes	N-(2-hydroxyethyl)piperazine-N'-2-ethane sulfonic acid
hexose-P	hexose phosphate
HK	hexokinase
kDa	kilo Dalton
mRNA	messenger RNA
MW	molecular weight marker
NAD^+	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	oxidised nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
PEP phosphatase	phosphoenolpyruvate phosphatase
P-ester	phosphate ester
PFK	phosphofructokinase
PGA	phosphoglyceric acid
PGI	phosphoglucose isomerase
PGK	phosphoglycerate kinase
PGM	phosphoglucomutase
Pi	inorganic phosphate
PK	pyruvate kinase
PMSF	phenylmethylsulfonylfluoride
PPi	pyrophosphate
PPi-PFK	pyrophosphate dependent phosphofructokinase
PVP	polyvinyl pyrrolidone
RNA	ribonucleic acid

Ru 1,5-P_2	ribulose-1,5-bisphosphate
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulfate
SE	standard error
SPS	sucrose phosphate synthase
SSC	saline-sodium citrate
SuSy	sucrose synthase
TPI	triose phosphate isomerase
TPT	triose phosphate transporter
Triose-P	triose phosphate
Tris/HCl	tris(hydroxymethyl)aminomethane
UDP	uridine diphosphate
UDPglc	UDP glucose
UGPase	UDP glucose pyrophosphorylase
UTP	uridine triphosphate
v/v	volume per volume
w/v	weight per volume
WT	wild type

1 General Introduction

Tomato was one of the first plants to be genetically modified utilising recombinant DNA techniques. It might have been expected, therefore, that it would become a model system for the study of many aspects of plant biology. Although there have been some studies examining carbohydrate metabolism in tomatoes utilising molecular biological techniques (**Ohyama *et al*., 1995; Klann *et al*., 1996; Chengappa *et al*., 1999; D'Aoust *et al*., 1999; Nguyen-Quoc *et al*., 1999**), in recent years other species, such as *Arabidopsis* and potato, have been much more widely used. The reasons for this are that potato, for example, produces a large, commercially important storage organ, whilst *Arabidopsis* has a short life cycle and a fully sequenced genome. It has been demonstrated that the tomato fruit are an excellent model for the investigation of the regulation of sink activity and strength **(Ho, 1996)**.

Tomato is, however, interesting in its own right as it produces a fruit, which has a very different metabolism to either a leaf or a potato tuber. Leaves have the capacity to fix carbon through photosynthesis and, therefore, produce starch in the chloroplast directly. Potato tubers on the other hand rely on sucrose, which is exported from leaves via the phloem for a source of carbon. The sucrose has to be metabolised and transported over the amyloplast membrane before being converted to, among other things, starch. Tomato fruits initially contain chloroplasts that are photosynthetically active, but these differentiate to non-photosynthetic chromoplasts during the ripening process. They can, therefore, at least initially fix carbon, but they also receive carbon in the form of sucrose from the phloem. This raises several questions, not least about whether it is carbon fixed in the fruit or in the leaf that is most important for the growth and development of the fruit, and how this alters during fruit development.

Because tomato is a very close relative of potato it is normally possible to repress the activity of specific enzymes using cDNA's isolated from potato. Many cDNA's from potato have been isolated for genes involved in carbohydrate metabolism, which could act as a resource for studies in tomato. In addition, the recent isolation of many expressed sequence tags (EST) from tomato allows the possibility of using genetic engineering techniques to repress the activity of many enzymes in tomato. As tomato is a diploid species that can be crossed, it is also possible to combine the reduction in activities of multiple enzymes simultaneously, something that is difficult in potato.

The conversion of sucrose to starch has been relatively well studied in tomato fruits (**Robinson *et al*., 1988; Yelle *et al*., 1988; Schaffer and Petreikov, 1997a).** It has been

found that sucrose concentrations are lower than both glucose and fructose (**Damon *et al*., 1988; Klann *et al*., 1996; Schaffer and Petreikov, 1997a**), whilst the wild type (WT) tomato relative *Lycopersicon chemielewskii* accumulates higher levels of sucrose than the other soluble sugars (**Yelle *et al*., 1988**). The reason for this accumulation of sucrose in the wild relative is due to a reduction in the activity of acid invertase (**Klann *et al*., 1996**)

There are two aims for this work. The first one was to examine whether the Micro-Tom tomato cultivar was a suitable candidate to act as a model system for the study of carbohydrate metabolism in tomato fruit generally and the second to elucidate the role of three enzymes are thought to influence the accumulation of starch in early development stage of tomato fruits (cp-FBPase, AGPase and GWD protein) by antisense technique.

2 Review of literature

2.1 Carbon Metabolism in Photosynthetic Tissue

Life on our planet obtains its substance and energy through the process of photosynthesis- by which photosynthetic organisms use the electromagnetic energy of sunlight to synthesize carbohydrates and other cellular constituents from carbon dioxide and water. Photosynthesis can be broadly divided into two phases: a light phase in which the electromagnetic energy of sunlight is trapped and converted to ATP and NADH, and a synthetic phase in which the ATP and NADH thus generated are used in part for biosynthetic reduction of assimilated carbon dioxide **(Calvin-Benson cycle; for review see Leegood, 1996)**. The overall reactions of the Calvin-Benson cycle can be described as the fixation of three molecules of carbon dioxide into triose phosphate (triose-P) with the incorporation of one Pi derived from hydrolysis of ATP. Light functions to regulate not only the source of reductant but also the synthetic and carbon reductive phases of photosynthesis and related biochemical processes of chloroplasts.

In most plants the major products of photosynthesis are starch (formed in the chloroplasts) and sucrose (formed in the cytosol). Both of these products are synthesized from photosynthetically generated dihydroxyaceton phosphate (DHAP). In the first case, DHAP is converted into hexose phosphates (hexose-P) by the concerted action of aldolase and chloroplastic fructose-1,6-bisphosphatase (FBPase), these hexose-P are in turn converted to starch following the reactions of chloroplastic isoforms of phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM) and those of ADP-glucose pyrophosphorylase (AGPase) and the starch polymerising enzymes starch synthase and starch branching enzyme. In sucrose synthesis DHAP, or a derivative thereof is transported to the cytosol where it is converted firstly to fructose 6-phosphate (Fru-6-P) through operation of cytosolic isoforms of aldolase and FBPase and then to sucrose via the route defined below. When the rate of photosynthesis exceeds the rate of sucrose export from the source tissue, sucrose initially accumulates in the vacuole, where it has little effect on the rate of triose-P export from the chloroplast. At saturation of the vacuolar sucrose capacity sucrose synthesis is inhibited and instead photosynthate is converted to starch, which is transiently stored in the chloroplast. The relative rates of sucrose and starch production in photosynthetically active tissues are maintained by tight and complex regulation patterns known as feedforward and feedback control mechanisms.

2.1.1 Feedforward control of photosynthesis

At the start of the photoperiod the rate of photosynthesis increases. This results in an increased cytosolic DHAP concentration due to a greater rate of export from the chloroplast via the triose-P translocator in exchange for Pi **(Heldt and Flügge, 1987),** and therefore the cytosolic 3-PGA/Pi ratio rises **(Gerhardt *et al.,* 1987; Neuhause and Stitt, 1989; Stitt *et al.,* 1984b,c)** (Fig. 1). These changes bring about an increase in the cytosolic concentration of the substrate of the cytosolic FBPase, Fru-1,6-P_2, which is nearly in equilibrium with triose-P. Simultaneous with the increase in the levels of Fru-1,6-P_2 is a rapid drop in the concentration of Fru-2,6-P_2 (a potent inhibitor of cytosolic FBPase) which relieves inhibition of the enzyme and thus increases flux through the reaction it catalyes. A consequence of the increased flux through FBPase is an increase in the cytosolic concentration of glucose 6-phosphate (Glc-6-P). This leads to an increased Glc-6-P/Pi ratio and causes potent allosteric activation of, one of the routes of sucrose synthesis that catalysed by, sucrose phosphate synthase (SPS) **(Huber and Huber, 1992)** resulting in an increased rate of sucrose synthesis. Furthermore the elevated Pi in the plasted results in an inhibition of the reaction catalysed by AGPase **(Preiss, 1988)** and thus restricts the partitioning of photoassimilate towards starch.

2.1.2 Feedback control of photosynthesis

During the day, the rate of sucrose synthesis increases with the rate of photosynthesis. If the rate of sucrose production exceeds its rate of export from the cell, sucrose will accumulate. However, in response to feedback signals, probably related to the absolute level of sucrose, the rate of synthesis is decreased via inhibition of SPS **(Stitt, 1990)** (Fig. 2). This inhibition leads to increased cytosolic levels of hexose-P, which result in a large increase in the Fru-2,6-P_2 level leading to inhibition of cytosolic FBPase. The inhibition of cytosolic FBPase results in increased cytosolic levels of triose-P, which prevent export of chloroplastic triose-P. Consequently, more carbon is retained in the chloroplast and enters the pathway of starch synthesis. Studies on mutants of *Clakia xantiana*, which have reduced levels of cytosolic PGI, support this theory **(Neuhause *et al.,* 1989).** These plants have a higher Fru-2,6-P_2 concentration than wild type (due to an increase in Fru-6-P concentration) and all of the above effects on metabolite concentrations were observed **(Krukeberg *et al.,* 1989; Neuhause *et al.,* 1989).** The reduced rate of sucrose synthesis additionally prevents Pi cycling, which has consequently been shown to result in an accumulation of 3-PGA in isolated chloroplasts **(Heldt *et al.,* 1977).** This is probably due to the fact that phosphoglycerate kinase (PGK) is particularly sensitive to the falling concentrations of ATP that occur during these conditions.

The elevated chloroplastic 3-PGA/Pi ratio stimulates starch synthesis by the allosteric activation of AGPase **(Preiss, 1988).**

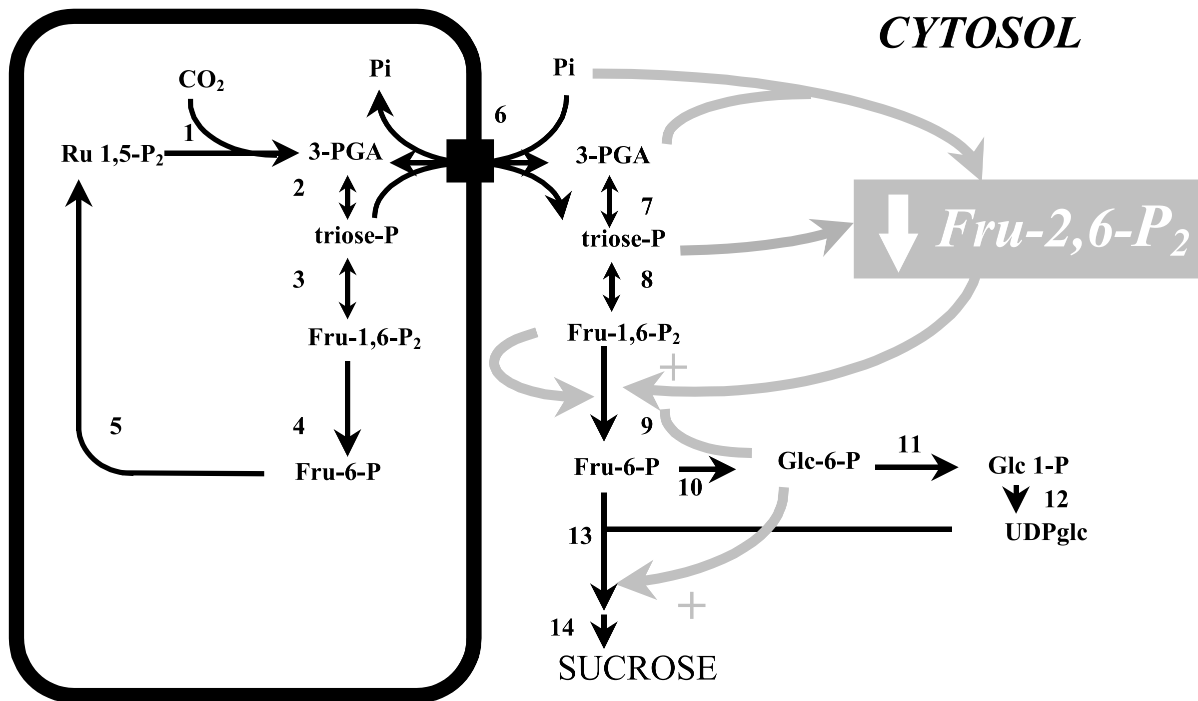


Figure 1: The role of Fru-2,6-P2 in feedforward control of sucrose synthesis.

+ represents allosteric activation. Reactions shown are catalysed by the following enzymes (note in some instances multiple reactions are represented by a single arrow): 1, Rubisco; 2, chloroplastic PGK and chloroplastic TPI; 3, chloroplastic Fru-1-6-P2 aldolase; 4, chloroplastic FBPase; 5, transketolase, sedoheptolase-1,7-bisphosphatase aldolase, sedoheptolase-1,7-bisphosphatase, phosphopentoepimerase, phosphoriboisomerase and phosphoribulokinase; 6, triose phosphate transporter; 7, cytoslic PGK and cytosolic TPI; 8, cytosolic Fru-1-6-P2 aldolase; 9, cytosolic FBPase; 10, cytosolic PGI ; 11, cytosolic PGM , 12, UGPase, 13, SPS, 14, sucrose phosphatase.

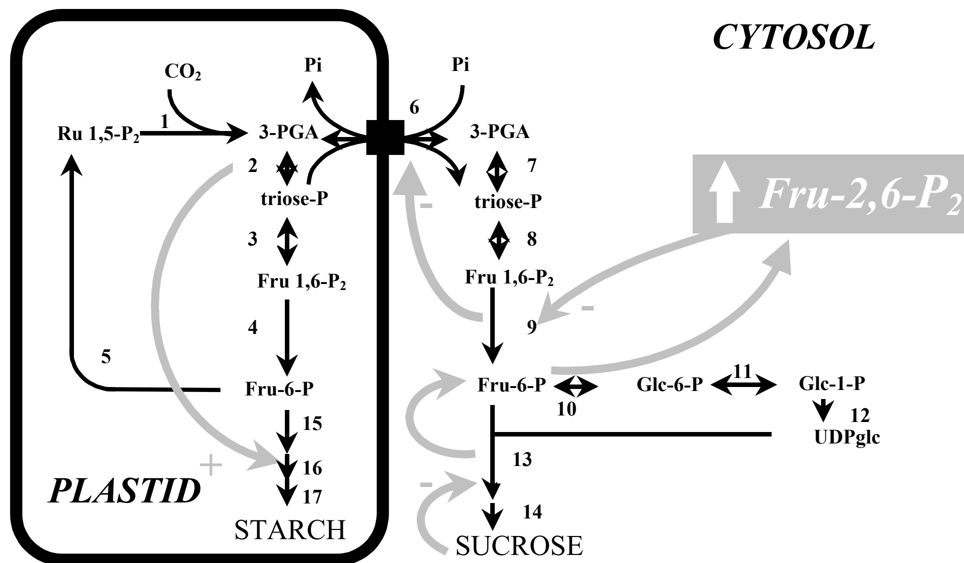


Figure 2: The role of Fru-2,6-P2 in feedback control of sucrose synthesis.

+ represents allosteric activation, - represents allosteric inhibition. Reactions shown are catalysed by the following enzymes (note in some instances multiple reactions are represented by a single arrow): 1, Rubisco; 2, chloroplastic PGK and chloroplastic TPI; 3, chloroplastic Fru-1-6-P2 aldolase; 4, chloroplastic FBPase; 5, transketolase; sedoheptolase-1,7-bisphosphatase aldolase; sedoheptolase-1,7-bisphosphatase; phosphopentoepimerase, phosphoriboisomerase and phosphoribulokinase; 6, triose phosphate transporter; 7, cytoslic PGK and cytosolic TPI 8, cytosolic Fru-1-6-P2 aldolase; 9, cytosolic FBPase; 10, cytosolic PGI; 11, cytosolic PGM; 12, UGPase; 13, SPS; 14, sucrose phosphatase; 15, choroplastic PGI and chloroplstic PGM; 16, AGPase; 17, starch synthase and branching enzyme.

In summary, the rate of carbon export from the chloroplast and therefore ultimately the rate of sucrose synthesis depends on a balance between feedforward mechanisms that decrease Fru-2,6-P_2 (and activate SPS) and feedback mechanisms that increase Fru-2,6-P_2 (and inhibit SPS). Similarly the accumulation of starch is a function of the relative activities of the enzymes which synthesize and degrade it. In leaves, starch is accumulated during the day and is nocturnally degraded to provide carbohydrate required for various anabolic reactions **(Beck and Zieger, 1989; Trethewey and Smith, 2000).** The metabolism of transitory starch is dynamic and regulation of this process results in alternating periods of net synthesis and degradation **(Stitt and Heldt, 1981).** Degradation of transitory starch is initiated by α-amylases at the surface of starch granule **(Beck and Zieger, 1989; Trethewey and Smith, 2000),** and involves the co-operative attack of phosphorolytic and hydrolytic activities **(Steup**

et al., 1983). The final starch degradation products glucose or triose-P are exported into the cytosol **(Trethewey and ap Rees, 1994a,b)** where they are metabolised to sucrose.

2.2 Carbohydrate Allocation from photosynthetic "source" to heterotrophic "sink Tissues"

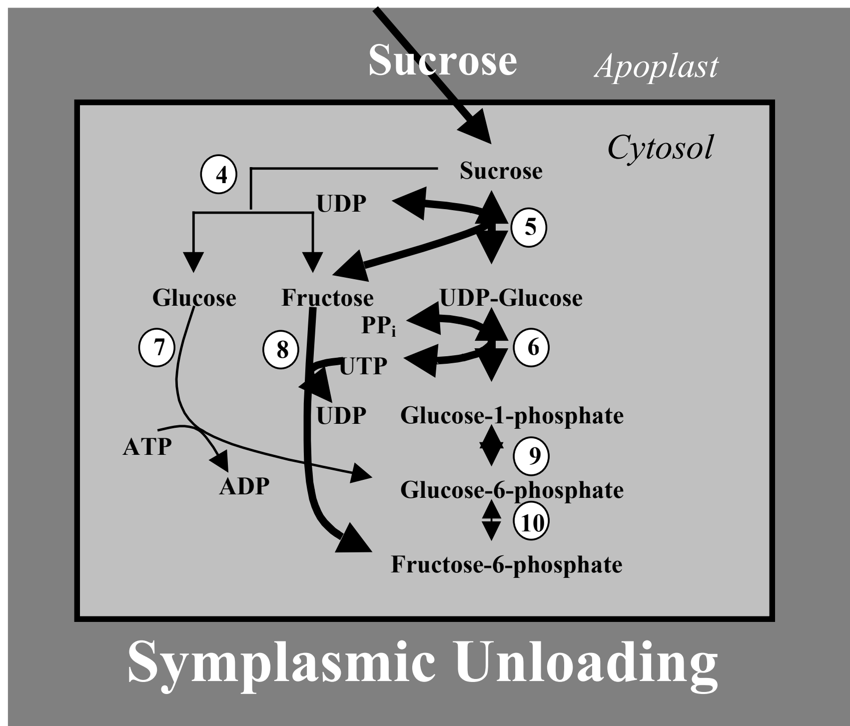
In photosynthetic tissues sucrose is predominantly exported from cells, most probably by facilitated diffusion and subsequently taken up by the phloem complex by a specific sucrose/H+ co transport mechanism **(Riesmeier *et al.,* 1994; Frommer and Sonnewald, 1995).** Once in the phloem complex sucrose is transported to cells in heterotrophic "sink" tissues. At least two distinct classes of sink tissues can be differentiated: (i) "utilisation sinks", highly metabolically active, rapidly growing tissues like meristems and immature leaves, and (ii) "storage sinks", such as tubers, seeds, roots or fruits which deposit imported carbohydrates as storage compounds (e.g. starch, sucrose, lipid or protein) **(Sonnewald and Willmitzer, 1992)**. However, the route of carbon transport to these and the mechanisms by which the different types of sink obtain carbon in the form of sucrose is the same so they will be considered together for the purposes of this report. Sucrose obtained through translocation, by sink tissues, can enter a cell directly via the symplasm (see Fig., 3A) or the apoplasm (whereby it is transported by specific sucrose or, following cleavage to its component hexoses, monosaccharide transporters (see Fig., 3B). In many plants the nature of the predominantly used route of sucrose unloading is heatedly debated. Several studies using asymmetrically labelled sucrose suggest that carbon obtained by heterotrophic cells moves primarily through the symplastic route and is not cleaved to glucose and fructose during transport. It seems likely that cells of many species receive most of their sucrose by such as route **(Patrick, 1990; Tegeder *et al.,* 1999; Lalonde *et al.,* 1999).** However, in certain tissues it is clear that sucrose must be supplied through the apoplasm. This is certainly the case in developing seeds in which protoplasmic concentrations between maternal and embryonic tissue simply do not exist. In potato tuber recent studies using a combination of confocal microscopy, autoradiography and biochemical analyses have provided definitive evidence that unloading in the potato tuber is predominantly apoplastic during stolon elongation and becomes primarily symplastic during initial phases of tuberisation **(Viola *et al.,* 2001).** This is in direct contrast to the situation observed in the developing tomato fruit in which sucrose unloading is predominantly symplastic during early, starch accumulating, stages of development **(Damon *et al.,* 1988; Ruan and Patrick, 1995)** and apoplastic during later, hexose accumulating stages **(Patrick, 1990; Ruan and Patrick, 1995).** The amount of

sucrose unloaded into tomato fruits differs with the age **(Walker and Ho, 1977)** and developmental stage of fruit. Being high during early periods of high growth and maintained albeit it at a much reduced level during the later phases of slow growth **(Walker and Ho, 1977).** It has been suggested that sucrose unloading may be controlled, at least in part, by the activity of the sugar transporters which may in turn be influenced by the activity of the enzymes of sucrose cleavage within the sink tissues **(D'Aoust *et al.,* 1999; N'tchobo, 1998).** Definitive proof in support of this suggestion is however still lacking and it is important to note that although the transport mechanism of the much studied potato sucrose transporter SUT1 has been characterised by expression in Xenopus oocytes **(Boorer *et al.,* 1996).** Its precise role in planta has yet to be fully elucidated. Since this is one of the best characterized transporters it therefore follows that much work is required before the factors controlling the intracellular movement of sugars can be fully resolved.

2.3 Mobilisation of Sucrose in sink tissues

Sucrose delivered to the sink tissue can be cleaved in one of three ways (i) in the apoplast, as described above, by the action of an acid invertase or in the cytosol by either (ii) alkaline invertase or (iii) sucrose synthase (SuSy). As indicated in Fig., 3A and B the primary route of sucrose cleavage mirrors the mechanism of unloading with invertase activities being high in during the early stages of tuber inititation whilst SuSy predominates in the developing tuber **(Appeldoorn *et al.,* 1999)**, whereas the opposite is true for the developing tomato fruit **(Damon *et al.,* 1988; Robinson *et al.,* 1988; DemnitzKing *et al.,* 1997).** The products of sucrose cleavage enter into metabolism by the concerted action of fructokinase (FK) and UDP-glucose pyrophosphorylase (UGPase) **(Zrenner *et al.,* 1993)** or FK and hexokinase (HK) **(Smith *et al.,* 1993; Veramendi *et al.,* 1999)** in the case of the SuSy and invertase pathways, respectively. Hexose phosphates produced by these pathways are then equilibrated by the action of cytosolic isoforms of PGI and PGM. Hexose phosphates are then partitioned between starch synthesis within the amyloplast and glycolytic pathway of the cytosol (and plastid).

A



B

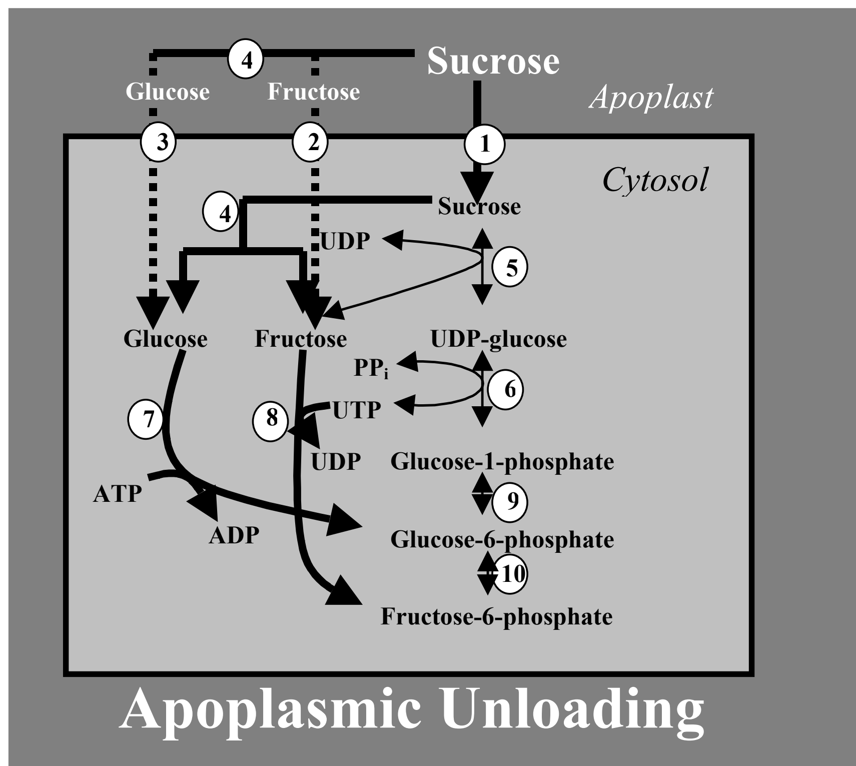


Figure 3: The predominant route of sucrose unloading and subsequent mobilization.

(A) Symplasmic unloading. (B) Apoplasmic unloading. The numbers denote the following enzymes: 1, Sucrose transporter; 2 and 3, Hexose transporter(s); 4, Invertase; 5, SuSy; 6, UGPase; 7, HK; 8, FK; 9, PGM and 10, PGI. The thickness of the arrow indicates the predominant flux.

2.4 Uptake of carbon into amyloplasts

The form in which carbon crosses the amyloplast membrane and enters into starch biosynthesis has been the subject of considerable debate. Categorical evidence that carbon enters the amyloplasts of a wide range of species, including the Solanaceous species tobacco and potato, in the form of hexose monophosphates (or nucleosides) rather than triose phosphates was provided by determination of the degree of randomisation of radiolabel in glucose units isolated from starch following incubation of various tissues with glucose labelled at the C1 or C6 position (**Keeling *et al*., 1988; Viola *et al*., 1991; Hatzfeld and Stitt, 1990; Fernie *et al*., 2001**). These data are in agreement with the observation that many heterotrophic tissues lack plastidial FBPase activity (**Entwistle and ap Rees, 1990**) and the failure to find expression of plastidial FBPase in potato tubers (**Kossmann *et al*., 1992**).

Although it is clear that triose phosphates are not the substrate taken up by amyloplasts to support starch synthesis there has been considerable debate as to whether Glc-1-P (**Naeem *et al*., 1997; Tetlow *et al*., 1994; Tyson and ap Rees, 1988**) or Glc-6-P (**Schott *et al*., 1995; Wischmann *et al*., 1999**) is the preffered substrate for uptake. Recently, particularly in cereals, the uptake of cytosolically produced ADP-glucose has also been much discussed (**Pozeuta-Romero *et al*., 1991a,b; ap Rees, 1995**). The results of many recent transgenic and immunolocalisation experiments have indicated that the substrate for uptake is most probably species specific. Clear evidence for the predominant route of carbon uptake in the tuber being in the form of both transgenic experiments (**Tauberger *et al*., 2000**) and the recent cloning of a Glc-6-P transporter (**Kammerer *et al*., 1998**). Whilst a wealth of experimental evidence indicates that in barley, wheat, oat and possibly maize the predominant form of uptake is as ADP-glucose (**Denyer *et al*, 1996; Thorbjornsen *et al*., 1996b; Shannon *et al*., 1998**). In tomato the form in which carbon crosses the amyloplast membrane is contentious. Studies comparing the ratio of ADP-glucose to UDP-glucose (**Beckles *et al*., 2001a**) and comparing the activity of AGPase that is confined to the plastid with that of other enzymes known to be confined to the plastid (**Beckles *et al*., 2001b**) suggest the absence of a cytosolic AGPase in this species. However, these are in contradiction to earlier immunolocalisation studies using antisera raised against AGPase that suggested the presence of an extra-plastidiary isoform of the enzyme in tomato fruit (**Chen *et al*., 1998**).

2.5 The synthesis of starch

Following uptake of carbon into the amyloplast, starch synthesis proceeds variously via (i) plastial PGM and plastidial AGPase, (ii) only via plastidial ADP-glucose or (iii) via no intermediate steps prior to the polymerising reactions of starch synthases and branching enzymes (**Smith *et al*., 1997**). The involvement of plastidial enzymes upstream of starch synthase being determined by the route of carbon import (see Fig., 2). The first reaction of heterotrophic plastidial starch metabolism within both the potato tuber (**Tauberger *et al*., 2000**), the pea embryo (**Hill and Smith, 1991**) and most probably the tomato fruit also is the interconversion of Glc-6-P and Glc-1-P catalysed by plastidial PGM. Compelling evidence for the involvement of this enzyme in pea starch synthesis was provided by studies on the rug3 mutant which revealed that this locus encodes a plastidial PGM and that mutation at this locus results in a severe depletion of starch levels in pea embryos (**Harrisson *et al*., 1998**). The next reaction on the path to starch synthesis, that catalysed by plastidial AGPase has received much attention for a number of years. This reaction is often considered to be the first committed step of starch synthesis it utilizes ATP and produces pyrophosphate (PPi), which is then hydrolysed by a specific pyrophosphatase to yield 2Pi. The hydrolysis of PPi serves to remove the AGPase reaction away from equilibrium. As discussed above, in many species including pea embryos, soybean cell suspension cultures and cauliflower buds AGPase appears to be located exclusively in the plastid (**Macdonald and ap Rees, 1983; Journet and Douce, 1985; Smith, 1988**) and this isoform thus plays an importance role in mediating the flux of carbon to starch. In keeping with this statement the removal or severe reduction of the AGPase activity in *Arabidopsis* or potato resulted in a dramatic reduction in the starch level in all tissues (**Lin *et al*., 1988a,b; Müller-Röber *et al*., 1992**).

Plant AGPases are multisubunit proteins and expression studies in which the potato tuber enzyme was expressed in *E.coli* revealed that maximal activity can only be achieved on expression of both the large and small subunit (**Iglesias *et al*., 1993**). Moreover they are allosterically regulated, being activated by 3-PGA and inhibited by Pi (**Preiss, 1988**), and there is clear evidence that changes in these metabolites are involved in the regulation of starch synthesis within leaves allowing the co-ordination of carbon assimilation, sucrose synthesis and starch synthesis (**Stitt, 1997**). The AGPase from potato tuber resembles that found in leaves with respect to its kinetic properties (**Sowokinos and Preiss, 1982; Ballicora *et al*., 1995**). There is also increasing evidence of a strong correlation between the 3-PGA and ADP-glucose levels and the rate of starch synthesis within potato tubers under a wide range of conditions (**Geigenberger *et al*., 1997; 1998**). Whilst there have been few direct studies of

the allosteric properties of AGPase from tomato it is likely that these will be similar to those found in potato.

Whilst the involvement of the above enzymes in starch biosynthesis are strictly species dependent, the starch polymerising activities are ever present and responsible for the formation of the two different macromolecular forms of starch, amylose and amylopectin. Starch synthases catalyse the transfer of the glucosyl moiety from ADP-glucose to the non-linear end of an α-1,4 glucan. The various starch synthases are able to extend 1,4-glucans in both amylose and amylopectin. At least four different classes of starch synthases exist, designated as GBSS (granule-bound starch synthase), SSI, SSII and SSIII which vary greatly in molecular weight, need for primers, substrate affinities and antigenic properties. It seems likely that most plant species contain the four different classes of starch synthase, however, the extent to which they contribute *in vivo* probably differs considerably between species (**Denyer *et al.*, 2001**). Starch branching enzymes are responsible for the formation of α-1,6 branch points within amylopectin. Although there are more than two isoforms present in most plant species, all isoforms can be separated into two classes – most simply designated as A and B forms (**Burton *et al.*, 1995**). The precise mechanism by which this is achieved is unknown, however it is thought to involve cleavage of a linear α-1,4 linked glucose chain and reattatchment of the chain to form an α-1,6 linkage (**Kossmann and Lloyd, 2000**). The combined action of starch synthases and branching enzymes play an important role in determining the structure of starch which will be described in detail below. Other enzymes of starch synthesis and degradation are less well understood. Disproportionating enzyme (D-enzyme) is able to synthesise α-1,4-glucans from maltose and has been suggested to be a candidate as a source of the malto-oligosaccharide primers required for starch synthesis. However several lines of evidence suggest this is unlikely to play a major role in starch synthesis *in vivo*. The maltose present in plant tissues is almost exclusively derived from starch (**Kossmann and Lloyd, 2000**) and transgenic plants exhibiting reduced D-enzyme expression had no effect on starch content (**Takaha *et al.*, 1998**). Furthermore, recent studies on an *Arabidopsis* mutant deficient in D-enzyme reveal a minor decrease in starch under certain conditions, however, they indicate that this enzyme primarily plays a role in the removal of malto-oligosaccharides during starch degradation (**Critchley *et al.*, 2001**).
Recent studies of 14-3-3 proteins within starch granules of *Arabidopsis* chloroplasts (**Sehnke *et al.*, 2001**) and of an AGPase from barley leaves (**Rodriguez-Lopez *et al.*, 2000**) indicate that enzymes other than those classically considered to constitute the starch synthetic pathway

may also contribute to this process. However, there is no evidence as yet for a physiological role for either of these proteins within plant systems.

2.6 Starch degradation

Starch is synthesised as a store for carbon. In leaves it is manufactured and degraded over a 24 hour period, being synthesised during the light period and degraded in the dark period; in storage organs, however, it can be stored for years, or even decades, prior to its mobilisation. Many enzymes have been isolated which can degrade starch yet, despite this, it is only recently becoming apparent which isoforms are actually important in this process. This is because many of the enzymes which can degrade starch (β-amylase, starch phosphorylase, α-amylase) are present as multiple isoforms, some of which are present within the plastid, others being extra-plastidial.

Recently two papers have been published showing unequivocally that two different enzymes are involved in mobilising starch in leaves. The first was mentioned above as being an *Arabidopsis* mutant affected in D-enzyme activity (**Critchley *et al.*, 2001**). The second was an isoform of β-amylase in potato that was repressed using an antisense construct (**Scheidig *et al.*, 2002**). Leaves from both of these plants did not degrade as much starch during the dark period as the controls, demonstrating a block in starch degradation.

In order to try and identify enzymes involved in starch degradation there have been several screens of mutant *Arabidopsis* populations to identify plants that do not degrade starch upon being shaded. Many mutants have been isolate and have been named *sex* mutants as they show a **s**tarch **ex**cess phenotype (**Caspar *et al.*, 1991**). Two of these showing such a phenotype (*sex1* and *sex4*) have been characterised in more detail. The *sex1* mutant will be discussed below in a section on starch phosphorylation. The mutation in *sex4* has not been identified, but the mutant plant has been shown to be deficient in a plastidial isoform of α-amylase (**Zeeman *et al.*, 1998**). It is known, however, that the mutation does not lie in the gene coding for this α-amylase as that is situated on chromosome 1, while the mutation lies on chromosome 4 (Dr. Samuel Zeeman, University of Berne, personnel communication). More work needs to be performed, therefore, to identify the genetic lesion.

2.7 Starch phosphorylation

Phosphate residues have often been associated with starch granules. The nature of these residues is, however, dependant on the species. Starch from potato tuber, for example, contains large amounts of phosphate that is covalently bound either to the C3 or C6 positions

of glucose residues. That from cereal endosperm contains almost no covalently bound phosphate, but large quantities of phospholipids which are associated with the granule. In this section I will concentrate solely on the covalently bound phosphate.

For many years it was speculated that covalently bound phosphate becomes incorporated into starch either through the action of starch phosphorylase, or AGPase (**Kossman and Lloyd, 2000; Lloyd *et al*., 1999**). The reason for this was that it no enzyme had been isolated which could phosphorylate starch directly. Recently however this has been accomplished. The cDNA coding for this protein was isolated using an early proteomic approach. It is known that many enzymes bind to starch granules, and that many of these are involved in starch metabolism. It was hypothesised that if one could identify the cDNA's coding for previously unidentified proteins which bind to starch granules they may well also be involved in starch metabolism. To achieve this, starch-granule-bound proteins were isolated, antibodies raised against them and these antibodies were used to screen a potato cDNA library. One of the clones isolated coded for a protein which is approximately 160 kDa in size. This protein was repressed in potato using an antisense construct, and two phenotypes were noted. The first was that the amount of covalently bound phosphate in the starch was greatly reduced, and that the plants were also inhibited in starch degradation in both leaves and tubers (**Lorberth *et al*., 1998**). It was not demonstrated for several years, however, that the protein could actually phosphorylase glucans, and therefore at the time it was called R1. To show that the R1 protein was indeed responsible for phosphorylating starch it was purified to homogeneity. It was then incubated with starch and various potential phosphate donor molecules. The amount of phosphorylation was measured after incubation and it was shown that the protein could indeed phosphorylate starch, and that it required ATP to do so. It was further shown that the mechanism of phosphorylation was a dikinase rather than a kinase. This means that the γ-phosphate of ATP is released as inorganic phosphate, while the β-phosphate is the one transferred to the glucan (**Ritte *et al*., 2002**). The protein is, therefore, a **g**lucan **w**ater **d**ikinase and has been renamed as GWD (**Ritte *et al*., 2003**).

Other evidence has demonstrated that the GWD protein has a similar effect in other species. This comes from the identification of the starch accumulating *sex1* mutant as being mutated in an *Arabidopsis* homolog of the GWD protein (**Yu *et al*., 2001**). It was also found that the phosphate content of the starch in the mutant *Arabidopsis* leaves was greatly reduced. This raises the question, however, as to why starch phosphorylation appears to affect starch degradation also. There is no clear answer to this. It may be that enzymes which degrade starch need covalently bound phosphate residues to act, or that they interact with the GWD

protein in some way during the degradation process. The possibility of answering these questions in the future are, however, now greater because of the identification of the function of the GWD protein

2.8 Glycolysis

Hexose phosphates are not only precursor (and products) of starch synthesis (and degradation) but also important substrates for glycolysis. Respiratory carbon metabolism which is an essential provider of both the energy and the required precursors to support biosyntheisis in the heterotrophic cell couples the partial oxidation of glucose to pyruvate during gylcolysis to the complete oxidation of pyruvate to carbon dioxide during operation of the Krebs cycle. In plants, oxidation of carbohydrate via glycolysis provides the majority of substrate for the operation of the Krebs cycle – with the oxidative pentose phosphate pathway, protein and lipid only making minor contributions to respiration (**ap Rees, 1980; Holtman *et al.*, 1994**). The glycolytic chain of reactions is often split into two parts (**for a review see Hopkins, 1995**), the first part comprising of the set of reactions by which substrates of glycolysis are converted to the common intermediate Fru-6-P and the second part comprising of steps following on from the conversion of Fru-6-P to Fru-1,6-P_2 by the action of either ATP- or PPi-dependent PFK. The interconversion of Fru-6-P to Fru-1,6-P_2 is often said to be the first committed step of glycolysis. In plants this step is very tightly regulated and is complicated by the presence of three enzymes involved in its interconversion the ATP-PFK which catalyse the production of Fru-1,6-P_2 and FBPase and the PPi-PFK (which is freely reversible) which catalyse the production of Fru-6-P. Moreover, the interconversion of Fru-6-P to Fru-1,6-P_2 in the cytosol is strongly influenced by the concentration of the signal metabolite Fru-2,6-P_2 which potently inhibits the FBPase and activates PPi-PFK (**Stitt, 1990**). Following the phosphorylation of Fru-6-P, the resultant bisphosphate is cleaved to form the triose-P DHAP and G3P by the action of aldolase. The triose-P are readily equilibrated by triose phosphate isomerase (TPI), whilst G3P subsequently converted to 1,3-BPGA, 3-PGA, 2-PGA, PEP and pyruvate via the actions of glyceraldehyde 3phosphate dehydrogenase (G3P DH), (PGK), phosphoglycerate mutase, enolase and pyruvate kinase (PK) respectively.

PEP can alternatively be brought into the Krebs cycle by a different route in which it is carboxylated by the action of PEP carboxylase yielding oxaloacetate which is subsequently reduced to malate by the action of malate dehydrogenase which is then taken up into the mitochondrion. That said the major link between glycolysis and the Krebs cycle is provided by the uptake of pyruvate and its subsequent decarboxylation and oxidation and finally

condensation of the resultant acetyl group with CoA to form acetyl CoA is all carried out by the large multienzyme complex known as pyruvate dehydrogenase (**Bryce and Thornton, 1996; Hopkins, 1995**). Acetyl CoA production by the pyruvate dehydrogenase complex thus fuels the operation of the Krebs cycle which in conjuncture with the respiratory electron transport chain provides for the majority of the energy requirements of the heterotrophic cell in addition to providing carbon skeletons for the biosynthesis of a wide range of primary and secondary metabolites and being an important source of reductant for the cell.

2.9 Fruit metabolism

Whilst the preceding chapters have largely considered organs as either photosynthetic or heterotrophic the situation in fruits is somewhat more complex. The tomato fruit is no exception to this generalisation with numerous studies investigating source sink interactions between leaves and fruits and the effect of crop yields of manipulating leaf photosynthetic activity by altering photon flux density, temperature, carbon dioxide concentration, nutrient and water supplies (**for a review see Ho and Hewitt, 1986**). However, there are many parts of the tomato plant other than the leaves that contain chlorophyll and capture light energy. Yet the photosynthetic contribution of these tissues to the maintenance and growth of the plant have received scant attention. The work described in this thesis is intended to investigate the impact of altering the activities of three enzymes associated with starch metabolism on the development and metabolism in the fruit. Whilst the enzymes involved in photosynthetic and heterotrophic starch synthesis are well known it is clear that their regulation and precise metabolic function within the fruit is not fully understood. The application of antisense technology approaches targeted at AGPase, FBPase and GWD and biochemical analysis of fruits, taken along a developmental axis, from the resultant transformant lines may well allow a better understanding both of the regulation of this important storage pathwaye in the fruit and of the relative importance of photosynthesis during early stages of fruit development.

3 Material and Methods

3.1 Chemicals

General chemicals were obtained from Boehringer Mannheim (Mannheim), Sigma Chemical company (St.Louis, Missouri, USA) or Merck (Darmstadt).

Bactotrypton (= Select Peptone 140), Select Yeast Extract, Meat Peptone, and Select Agar were obtained from GibcoBRL Life Technologies GmbH (Paisley, Scotland, UK). Antibiotics were purchased from Sigma Chemical company (St. Louis, Missouri, USA) or Boehringer Mannheim (Mannheim) except for Betabactyl® (Smithkline Beecham Pharma, Munich).

Restriction enzymes and buffers were obtained either from Boehringer Mannheim (Mannheim) or from New England Biolabs (Beverly, Massachusetts, USA), Ready-to-GoTM T4-DNA-Ligase was brought from Pharmacia Biotech (Freiburg).

[α-32P]dCTP (110 TBq mmol-1) were purchased from Amersham Buchler (Braunschweig, Germany)

The starch determination kit (UV method; Cat. No. 207 748) and, except where noted otherwise, all biochemical enzyme purchased from Boehringer Mannheim (Mannheim).

Adenine and uridine nucleotides, NAD^+, NADH, $NADP^+$, fructose 6-phosphate, glucose 6-phosphate, glucose 1-phosphate, phosphoenolpyruvate and 3-phosphoglycerate were obtained from Boehringer Mannheim (Mannheim). All other substrates were purchased from Sigma Chemical company (St. Louis, Missouri, USA).

Rainbow TM coloured protein molecular weight marker (14 300-220 000 Da) was purchased from Amersham Buchler (Braunschweig), all other chemical for PAGE and protein determination were obtained from BioRad (Richmond, California, USA).

The peptide antibody recognising the GWD protein was kindly provided by Dr. James Lloyd Plant Research Department, Risø National Laboratory, DK-4000 Roskilde, Denmark.

3.2 Vectors and Bacterial Strains

3.2.1 Vectors

pBluescript II SK+/-	Stratagene, La Jolla, CA, USA.
pBluescript II KS+/-	Stratagene, La Jolla, CA, USA.
pBinAR	**(Höfgen and Willmitzer, 1990).**

3.2.2 Bacterial

Strains *Escherichia coli*

Xl-1 blue *Startagene*, La Jolla, CA, USA **(Bullock *et al.*, 1987)**.

DH5α *Gibco BRL*, Gaithersburg, USA **(Raleigh *et al.*, 1989)**.

Strains *Agrobacterium tumefaciens*

GV2260 **(Deblaere *et al.*, 1985).**

GV3101 **(Koncz und Schell, 1986)**.

pBluescript Plasmids which include fragment cDNA's encoding for FBPase, AGPase and GWD were kindly provided by Dr. Jens Koßmann, Max-Planck Institute of Molecular Plant Physiology, Golm.

Aqueous plasmid stocks were kept at -20 °C prior to use. Bacterial glycerol stocks were generated as described **Sambrook *et al.*, 1989** and stored at -80°C.

3.3 Transformation and Cultivation of Bacteria

Competent *E.coli* XL1 Blue cells were prepared and transformed by heat-shock as described by **Hanahan (1983)**. The cells were grown at 37°C on YT-medium plus appropriate selective antibiotic as described by **Sambrook *et al.*, (1989)**.

Competent *Agrobacterium Tumifaciens* cells were prepared according to **Höfgen and Willmitzer (1990)** and transformed by electroporation according to **Miller *et al.*, (1988)**. The cells were grown at 28°C on YEP-medium plus appropriate selective antibiotic according to **Vervliet *et al.*, (1975)**.

3.4 DNA manipulations

DNA manipulations were performed essentially as described by **Sambrook *et al.*, 1989**. For construction of the cp-FBPase antisense gene, cDNA from potato (**Kossmann *et al.*, 1992**) was digested with the restriction enzymes *ASP718* and *BamH1* resulting in two DNA fragments, one of approximately 800bp ant the other of 400bp. The 800bp fragment was isolated from an agarose gel using a commercially available kit (Qiagen), and was ligated in antisense orientation with respect to the patatin B33 promoter (**Rocha-Sosa *et al.*, 1989**) in the *ASP718/BamH1* sites of the plant transformation vector pBinARB33 producing the vector pBinB33cp-FBPase.

For construction of the AGPase antisense gene, cDNA from potato (**Müller-Röber *et al.*, 1990**) was digested with the restriction enzymes *Eco*R1 and *Sma*I resulting one DNA

fragment approximately 1.6kp. The 1.6kp fragment was isolated from an agarose gel using a commercially available kit (Qiagen), and was ligated in antisense orientation with respect to the CaMV 35S promoter in the *Eco*R/*Sma*I sites of the plant transformation vector pBinAR35S (**Höfgen and Willmitzer, 1990**), producing the vector pBin35S AGPase.
For construction of the GWD antisense gene, cDNA from potato (**Kossmann *et al*., 1991**) was digested with the restriction enzymes *ASP718* and *BamH1* resulting one DNA fragment, approximately 1.9kp. The 1.9kp fragment was isolated from an agarose gel using a commercially available kit (Qiagen), and was ligated in antisense orientation with respect to the CaMV 35S promoter in the *ASP718/BamH1* sites of the plant transformation vector pBinAR35S (**Höfgen and Willmitzer, 1990**), producing the vector pBin35S GWD.

3.5 Cloning

Preparation and restriction of plasmids, cloning, and gel electrophoresis were performed according to **Sambrook *et al*., (1989)**. Ligations were performed using the Ready-to-GoTM T4-DNA-Ligase system (Pharmacia Biotech; Freiburg) according to the manufacturer's protocol. DNA fragment were eluted from the gel and purified using Microcon Columns (Amicon Inc.; Bevery, Massachusetts, USA) according to the manufacturer's protocol.

3.6 Plant Material

Wild-type (WT) Micro-tomato (*Lycopersicon esculentum* cv. Micro-Tom) seeds were a kind gift of Dr. Avraham Levy (The Weizmann Institute of Science, Rehovot, Israel), whilst seeds of wild-type Moneymaker (*Lycopersicon esculentum* L. cv. Moneymaker) were kindly provided by Dr. Jens Koßmann, Max-Planck Institute of Molecular Plant Physiology, Golm. Seeds were sown individually in small pot (5cm diameter) in the case of Micro-Tom and in a big pot (10 cm diameter) in the case of Moneymaker in growth chamber. After two weeks the plants were transported into a glasshouse and grown illumination (16h light: 8h dark regime (approximately 250µmol photons m^{-2} sec^{-1}) at 22°C temperature (20-24°C) with a relative humidity of 60-70%. Individual flowers were tagged at anthesis to accurately follow fruit ages through development, with only ten fruits per plant being allowed to develop.

3.7 Sampling of fruits

Micro-Tom and Moneymaker fruits were harvested at five days intervals between 20-60 DAF in the case of Micro-Tom and 25-70 DAF in the case of Moneymaker, which covered the transition from green to fully ripe red fruit. Harvested fruits were cut in two parts with a

scalpel blade and the pericarp was separated from the placental tissue. In the case of Micro-Tom the placenta was then further separated from the developing seeds and jelly, and the both pericarp and placental tissues were frozen separately in liquid nitrogen, but in the case of Moneymaker only the pericarp was immediately frozen in liqued nitrogen. All samples were kept at –80°C until use.

3.8 Transformation and Cultivation of tomato

Transformation of three antisense construct (αcp-FBPase, α-AGPase anḍ α-GWD protein) has been carried out using (*Lycopersicon esculentum* L. cv. Moneymaker) instead of (*Lycopercicon esculentum* cv. Micro-tom) which can not be used for transformation of these antisense construct.

Transformation of tomato (*Lycopersicon esculentum* cv. Moneymaker) plants was achieved by *Agrobacterium Tumifaciens* mediated gene transfer following the method of **Rocha-Sosa *et al*., (1989)**. The selection of transgenic plants was performed on medium containing Kanamycin **(Dietze *et al*., 1995)**.

3.9 Selection of plants with reduced cp-FBPase AGPase and GWD protein

Plants were maintained in tissue culture on MS-Medium **(Murashing and Skoog, 1962)** containing 2% (w/v) sucrose, 0.8% (w/v) Select Agar and 125µg/ml Ticarcillin Disodium/Potassium Clavulanate (Duchefa) (Timentin) under the following conditions: 22°C, 56-70% relative humidity, 3000 Lux, and a 16h light, 8h dark regime. Regenerates were screened for expression of the transgene by determining enzyme activity in the case of α-cp-FBPase and, by determining enzyme activity as well as starch content in the case of α-AGPase and by determining western blot analysis in the case of α-GWD protein.

3.9.1 Selection of plants with reduced cp-FBPase activity

In order to select plants with reduced cp-FBPase activity, 25 days old green fruits were harvested from sixty independent transgenic lines growing in soil. Soluble proteins were extracted from the pericarp of all lines and FBPase activity was determined in all of them. Three lines (#19, #33 and #34) showed significant reduction in total FBPase activity and were chosen for further study. Seeds from these plants were sterilized and germinated on MS media (**Murashige and Skoog, 1962**) containing 50mg l^{-1} kanamycin. Seeds that were able to grow on this media were presumed to contain the transgene and were planted in soil for further

analysis. In those fruits showing reduced FBPase activity the amount of the cp-FBPase was examined using western blot analysis.

3.9.2 Selection of plants with reduced AGPase activity

In order to select plants with reduced AGPase activity, 25 days old green fruits were harvested from forty independent transgenic lines growing in soil. Soluble proteins were extracted from the pericarp of all lines and AGPase activity was determined in all of them. Three lines (#2, #7 and #11) showed reductions in AGPase activity as well as in starch content and were chosen for further study. Seeds from these plants were sterilized and germinated on MS media (**Murashige and Skoog, 1962**) containing 50mg l^{-1} kanamycin. Only seeds of transgenic lines (#7) were able to grow on this media, while seeds both lines (#2 and #11) were not able to grow in this media, therefore, both seeds from WT control and all of transgenic lines were sown directly in soil for further analysis.

3.9.3 Selection of plants with reduced GWD protein levels

In order to select plants with reduced GWD expression, plant leaves were kept for 72 hour in darkness. Leaf blades in different stages of development were collected, and de-stained in 80% (v/v) ethanol at 80°C. After chlorophyll was removed, the leaf blades were stained in lugol's solution for the absence or presence of starch. All plants with lowered levels of GWD expression displayed a starch excess phenotype in leaves. Out of 30 independent transgenic plants screened only three lines (#16, #17 and #20) displayed a starch excess phenotype in leaves. In these lines the level of GWD expression was examined using western blot analysis. Seeds from all of these transgenic lines were sterilized and germinated on MS media **(Murashing and Skoog, 1962)** containing 50mg l^{-1} kanamycin. Seeds that were able to grow on this media were presumed to contain the transgene and positive transformants were planted in soil for further analysis.

3.10 Western Blot Analysis

Soluble proteins from the pericarp of 25 DAF old tomato fruits and leaves were denatured in buffer containing SDS **Laemmli (1970)**. 25µg of soluble were separated by SDS-PAGE on either a 8% gel in the case of GWD protein or 10% gel in the case of FBPase and AGPase. The proteins were blotted at 4°C according to **Khyse-Andersen (1984)** on a nitrocellulose membrane (BA 85, 0.45µm; Schleicher und Schüll, Dassel) using a semi-dry electroblotting apparatus (MultiphorII, LKB, Bromma, Sweden). The blots were developed with rabbit serum

followed by alkaline phosphatase-conjugated goat anti-rabbit serum (Amersham) according to **Bhattacharyya *et al*. (1990)**.

3.11 RNA (Northern) Blot Analysis

Total RNA was isolated from frozen developing tomato fruits by a modification of the method of **Hughes and Galau (1988)**. Plant tissue (5 to 10g) was ground to a fine powder in liquid nitrogen and sprinkled into 55 ml of ice-cold buffer (200mM Tris-HCL, pH 8.5, 300mM LiCl, 10mM EDTA, 1,5% (w/v) lithium dodecylsulfate, 1% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P-40, 5% (w/v) insoluble PVP, 90mM β-mercaptoethanol, 10mM DTT, 0,5% (v/v) DEPC) and stirred to ensure immediate contact with the buffer. After stirring for 5 to 10 min, 46 ml of 3M ammonium acetate was added, and the extract was spun at 2500 **g** for 10 min at 4°C. RNA was precipitated from the supernatant with one-tenth volume of isopropanol and was centrifuged at 2500 **g** for 10 min at 4°C. The pellet was resuspended in 5 to 10 ml of H_2O and purified by phenolchloroform extraction; this process was repeated until the preparation appeared clean. RNA was precipitated with ¼ volume of 10 M LiCl on ice for 2 to 12 hours, and then centrifuged at 2500 **g** for 10 min at 4°C. The RNA pellet was re-suspended in DEPC-water.

40 µg RNA was denatured in 40% (v/v) formamide, separated on a 1.5 % (w/v) agarose gel containing formaldehyde **(Lehrach *et a*l., 1977)** and blotted onto nylon membrane (porablot NY plus, *Macherey-Nagel*, Düren, Germany), by means of capillary transfer using 20*SSC as the buffer (1*SSC is 0.15M NaCl, 0.015 M sodium citrate). The RNA was fixed to the membranes using an UV-crosslinker (*Stratagen*e, La Jolla, USA). Hybridization of membranes was performed with ^{32}P-labelled probes in 0.25M sodium phosphate (pH 7,2), 1mM EDTA, 1% (w/v) BSA and 7% (w/v) SDS. The filters were washed twice with 0.1 % SSC and 0.5% (w/v) SDS for 15 min at 68°C. The filters were subjected to autoradiography between intensifying screens at –80°C.

cDNA clones coding for plastidial transporters were either tomato EST's (TPT, EST No.cLEM23J19; ADP/ATP transporter, EST No.cLEM8I17) purchased from the Clemson University Genomics Institute (Clemson, South Carolina, USA) or in the case of Glc-6-P transporter, a potato clone which was the gift of Dr. Andreas Weber (Michigan State University, East lansing, Michigan, U.S.A). The plasmids were cut with sutable restriction enzymes and fragments isolated from a gel using the QIAquick kit (Qiagen) according to the manufacturers instructions. Radioactively labelled probes were made by the random primed

method using a commercially available kit (Roche) according to the manufacturer instructions.

3.12 Determination of enzyme Maximum Catalytic Activities

3.12.1 Extraction Procedures and Assay Condition

Plant material was ground under liquid nitrogen to a fine powder in a pestle and mortar. Twice as much extraction buffer (50mM Hepes-KOH (pH 7.4), 5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 10%(v/v) Glycerol, 0.1% (v/v) Triton X-100, 5mM DDT, 2mM ε-Amino-caproic acid, 2mM Benzamidine, and 0.5mM PMSF according to **Trethewey *et al.* (1998)**, was added as weight of sample and the buffer and powder were mixed together. The samples were centrifuged at 2800 g and 4°C for 15 min and the supernatant was recovered. This was de-salted using NAP-5 columns (Pharmacia) and the resulting plant extract was either assayed for enzyme activity immediately in the case of AGPase and FBPase, or frozen in aliquots in liquid nitrogen before being stored at –80°C until use. Total protein content was determined by the method of **Bradford (1976)**

Extracts were kept at 4°C prior to assaying. If not noted otherwise, enzyme assays were carried out at 25°C in a final reaction volume of 300µl according to the accompanying references. The change in absorbance was continuously followed at 340nm using an Anthose ht II microtiter-plate reader (Anthos Labtec Instruments, Hanau).

Activities of Sucrose Phosphate Synthase and acidic Invertase were determined in stopped assays.

All coupling enzymes provided as ammonium sulfate suspension were desalted by centrifuging for 1 min, the supernatant being discarded and the sediment dissolved in the corresponding reaction buffer.

3.12.2 Phosphoglucoisomerase (EC 5.3.1.9)

Phosphoglucoisomerase was assayed in the direction of glucose-6-phosphate as described by **Burrell *et al.*, (1994)**. The assay consisted of 5µl de-salted extract in 75mM glycylglycine (pH 8.5), 10mM $MgCl_2$, 0.5mM NAD^+, 0.5U/ml glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by the addition of fructose 6-phosphate to a final concentration of 1mM.

3.12.3 Phosphoglucomutase (EC 5.4.2.2)

Phosphoglucomutase was assayed in the direction of glucose-6-phosphate formation **(Takamiya and Fukui, 1978)**. The assay consisted of 5µl de-salted extract in 50mM Hepes-KOH (pH 7.8), 5mM $MgCl_2$, 2mM NAD^+, 100µM glucose-1,6-bisphosphate, 0.5U/ml glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by the addition of glucose 1-phosphate to a final concentration of 2mM.

3.12.4 Hexokinase (EC 2.7.1.1)

Hexokinase was assayed in the direction of glucose-6-phosphate production as described by **Veramendi *et al.*, (1999)**. The assay consisted of 5µl de-salted extract in 50mM Tris/HCl (pH 8.0), 4mM $MgCl_2$, 0.33mM NAD^+, 2mM ATP, 2.5U/ml glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by the addition of glucose to a final concentration of 1mM.

3.12.5 Fructokinase (EC 2.7.1.4)

Fructokinase was assayed in the direction of glucose 6-phosphate as described by **Renz *et al.*, (1993)**. The assay contained 10µl de-salted extract in 50mM Tris/HCl (pH 8.0), 4mM $MgCl_2$, 0.33mM NAD^+, 2.5mM UTP, 2.5U/ml glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) and 1.75 U/ml phosphoglucoisomerase (Yeast). The reaction was started by the addition of fructose to a final concentration of 1mM.

3.12.6 UDP-glucose Pyrophosphorylase (EC 2.7.7.9)

UDP-glucose pyrophosphorylase was assayed in the direction of glucose 1-phosphate formation **(Zrenner *et al.*, 1993)**. The reaction mixture contained 10µl de-salted extract in 100mM Tris/HCl (pH 8.0), 2mM $MgCl_2$, 0.25mM NAD^+, 2mM UDP-glucose, 20µM glucose-1,6-bisphosphate, 2.5U/ml glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) and 3U/ml phosphoglucomutase (rabbit muscle). The reaction was started by the addition of tetrasodium pyrophosphate to a final concentration of 2mM.

3.12.7 Sucrose Synthase (EC 2.4.1.13)

Sucrose synthase was assayed in the direction of sucrose production **(Sweetlove *et al.*, 1996)**. The assay consisted of 5µl de-salted extract in 100mM Hepes-NaOH (pH 7.5), 4mM $MgCl_2$, 0.2mM NADH, 40mM UDP-glucose, 1mM phosphoenolpyruvate, 10U/ml Pyruvate kinase

and 2U/ml lactate dehydrogenase. The reaction was started by the addition of fructose to a final concentration of 10mM.

3.12.8 Enolase (EC 4.2.1.11)

Enolase was assayed in the direction of phospho*enol*pyruvate production as described by **Burrell *et al*., (1994)**. The assay consisted of 10µl de-salted extract in 100mM Hepes-NaOH (pH 7.5), 10mM $MgCl_2$, 0.2mM NADH, 2.7mM ADP, 5U/ml Pyruvate kinase and 6U/ml lactate dehydrogenase. The reaction was started by the addition of 2-phosphoglycerate to a final concentration of 0.5mM.

3.12.9 Triose Phosphate Isomerase (EC 5.3.1.1)

Triose phosphate isomerase was assayed in the direction of dihydroacetone phosphate formation **(Burrell *et al*., 1994)**. The assay consisted of 10µl de-salted extract in 100mM Hepes-NaOH (pH 8.0), 0.2mM NADH, 5mM EDTA, 1U/ml glycerol 3-phosphate dehydrogenase. The reaction was started by the addition of glyceraldehydes 3-phosphate to a final concentration of 1.5mM.

3.12.10 Phosphoglycerate Kinase (EC 2.7.2.3)

Phosphoglycerate kinase was assayed in the direction of formation 1,3-bisphosphoglycerate according to **Burrell *et al*., (1994)**. The assay consisted of 5µl de-salted extract in 100mM Hepes-NaOH (pH 7.6), 2mM $MgSO_4$, 0.3mM NADH, 1mM EDTA, 6.5mM glycerate 3-phosphate, 3.32U/ml glycerate 3-phosphate dehydrogenase. The reaction was started by the addition of ATP to a final concentration of 1mM.

3.12.11 Phosphofructokinase (EC 2.7.1.11)

Phosphofructokinase was assayed by the production of fructose-1,6-bisphosphate (**Burrell *et al*.,1994)**. The assay consisted of 25µl de-salted extract in 100mM Tris/HCl (pH 8.0), 5mM $MgCl_2$, 0.1mM NADH, 5mM fructose 6-phosphate, 1U/ml aldolase, 1.36U/ml glycerol 3-phosphate dehydrogenase and 2.6U/ml triose phosphate isomerase. The reaction was started by the addition of ATP to a final concentration of 1mM.

3.12.12 Pyruphosphate dependent Phosphofructokinase (EC 2.7.1.90)

Pyruphosphate dependent Phosphofructokinase activity was measured in the glycolytic direction by following the production of fructose-1,6-bisphosphate **(Scott *et al*., 1995).** The

assay consisted of 10µl of de-salted extract in 75mM Hepes-NaOH (pH 7.5), 2mM Mg-acetate, 0.15mM NADH, 10µM fructose-2,6-bisphosphate, 7.5mM fructose 6-phosphate 1U/ml aldolase, 1.36U/ml glycerol 3-phosphate dehydrogenase (rabbit muscle) and 2.6U/ml triose phosphate isomerase (rabbit muscle). The reaction was started by the addition of tetrasodium pyrophosphate to a final concentration of 0.25mM.

3.12.13 Glyceraldehyde 3-Phosphate dehydrogenase (EC 1.2.1.12)

Glyceraldehyde-3-phosphate dehydrogenase was assayed in the direction of glyceraldehydes 3-phosphate production as described by **Plaxton (1990)**. The assay consisted of 5µl de-salted extract in 100mM Hepes-NaOH (pH 8.0), 8mM $MgSO_4$, 0.3mM NADH, 1mM EDTA, 2mM DTT, 6mM 3-phosphoglycerate and 4U/ml phosphoglycerate kinase. The reaction was started by the addition of ATP to a final concentration of 2mM.

3.12.14 Pyruvate Kinase (2.7.1.40)

Pyruvate kinase was assayed in the direction of pyruvate formation **(Burrell *et al.*, 1994)**. The assay consisted of 10µl de-salted extract in 50mM MOPS (pH 7.0), 15mM $MgCl_2$, 0.15mM NADH, 100mM KCl, 5mM phosphoenolpyruvate and 6U/ml lactate dehydrogenase. The reaction was started by the addition of ADP to a final concentration of 5 mM.

3.12.15 Phosphoenolpyruvate Phosphatase (3.1.3.60)

Phosphoenolpyruvate phosphatase was assayed in the direction of pyruvate formation **(Duff *et al.*, 1989a)**. The assay used was the same as that for pyruvate kinase except ADP was omitted. The reaction was started by the addition of phosphoenolpyruvate to a final concentration of 5mM.

3.12.16 Fructose-1, 6-bisphosphatase (EC 3.1.3.11)

Fructose-1,6-bisphosphatase was assayed in the direction of fructose-6-phosphate production according to **Kruger and Beevers (1984)**. The assay consisted of 40µl de-salted extract in 20mM Hepes-NaOH (pH 7.0), 5mM $MgCl_2$, 0.5mM NAD^+, 1U/ml phosphoglucoisomerase and 1U/ml glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by the addition of fructose-1,6-bisphosphate to a final concentration of 0.5mM.

3.12.17 ADP-glucose Pyrophosphorylase (EC 2.2.7.27)

ADP-glucose pyrophosphorylase was assayed in the direction of glucose-1-phosphate formation as described by **Müller-Röber *et al*., (1992)**. The assay consisted of 30µl de-salted extract in 80mM Hepes-NaOH (pH 7.4), 10mM $MgCl_2$, 0.02%(w/v) fatty-acid free BSA, 0.6mM NAD^+, 10µM glucose-1,6-bisphosphate, 10mM 3-phosphoglycerate, 3mM DTT, 1mM ADP-glucose 2.5U/ml phosphoglucomutase (rabbit muscle) and 1U/ml glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by the addition of tetrasodium pyrophosphate to a final concentration of 2mM.

3.12.18 Acid Invertase (EC 3.2.1.26)

Acid Invertase was assayed in the direction of sucrose degradation to produce glucose and fructose as described by **Zrenner *et al*., (1996)**. The reaction mixture consisted of 20mM acetate buffer (pH 4.7), 100mM sucrose and 30µl of desalted enzyme extract in total volume of 100µl. The reaction mixture was incubated at 25°C for 60 min. After 1h, 25µl of 1M Tris/Hcl (pH 8.0) was added to the solution to be neutralized. The reaction mixture was stopped by heating at 95°C for 3 min. Control sample was prepared by heating the reaction mixture for 3 min in the presence of 25µl of 1M Tris/Hcl (pH 8.0) without period of incubation. For both control and assay samples glucose and fructose was measured directly in 25µl of the reaction mixture by the method of **Stitt *et al*., (1989)** as described below.

3.13 Determination of Soluble Sugars and Starch Content

Starch and soluble sugars glucose, fructose and sucrose were extracted as described by **Trethewey *et al.*, (1998)** and determined photometrcally. The change in absorbance was continuously followed at 340nm using an Anthos hat II microtiter-plate reader (Anthos Labtec Instrument, Hanau).

Soluble sugars were determined modified from **Stitt *et al*., (1989)**. The reaction mixture consisted of 5µl ethanolic extract and 250µl of 100mM imidazol, 5mM $MgCl_2$, 2mM $NADP^+$, 1mM ATP and 2U/ml glucose 6-phosphate dehydrogenase (yeast). To start the reaction**,** 5µl of the respective enzymes were sequentially added: for glucose 1U/ml hexokinase (yeast overproducer), for fructose 0.5U/ml phosphoglucoisomerase (yeast), for sucrose a 1:5 dilution of saturated solution of Invertase (β-fructosidase from yeast).

Starch content was measured according to **Trethewey *et al*., (1998)** using a commercially available starch determination kit (Boehringer Mannheim, Mannheim). The assay is based on

the enzymatic hydrolysis of starch by α-amyloglucosidase and the determination of glucose in a coupled assay with hexokinase and glucose 6-phosphate dehydrogenase.

3.14 Determination of Metabolic Intermediates

Trichloroacetic acid extracts of pericarp material for the determination of metabolic intermediates were prepared as described by **Trethewey *et al.*, (1998**). The intermediates were determined photometrically in a final volume of 700µl according to **Lytovchenko *et al.*, (2002)** using a Dual-wavelength spectrophotometer (ZWSII; Sigma, Berlin). Extraction procedure and assays were evaluated according to **(Fernie *et al.*, 2001)**.

Pyruvate and phosphoenolpyruvate were sequentially determined in 50mM Hepes-KOH (pH: 7.4), 5mM $MgCl_2$, 50µM NADH and 1mM ATP. The reaction for pyruvate was started by addition of 0.6U lactate dehydrogenase (hog muscle), for phosphoenolpyruvate by addition of 2U pyruvate kinase (rabbit muscle).

Glucose 6-phosphate, glucose 1-phosphate, and fructose 6-phosphate, were determined in 50mM Hepes-KOH (pH: 7.4), 5mM $MgCl_2$ and 250µM $NADP^+$. The reactions were sequentially started by addition of 0.2U glucose 6-phosphate dehydrogenase (yeast), 0.4U phosphoglucomutase (rabbit muscle) and 0.4U phosphoglucoisomerase (yeast).

3-phosphoglycerate was assayed in 50mM Hepes-KOH (pH: 7.4), 5mM $MgCl_2$, 50µM NADH, 1.5mM ATP and 5U/ml 3-phosphoglycerate kinase (yeast). The reaction was initiated by addition of 5U glyceraldehydes 3-phosphate dehydrogenase (rabbit muscle).

Inorganic phosphate was measured after extraction of the metabolite fraction in 700µl 3.5% perchloric acid as described by **Sharkey and Vanderveer (1989**). The extracts were neutralized to pH 6 to 7 by adding a solution of 2N KOH, 150mM Hepes (to help stabilize the pH), and 10mM KCl (to help the precipitation of $KClO_4$). The phosphate assay was the malachite green enhanced-molybdate assay. An assay solution of 2g l^{-1} malachite green (Sigma M9636) and 10mM ammonium molybdate in 0.8 M HCl was made up at least two days prior to assay. This solution was filtered through Whatman No. 1 filter paper. Plant (10-50 µl) was added to 800µl of molybdate reagent. After 1 min, 100 µl 1M trisodium citrate was added to the assay. After 1 further min, 100µl of 1% Extran 1000 detergent was added to the assay. The optical density at 650 nm was read after 30 min and compared with standards made with dried KH_2PO_4.

3.15 Analysis of fruit yield and flowers

3.15.1 Analysis of fruit weight

This trait was measured directly after harvesting when fruits were fully ripe (after 65 DAF). The weight of fruits was carried out on a balance.

3.15.2 Analysis of fruit size

This trait was also measured directly after harvesting when fruits were fully ripe (65 DAF). The size of fruits was carried out by a diameter.

3.15.3 Fruit setting

This trait was calculated by using the following formula:

Fruit setting% = No.of fruits that set/No.of flowers that anthesized *100

3.15.4 Date of 50% flowering

This trait was recorded as a number of days from date of planting to date of flowing 50% of plants.

3.16 Statistical Analysis of Data

t-tests were performed using the algorithm included into Microsoft Excel 2000. The expression ‘significant’ is used only when an alteration has been confirmed to be statistically significant (($P \leq 0,05$) and ($P \leq 0,01$) with the Student’s *t*-Test.

4 Analysis of Carbohydrate Metabolism in Micro-Tom Fruits

4.1 Introduction

The *Arabidopsis* model system has been contributed much to the remarkable advances in plant molecular biology during the last decade. The major reasons for the success of *Arabidopsis* are its small size, short life cycle, small genome (**Leutwiler *et al*., 1984**) and easy of transformation (**Bechtold *et al*., 1993**) These features facilitate the genetic dissection of any trait through the screening of large populations saturated in mutants for the various genes involved in the trait. Nevertheless, despite the considerable advantages of *Arabidopsis*, the knowledge acquired in this species cannot always be applied to other plant species. Having a silique type of fruit makes *Arabidopsis* a good model for species of the Brassicaceae but not for those with a fleshy fruit.

Tomato (*Lycopersicon esculentum*) offers a good model for other crop species whose fruit is also a fleshy berry. It is one of the most important crops in the fresh vegetable market as well as in the food processing industry (**Rick and Yoder, 1988; Hille *et al*., 1989**). It is well characterised genetically; it has a relatively small diploid genome (n=12) and is readily transformable (**McCormick *et al*., 1986**). One disadvantage of tomato is that the plants have a large size and relatively long live cycle.

A new cultivar (Micro-Tom) has, however, recently been developed that overcomes these problems (**Meissner *et al*., 1997**). The plants of this variety grow to a similar size as *Arabidopsis* and have a considerably shorter life cycle than other tomato varieties, routinely producing seed within twelve weeks of being planted.

4.2 Aim of the work

We wish to study tomato fruit carbohydrate metabolism in the Micro-Tom cultivar using genetic engineering techniques. As an initial study we decided to examine the activity of enzymes in untransformed fruit during development to see whether this new variety is equivalent to other varieties that have been studied previously, and as such to ascertain if it represent a useful model for fruit carbohydrate metabolism.

4.3 *Results*

4.3.1 Development of fruit of tomato cultivar Micro-Tom

The development of fruit of the Micro-Tom cultivar are shown in Fig. 4. The fruits were small and green at 20 DAF and remained green until 45-50 DAF when they reached the breaker stage. By 60 DAF they were fully ripe.

Figure 4: Developmental series of tomato fruits from Micro-Tom cultivar.

DAF = Days after Flowering.

4.3.2 Starch and soluble sugars in developing fruits of Micro-Tom

Starch and soluble sugar contents were determined in both the pericarp and placental tissues between 20-60 DAF Starch accumulated transiently in the fruits of the Micro-Tom variety in both the pericarp and placental tissues (Fig. 5A). In both these tissues the starch content was approximately 45µmol hexose (g FW)$^{-1}$ at 20 DAF. In the pericarp the decrease was quickly to under 10µmol hexose (g FW)$^{-1}$ at 30 DAF, before decreasing more slowly to barely detectable amounts at 45 DAF. In the placental tissue the decrease was slower and more linear, reaching barely detectable amounts at 60 DAF. There were no significant differences in starch concentrations between the pericarp and placental tissues between 25 and 35 DAF. After that the concentrations were significantly decreased in the pericarp in comparison with the placenta.

Sucrose levels remained relatively constant in the different tissues throughout fruit development, although they were generally greater in the placental tissue in comparison with the pericarp (Fig. 5B). There were no significant differences in sucrose concentrations between the pericarp and placenta, except for three time points. At 20, 25 and 30 DAF in the placental tissue there were significantly increased sucrose in comparison with the pericarp. In both tissues the concentration stayed below 7µmol hexose (g FW)$^{-1}$ throughout development.

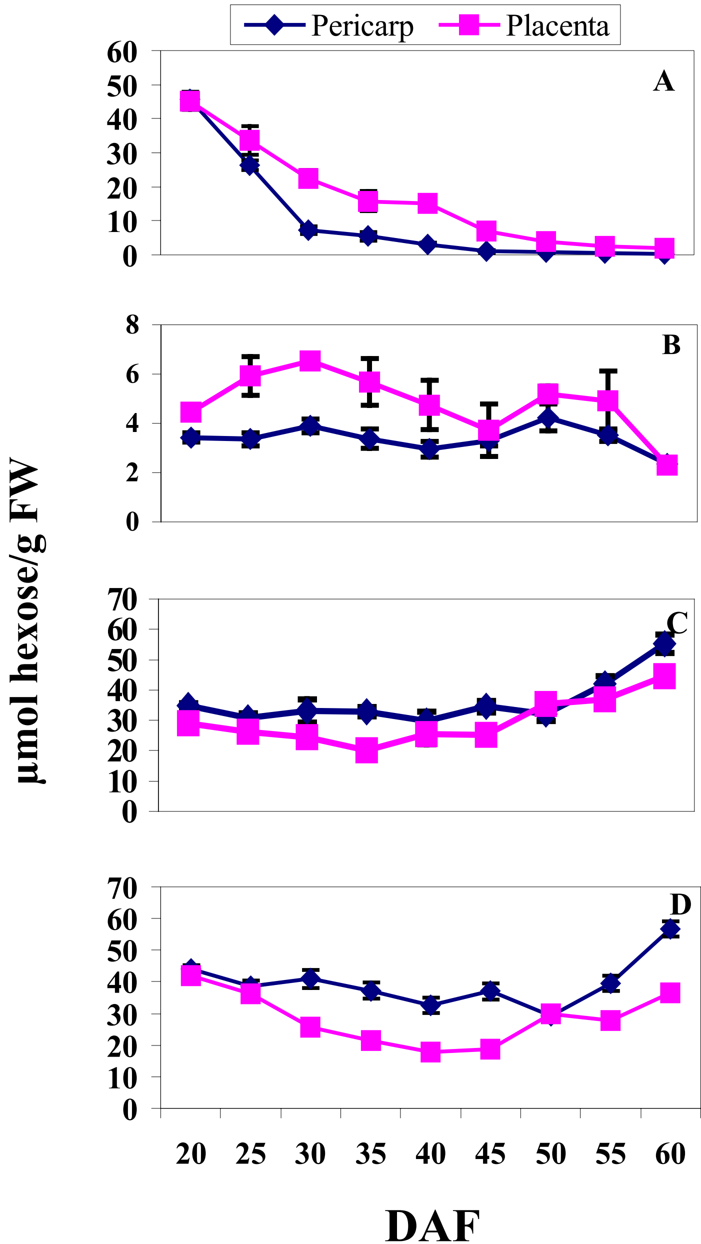


Figure 5: Starch and soluble sugar contents in pericarp and placental tissues of tomato cultivar Micro-Tom during development. (A) Starch. (B) Sucrose. (C) Fructose. (D) Glucose. Data represent the mean of five independent measurements + SE.

Changes in both fructose (Fig. 5C) and glucose (Fig. 5D) concentrations showed a similar pattern. In the pericarp they remained relatively constant between 20-50 DAF, before increasing at the end of development. Fructose levels also remained relatively constant in the placenta between 20-45 DAF whilst over this time period glucose levels decreased slightly. After 45 DAF the concentrations of both fructose and glucose increased. Both glucose and fructose levels were generally lower in the placenta than in the pericarp, but in both tissues their concentrations were an order of magnitude higher than the concentration of sucrose. Fructose concentrations were significantly increased in the pericarp in comparison with the placenta, but only at four time points (20, 35, 45 and 60 DAF). Glucose concentrations were also significantly increased in the pericarp in comparison with the placenta, but at six time points (30, 35, 40, 45, 55 and 60 DAF).

4.3.3 Changes in activities in enzymes involved in conversion of sucrose to starch

Sucrose synthase (SuSy) activity was initially about twice as high in the pericarp than in the placenta (Fig 6A). The Susy activity was significantly reduced in the placenta in comparison with the pericarp until 40 DAF, but at 60 DAF the activity was significantly increased in the placenta in comparison with the pericarp. The activity in both tissues decreased over time.
Acid-invertase activity was significantly greater in the placenta than in the pericarp at six time points (20, 25, 40, 45, 50 and 55 DAF), but at 60 DAF the activity was significantly increased in the pericarp in comparison with the placenta (Fig 6B). The activity in both tissues increased slightly between 20-55 DAF, before increasing quickly at 60 DAF.
UDP-glucose pyrophosphorylase (UGPase) activity was very high in both tissues (Fig 6C). It increased initially in both tissues until 45 DAF, after which it decreased slightly. The activity was significantly reduced in the placenta in comparison with the pericarp but only at three time points (30, 35, and 50 DAF).
Phosphoglucomutase (PGM) activity was significantly greater in the pericarp at all time points than the placenta. The activity stayed approximately the same until 40-45 DAF, after which it decreased (Fig 6D).
ADP-glucose pyrophosphorylase (AGPase) activity was also significantly greater in the pericarp than in the placenta but only at three time points (20, 35 and 45 DAF). In the placental tissue it decreased from a high activity to a low one between 20 and 30 DAF, after which it stayed at the low activity throughout the rest of development. The activity in the pericarp, on the other hand, decreased over the entire developmental stage (Fig. 6E).

4.3.4 Changes in activities in enzymes involved in glycolysis or the Calvin cycle

Hexokinase (HK) activity increased in a similar manner in both pericarp and placental tissues until 45 DAF, after which it decreased (Fig. 7A). The activity was significantly lower in the placenta than in the pericarp but only at two time points (30 and 35 DAF).

Fructokinase (FK) activity was initially significantly higher in the pericarp than in the placenta until 40 DAF, but after 50 DAF no differences could be detected. The activity decreased after 45 DAF in both tissues (Fig. 7B).

Phosphoglucose isomerase (PGI) activity increased in both pericarp and placental tissues until 45 DAF, wherafter it decreased (Fig. 7C). The activity was significantly lower in the placenta than in the pericarp at four time points (40, 45, 50 and 60 DAF), but at 25 DAF the activity was significantly increased in the placenta in comparison with the pericarp.

Fructose 1,6 bisphosphate (FBPase) activity was significantly higher in the placenta than in the pericarp at all time points except at 20 DAF. The activity of this enzyme decreased over time (Fig. 7D).

Phosphofructokinase dependent pyruphosphate (PP_i-PFK) activity decreased rapidly in both tissues from an initial relatively high activity to a basal activity at 35 DAF. Thereafter the activity remained relatively constant (Fig 7E). The activity was significantly increased in the placenta in comparison with the pericarp at three time points (20, 25 and 30 DAF).

Phosphofructokinase (PFK) activity was initially relatively high in both tissues and increased slightly until 45 DAF, after which it decreased (Fig. 7F). Its activity in the placenta was significantly lower than in the pericarp but only at two time points (30 and 35 DAF)

Triose phosphate isomerase (TPI) activity increased in both tissues until 40-45 DAF, after which it decreased (Fig 7G). The activity in the placenta was significantly lower than in the pericarp but only at three time points (30, 35 and 50 DAF).

Glyceraldehyde 3-phosphate dehydrogenase (G3P DH) activity was significantly greater in the placenta than in the pericarp at all time points except at 20 DAF. In the placenta it increased until 35 DAF, after which it decreased. In the pericarp it decreased from the first time point until 50 DAF after which it increased slightly (Fig. 7H).

Phosphoglycerate kinase (PGK) activity was significantly greater in the pericarp than in the placenta until 50 DAF. In both tissues it rose between 20-45 DAF, after which it decreased (Fig 7I).

Enolase activity was significantly lower in the placenta than in the pericarp at all time points except two time points (50 and 55 DAF). In the pericarp, however, the activity was greater and stayed relatively stable until 40 DAF after which it decreased (Fig. 7J).

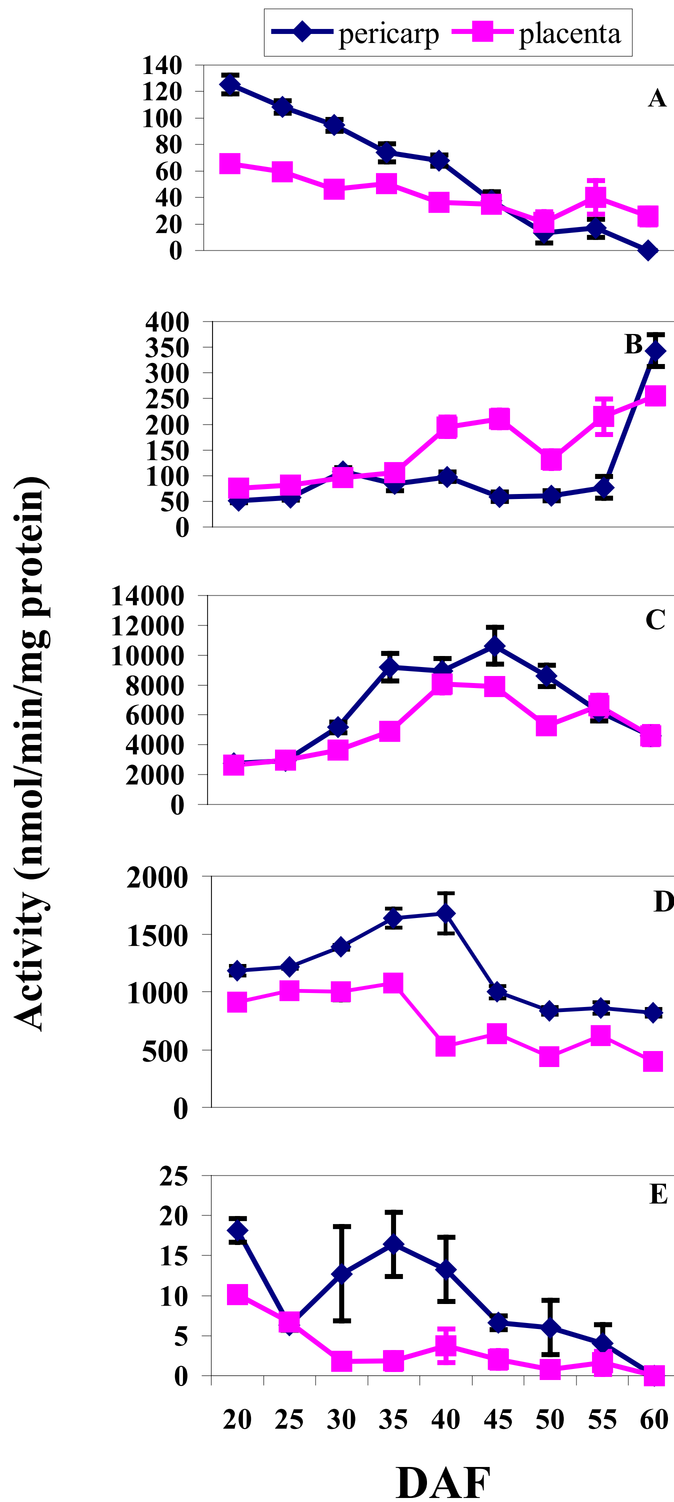


Figure 6: Activities of enzymes involved in the conversion of sucrose to starch in the pericarp and placental tissues of fruit of the tomato cultivar Micro-Tom. (A) SuSy. (B) Acid invertase. (C) UDPase. (D) PGM. (E) AGPase. Data represent the mean of five independent measurements + SE.

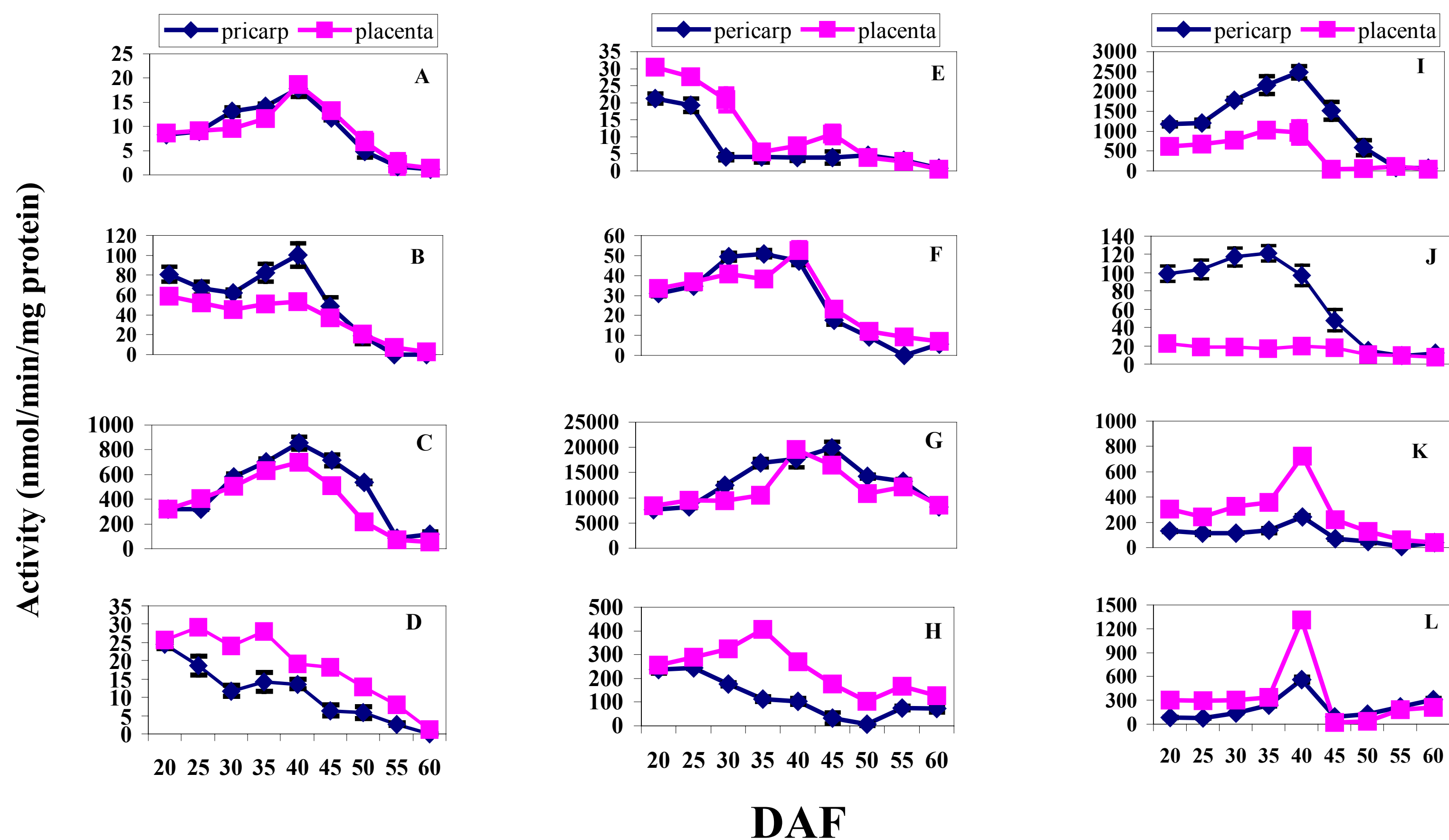


Figure 7: Activities of some glycolytic and clavin cycle enzymes in pericarp and placental tissues of fruit from the tomato cultivar Micro-Tom during its development.(A) HK. (B) FK. (C) FGI. (D) FBPase. (E) PPi-PFK. (F) PFK. (G) TPI. (H) G3P DH. (I) PGK. (J) Enolase. (K) PK. (L) PEP phosphatase. Data represent the mean of five independent measurements+ SE.

Pyruvate kinase (PK) activity was significantly greater in the placenta than in the pericarp at all time points except one time point (at 60 DAF). It remained constant until 35 DAF. Between 35-40 DAF the activity increased, before decreasing afterwards (Fig 7K).

Phosphoenolpyruvate phosphatase (PEP phosphatase) activity was also significantly greater in the placenta than in the pericarp at all time points except one time point (at 55 DAF). Generally, the alterations in activities of PEP phosphatase was similar to those of PK (Fig. 7L).

4.3.5 RNA blots of plastidial transporters

We wanted to examine how the accumulation of mRNA coding for of various plastidial transporters changed during development of the fruit. mRNA coding for the triose phosphate transporter (TPT) accumulated most in young, green, fruit. As the fruit developed the amount of mRNA decreased, but was still present throughout most of the developmental period (Fig. 8A and B).

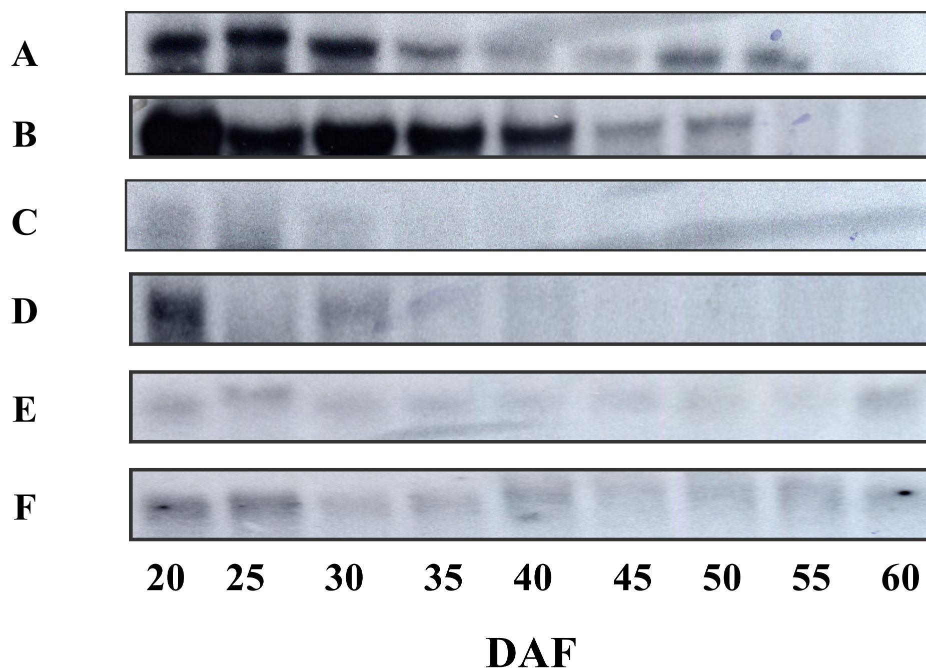


Figure 8: RNA blot analysis of some plastidial transporters throughout fruit development in the tomato cultivar Micro-Tom. TPT in (A) pericarp and (B) placental tissues. Glc-6-P transporter in (C) pericarp and (D) placental tissues. ATP/ADP transporter in (E) pericarp and (F) placental tissues.

The accumulation of mRNA coding for the Glc-6-P transporter was greatest between 25-30 DAF. There appeared to be no expression after 40 DAF (Fig. 8C and D). The expression in the placental tissue was greater than in the pericarp.

The ATP/ADP transporter was only expressed at low levels, but was present throughout development (Fig. 8E and F).

4.4 Discussion and conclusion

This study was initiated to examine carbohydrate metabolism in a new variety of tomato that is beginning to be used as a model system. Much of the data is, therefore, descriptive, but it also leads to some novel conclusions. The first of these is that the metabolism in the pericarp is different to that in the placenta. The placenta accumulates starch over a longer period than the pericarp, and has different concentrations of soluble sugars within it. This is presumably because the different tissues serve different roles. The placental tissue acts as a conduit for nutrients going to the developing seeds, while the pericarp protects the seeds within the fruit. The starch that accumulates over a longer period in the placenta may act as a nutrient reserve in case the flow of sucrose coming to it from the leaves becomes disrupted. The chloroplasts in the pericarp, on the other hand, differentiate to chromoplasts during ripening, and the starch is presumably degraded as a source of soluble sugars to make the fruit more palatable for the dispersal of seeds.

There were also differences in the activities of enzymes measured in the pericarp in comparison with the placental tissue. Although all of the enzyme activities shown were calculated on the basis of the amount of protein in the extract, it is also possible to do so based on the fresh weight. Although they showed minor differences with the data presented, these were not large enough to alter the conclusions. The pericarp consistently had increased activities of several enzymes in comparison with the placenta. These were SuSy, PGM, enolase, PGK and UGPase. With the exception of enolase all of these enzymes are closely associated with the degradation of sucrose. It is interesting to note that the pericarp had a lower concentration of sucrose in comparison with the placenta during development. It may be that the higher activities of these enzymes led to it being metabolised faster to its lower concentration.

The conversion of sucrose to starch has been relatively well studied in tomato fruits from varieties other than Micro-Tom (**Robinson *et al.*, 1988; Yelle *et al.*, 1988; Schaffer and Petreikov, 1997a**) and it is, thus, possible to compare the data from this study with that from

those. As in this study, it has often been found that sucrose concentrations are lower than both glucose and fructose (**Damon *et al.*, 1988; Klann *et al.*, 1996; Schaffer and Petreikov, 1997a**), although the wild tomato relative *Lycopersicon chmielewskii* accumulates higher levels of sucrose than the other soluble sugars (**Yelle *et al.*, 1988**). The reason for this accumulation of sucrose in the wild relative is due to a reduction in the activity of acid invertase (**Klann *et al.*, 1996**). The invertase activity in this study increased dramatically in the final stages of fruit development, especially in the pericarp. This again is similar to what has been found previously in wild-type tomato fruits (**Klann *et al.*, 1996**) indicating that the Micro-Tom cultivar is not significantly altered in this respect.

SuSy activity is often considered to be a major determinant of sink strength, although the evidence in tomato for this is contradictory. One study found repression of SuSy using genetic engineering techniques let to a decrease in a fruit set (**D'Aoust *et al.*, 1999**), whilst a second found no effect (**Chengappa *et al.*, 1999**). This might be because the first study used a constitutive promoter to reduce SuSy activity, whilst the second used a fruit specific promoter. In this study SuSy activity decreased during development in both the pericarp and placental tissues. Although there is variation between different studies as to what occurs to SuSy activity during fruit development, this type of pattern is not unusual (**for example Robinson *et al.*, 1988; Klann *et al.*, 1996**) and furthermore parallels the switch from symplastic to apoplastic unloading that occurs during development (**Ruan and Patrick, 1995**).

AGPase activity has often been correlated with starch accumulation in tomato fruits. In this study that was also the case, with AGPase activity being below detectable levels after 30 DAF in the placenta and decreasing in activity in the pericarp. At this time point there is net degradation of starch, and it might be expected, therefore, that enzymes involved directly in its synthesis would be down -regulated.

Although not so well studied in tomato as enzymes involved in sucrose to starch conversion, we also measured some enzymes involved in glycolysis or the Calvin cycle. Most glycolytic enzymes showed a peak of activity at about 40 DAF. Some of these, specifically HK, FK, PGI, PFK, TPI, G3P DH, and PGK, showed a gradual increase and decrease. Two others though, PK and PEP phosphatase showed a dramatic increase in activity between 35 and 40 DAF, which then declined very quickly to a lower level at 45 DAF. It is interesting that PK showed this sudden increase in activity as it is thought to exert significant control over flux through the glycolytic pathway (**Plaxton, 1990**). The peaking of activities of glycolytic

enzymes at 45 DAF is presumably due to climacteric respiration. It may be that the sudden up-regulation of PK just before this point indicates that this enzyme is important in increasing flux through glycolysis to generate ATP for climacteric respiration.

It has been speculated that climacteric respiration in fleshy fruits, such as tomato, only occurs when they are detached from the plant. This idea was based on studying internal CO_2 concentrations in both tomato and muskmelon, and not finding expected increases when the fruits were attached to the plant (**Saltveit, 1993; Shellie and Saltveit, 1993**). It has been argued, however, that the interpretation of these studies was incorrect as; they did not take into account the effect of photosynthesis on internal CO_2 concentration (**Knee, 1995**). Our data are consistent with tomatoes acting as climacteric fruits when attached to the vine as they show an up-regulation of glycolysis just prior to the onset of ripening.

We also decided to study the expression of some transporters that are present in the plastidial membrane. Expression of the TPT has previously been studied in tomato fruits (**Schünemann *et al.*, 1996; Büker *et al.*, 1998**). In these studies it was shown that both mRNA coding for the TPT protein, and the protein itself, accumulated in both green and red fruits. Our data do not disagree with these findings, showing maximal accumulation of mRNA in green fruits, with less accumulation during development. The expression of the Glc-6-P transporter has not previously been studied in tomato fruits. In maize, mRNA coding for it has been demonstrated to be present only in tissues containing non-green chloroplasts (**Kammerer *et al.*, 1998**). Our data indicate that in tomato fruits, the mRNA coding for the Glc-6-P transporter is expressed maximally in tissues containing green chloroplasts, with reduced amounts in red fruits. This is opposite to that found in maize, but indicates the difference of fruit of chloroplasts in comparison with those in leaves. The sole source of sugars in leaf chloroplasts comes directly from photosynthesis, while fruit chloroplasts can import Glc-6-P from the cytoplasm also (**Büker *et al.*, 1998**). This Glc-6-P is the result of the catabolism of imported sucrose in the cytosol. The expression of the Glc-6-P transporter appears to correlate with accumulation of starch in the pericarp and placental tissues. It is tempting to speculate that the Glc-6-P transporter is expressed at times of maximal starch accumulation to supplement carbon being fixed through photosynthesis in the chloroplast. This is especially so as it is much more strongly expressed in the placental tissues, which are in the centre of the fruit and will, therefore, receive less light for photosynthesis. This tissue would have to rely more on sucrose to supply starch synthesis, than on any photosynthate it may be able to produce. This is of course true for all pathways that would utilise Glc-6-P in the plastide, but

starch constitutes the major sink for carbon in plastids and so would have the greatest influence. The ATP/ADP transporter was weakly expressed throughout development of the fruits. This transporter has been demonstrated to have a great influence on the rate of starch accumulation in potato tubers (**Tjaden *et al.*, 1998**), but ATP is used in practically, all biosynthetic pathways, so maintaining a constant into the plastids would be expected.

One aim of this study was to examine whether the Micro-Tom tomato cultivar was a suitable candidate to act as a model system for the study of carbohydrate metabolism in tomato fruit generally. It might be that the mutations leading to the dwarf phenotype lead to pleotropic effects on the metabolism of the fruit, which would make it an unsuitable candidate. All the data in this study indicate that the metabolism of the Micro-Tom cultivar is not greatly altered in comparison with reports of other cultivars, showing that suitable for such studies.

From the previous data presented in this investigation, it can be concluded that: (A) The metabolism in the pericarp is different to that in the placenta. (B) Starch was degraded more slowly in the placenta in comparison to the pericarp, while soluble sugars accumulated to a greater extent in the pericarp. (C) There were also differences in the activities in enzymes involved in conversion of sucrose to starch measured in the pericarp in comparison with the placental tissue. (D) The pericarp consistently had increased activities of several enzymes SuSy, PGM, enolase, PGK and UGPase. SuSy, PGM and UGPase in comparison with the placenta. (E) The activities of glycolytic enzymes tended to peak at 40 DAF. (F) Two of these, PEP phosphatase and PK, showed a dramatic increase in activity just before this peak possibly indicating a role in up-regulating glycolysis to generate ATP for climacteric respiration. (G) Both the TPT and Glc-6-P transporter were expressed greatest in green fruits, before declining. (H) The expression of the TPT was greater than that of the Glc-6-P transporter. (I) The ATP/ADP transporter was expressed to a low level throughout fruit development.

5 Analysis of the Function of Chloroplastic Fructose 1,6-bisphosphatase in Tomato Fruit

5.1 Introduction

Fructose-1,6-bisphosphatase (FBPase) catalyses the inter-conversion of Fru-1,6-P_2 and Fru-6-P. In green plant tissues there are two isoforms, one situated in the plastid (cp-FBPase), and one in the cytosol (cy-FBPase). The plastidial isoform is an important enzyme in control of the Calvin cycle, and its repression in potatoes leads to an inhibition of photosynthesis and a reduction in growth (**Kossmann *et al.*, 1994**). The cy-FBPase isoform, on the other hand, is involved in gluconeogensis and sucrose synthesis. Inhibition of this isoform leads to increases in starch and decreases in sucrose synthesis (**Zrenner *et al.*, 1996; Strand *et al.*, 2000**).

Green tomato fruits contain photosynthetically active chloroplasts, which differentiate to chromoplasts during the ripening process. The cp-FBPase is present in green, but not in red fruits, which correlates with a switch from the fruits being photosynthetically active to becoming inactive (**Büker *et al.*, 1998**). Fruits obtain sugars both directly from photosynthesis, and through import from source leaves *via* the phloem. The triose phosphate and glucose phosphate transporters are active in tomato chloroplasts (**Büker *et al.*, 1998**), indicating that they could in principle both import and export sugars. It is not clear, therefore, what the role of fruit photosynthesis in fruit metabolism is, although it has been estimated to contribute between 10-15% of carbon skeletons in green fruits (**Tanaka *et al.*, 1974**).

5.2 Aim of the work

The aim of this work is to study the role of cp-FBPase in tomato fruit metabolism. To accomplish this, tomato plants were transformed with a construct designed to repress cp-FBPase activity solely in the fruit. Transgenic lines were isolated with reduced amounts of cp-FBPase protein, which showed a reduction in total FBPase activity also. Fruits from these plants were analysed for alterations in carbohydrate metabolism.

5.3 Results

5.3.1 Recovery of Plants with Reduced FBPase Activity in the Pericarp of Tomato Fruit.

The pericarps from 25 days after flowering (DAF) old tomato fruits were analysed for FBPase activity in sixty transgenic lines. Three lines (#19, 33 & 34) were selected which showed

reductions in FBPase activity (see Material and Methods). These cp-FBPase antisense plants were phenotypically identical to the untransformed control (Fig. 9).

Figure 9: Aerial parts of plants in both WT control and α-cp-FBP-transgenic lines after 8 weeks growth in the glasshouse. From left to right: untransformed WT control, α-cp-FBP#19, α-cp-FBP#33, α-cp-FBP#34 and α-cp-FBP#34. The α-cp-FBP plants are phenotypically identical to the untransformed WT control.

Fructose-1,6-bisphosphatase (FBPase) activity was studied throughout fruit development in the pericarp of these plants. In young (25 DAF), green, fruits, there was a significant reduction in total FBPase activity in all three transgenic lines in comparison with the WT control (Fig. 10A). At that time point the reduction in activity of line #34 was 25%, and of the other two lines 50%. The activity in all lines decreased during the ripening process, and was significantly lower than the WT control in lines #19 and #33 until 55 DAF when there was no significant difference.

Immunoblots using an antibody raised against wheat cp-FBPase **(Hagelin *et al.*, 1996),** but which recognizes the tomato cp-FBPase also, indicated that it was completely eliminated in 25 DAF fruit of the lines #19 and #33, and greatly reduced in the line #34 (Fig. 10B). To demonstrate that the antisense effect was fruit specific, FBPase activity was also determined

in the leaves of the transgenic plants. No differences between the transgenic plants and the WT control were found (Fig. 10C).

5.3.2 Starch and soluble sugar contents in the pericarp of the WT and transgenic lines

Starch and soluble sugar contents were determined in the pericarp between 25-70 DAF. There was net degradation of starch over the ripening period (Fig. 11A). In the control the concentration was approximately 8µmol hexose (g FW)$^{-1}$ at 25 DAF, and was less that 1µmol hexose (g FW)$^{-1}$ at the final sample point. The starch contents in lines #19 and #33 were not significantly different in comparison with the control over the entire sampling period, but those of line #34 were significantly ($P \leq 0,05$) reduced until 45 DAF, when there was no difference.

Glucose concentrations were significantly ($P \leq 0,05$) increased in the three transgenic lines in comparison with the WT control, but only between 25 and 35 DAF. At these time points the concentrations in the transgenic fruits were between 60-80 µmol hexose (g FW)$^{-1}$, while in the WT control fruits they were between 40-50 µmol (g FW)$^{-1}$ (Fig. 11B). After this point there were no significant differences with the concentration of glucose increasing to just less than 100µmol hexose (g FW)$^{-1}$ at the final sampling point.

Fructose concentrations showed a similar pattern to glucose concentrations (Fig. 11C). They were initially about threefold greater in the transgenic lines being generally between 50-70 µmol hexose (g FW)$^{-1}$ in comparison to under 20µmol hexose (g FW)$^{-1}$ in the WT control fruits. The fructose concentration in the WT control fruits then increased to similar levels as in the transgenic plants by 45 DAF, and after this point the fructose concentration stayed relatively constant at between 60-80 µmol hexose (g FW)$^{-1}$ in all the plants.

There were no significant differences in sucrose concentrations between the transgenic lines and WT control, except for two time points in one transgenic line. At 30 and 35 DAF in line #19 (Fig. 11D) there was greatly increased sucrose in comparison with the WT control. In all the other lines, however, the sucrose concentration decreased from about 3µmol hexose (g FW)$^{-1}$ at the first sampling point, to about 1µmol hexose (g FW)$^{-1}$ at the final sampling point.

5.3.3 Changes in activities in enzymes involved in conversion of sucrose to starch

Sucrose synthase (SuSy) activity decreased in pericarp tissue from WT control fruits over time from 176nmol min^{-1} (mg protein)$^{-1}$ at 25 DAF to 62nmol min^{-1} (mg protein)$^{-1}$ in the

oldest fruit. In the transgenic lines the activity was significantly decreased at three time points (45, 55 and 65 DAF) in one transgenic line (#33; Fig 12A).

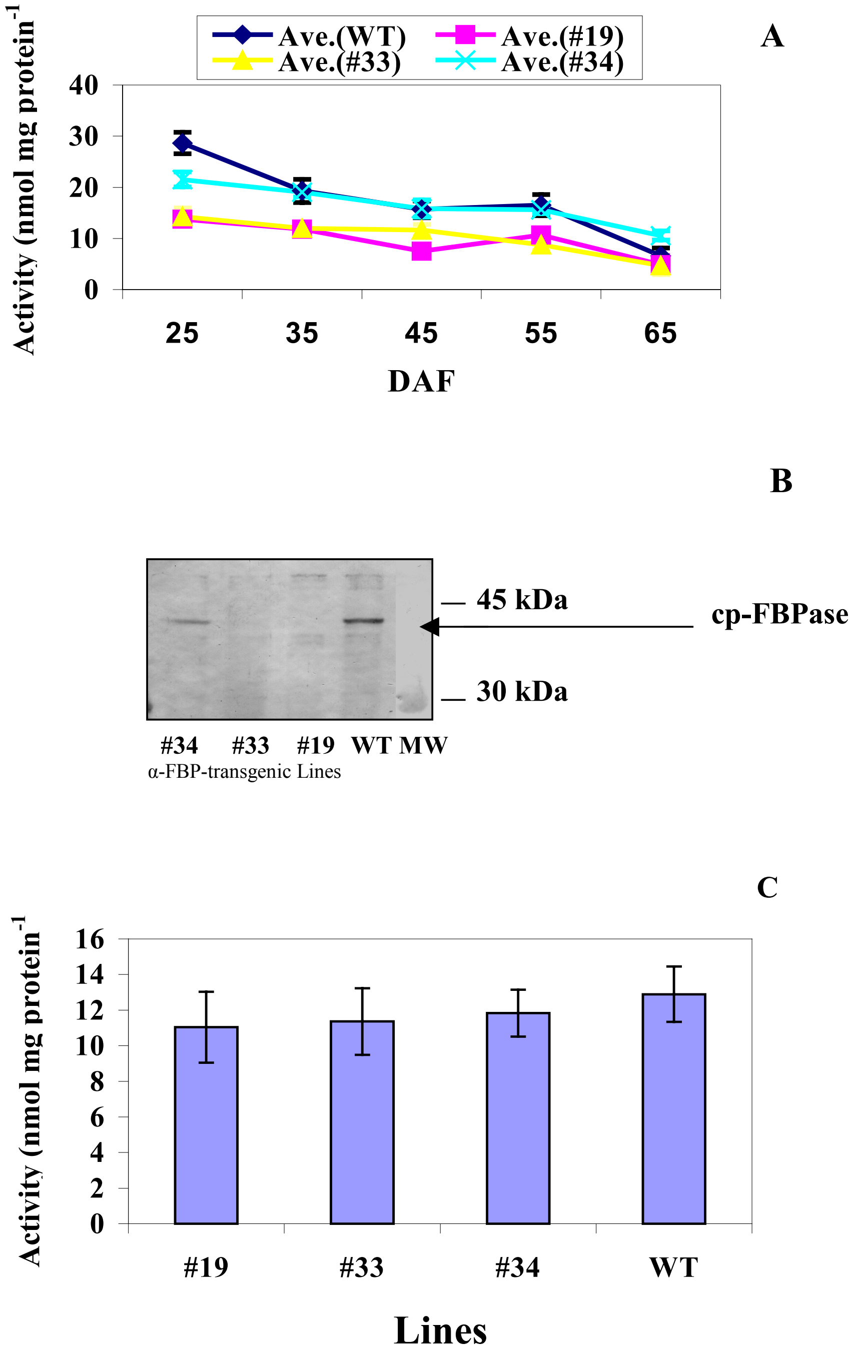


Figure 10: FBPase activity during developmental stage (A), Western blot analysis in green (25 DAF) (B) in the pericarp of WT and α-cp-FBP-transgenic lines [total soluble fruit protein (25µg) was subjected to SDS-PAGE on a 10% (w/v) gel] **and FBPase activity in the leaves of WT control and α-cp-FBP-transgenic lines (C).** Data represent the mean of five independent measurements + SE.

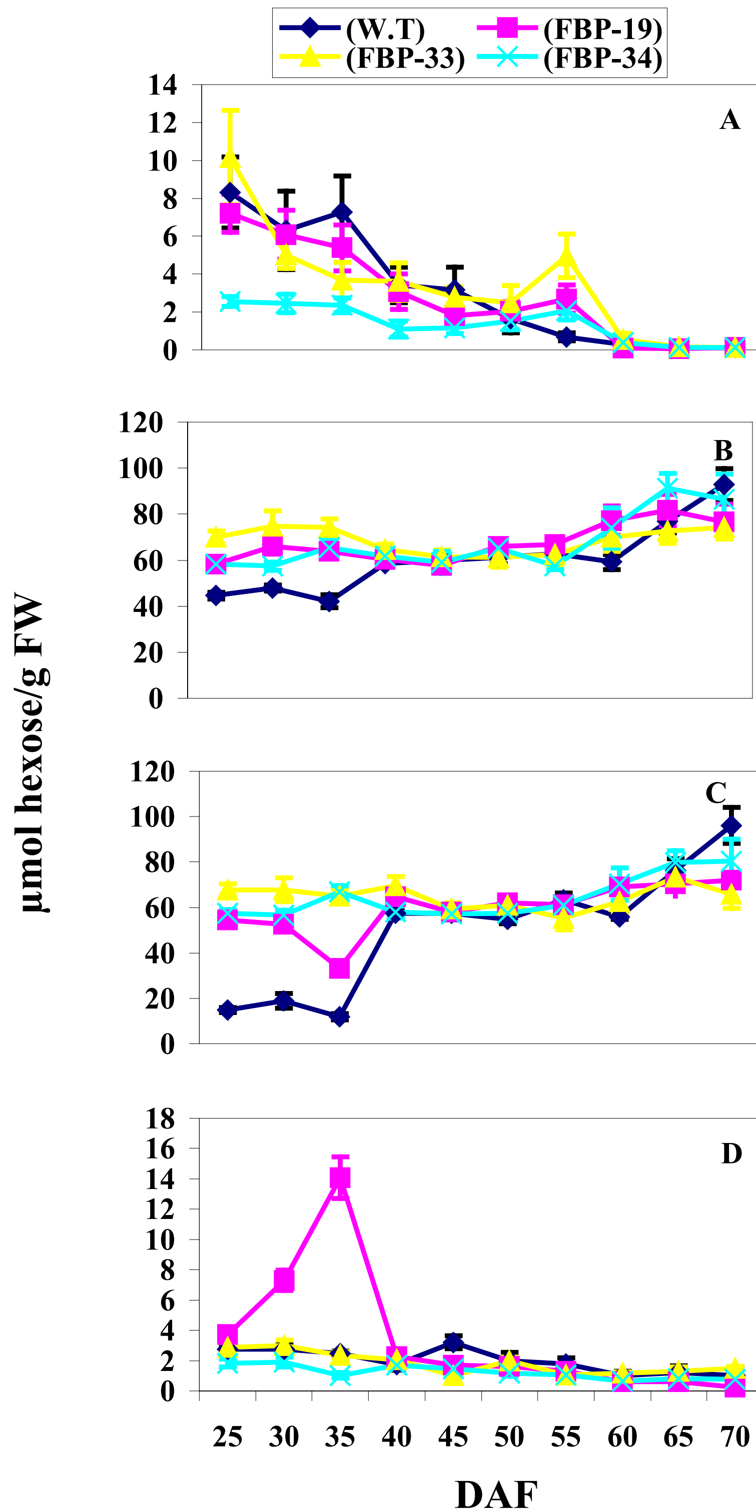


Figure 11: Starch and soluble sugar contents in pericarp of WT and α-FBP-transgenic lines in tomato cultivar Moneymaker during development. (A) Starch. (B) Glucose. (C) Fructose. (D) Sucrose. Data represent the mean of five independent measurements + SE.

UDP-glucose pyrophosphorylase (UGPase) activity was high in both the WT control and transgenic lines, and in the WT control the activity increased between 25 and 45 DAF, after which it decreased (Fig 12B). The activity in the fruits of the transgenic lines was significantly lower than the control at 25 DAF (line #33), 45 DAF (all lines) and 55 DAF (line #34; Fig 12B).

Phosphoglucomutase (PGM) activity decreased over the entire developmental period in the WT control from 1768nmol min^{-1} (mg protein)$^{-1}$ at 25 DAF to 897nmol min^{-1} (mg protein)$^{-1}$ at 65 DAF. The PGM activity was significantly reduced in the transgenic lines at 25 DAF (all lines), 35 DAF (line #19), 55 DAF (lines #19 and #34) and 65 DAF (lines #33 and #34; Fig 12C).

ADP-glucose pyrophosphorylase (AGPase) activity was significantly greater in the WT control at all time points up until 55 DAF than in all of the transgenic lines (Fig 12D).

5.3.4 Concentration of Metabolic Intermediates in the pericarp of the WT control and transgenic lines

The concentrations of several metabolites were measured in trichloroacetic acid extracts of the transgenic lines from 30DAF fruits. The metabolites determined were glucose 6-phosphate (Glc-6-P), glucose 1-phosphate (Glc-1-P), fructose 6-phosphate (Fru-6-P), 3-phosphoglyceric acid (3-PGA), phosphoenolpyruvate (PEP), pyruvate and inorganic phosphate (Pi). The data are presented in Table 1. The concentration of 3-PGA was increased in all lines, significantly ($P \leq 0.05$) so in lines #33 and #34. In addition the concentration of both Glc-1-P and Pi were significantly ($P \leq 0.05$) reduced in line #19. There were no other significant reductions of metabolite concentrations. The ratios of hexose phosphates (hexose-P) to 3-PGA, phosphate esters (P-ester) to Pi and 3-PGA to Pi were calculated also. The ratio of hexose-P to 3-PGA was reduced significantly ($P \leq 0.05$) in lines #33 and #19, but that of P-ester to Pi was increased, significantly in line #19. The ratio of 3-PGA to Pi was significantly ($P \leq 0.05$) increased in lines #19 and #34.

5.3.5 Analysis of fruit yield

Fruit were harvested, and their weights and sizes determined, after 65 DAF. Some fruits of the transgenic line can be seen in comparison with the WT control in Fig. 13. Both the average weights and sizes of fruits of all of the transgenic lines were significantly ($P \leq 0.05$) reduced in

comparison with the WT control (Table 2). This reduction was between 15-20% with respect to the weights of the control fruits and 9-11% with respect to the sizes of the control fruits.

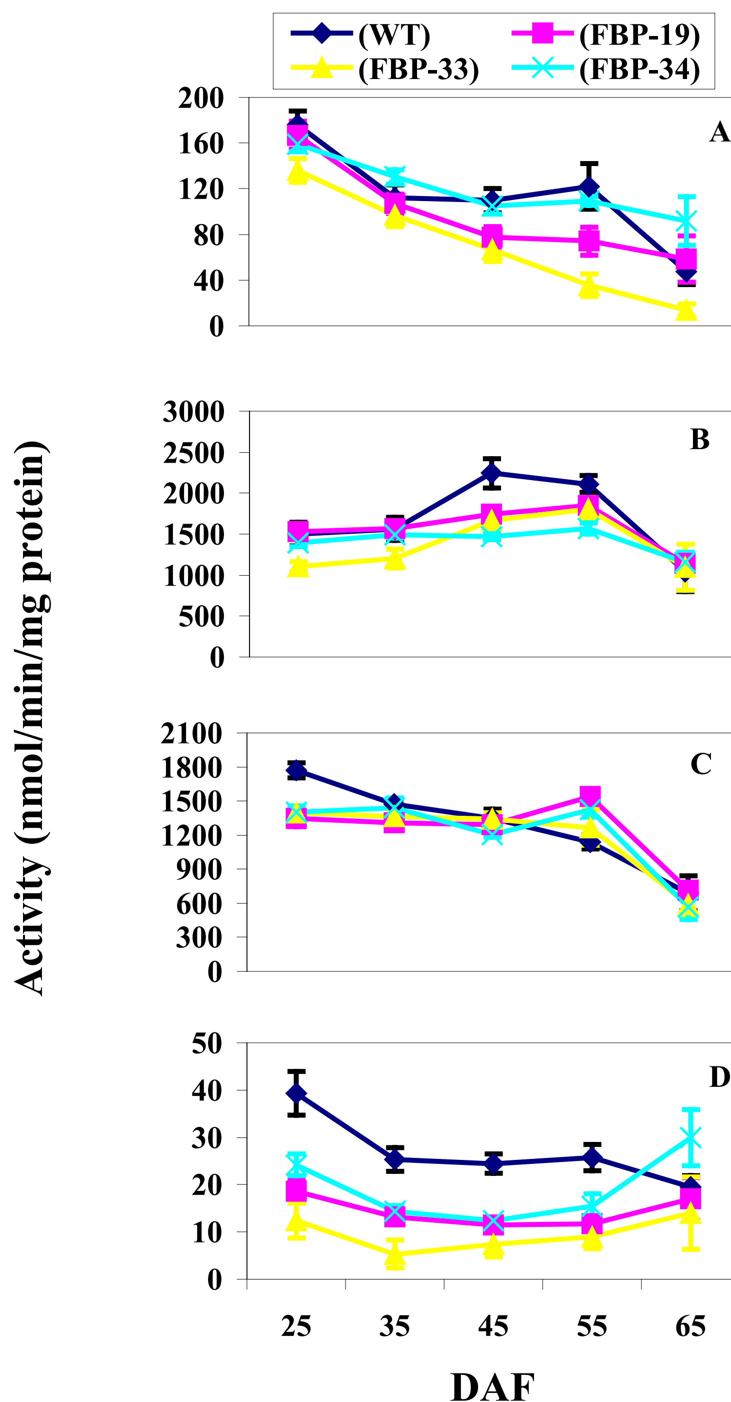


Figure 12: Activities of enzymes involved in the conversion of sucrose to starch in pericarp of the WT control and αcp-FBP-transgenic lines of fruit of the tomato cultivar Moneymaker. (A) SuSy. (B) UGPase. (C) PGM. (D) AGPase. Data represent the mean of five independent measurements + SE.

Table 1: Metabolite concentrations in the pericarp of 30 DAF old WT control and αcp-FBP-transgenic fruits.

Conc. nmol.(g FW)$^{-1}$	Lines			
	WT	#19	#33	#34
G6P	44.6 ± 2.5	48.5 ± 4.1	49.8 ± 4.3	52.9 ± 3.8
G1P	5.6 ± 0.3	**4.8 ± 0.2**	4.7 ± 0.7	6.1 ± 0.4
F6P	15.0 ± 1.0	14.8 ± 1.3	15.5 ± 1.5	17.2 ± 0.8
Total Hexose-P	65.2 ± 3.1	68.1 ± 4.4	70.0 ± 5.8	76.2 ± 6.3
3-PGA	15.0 ± 1.0	18.4 ± 1.0	**19.3 ± 2.2**	**19.7 ± 1.5**
PEP	4.5 ± 0.6	3.5 ± 0.4	4.6 ± 0.4	5.1 ± 0.8
Pyruvate	2.8 ± 0.4	3.9 ± 0.5	3.9 ± 0.5	3.2 ± 0.5
Pi	1.7 ± 0.1	1.2 ± 0.1	1.5 ± 0.2	1.6 ± 0.1
Ratio				
Hexose-P/3-PGA	4.5 ± 0.5	**3.7 ± 0.1**	**3.6 ± 0.1**	3.9 ± 0.2
P-ester/Pi	50.6 ± 5.3	**79.4 ± 12.6**	59.8 ± 6.8	60.2 ± 2.6
3-PGA/Pi	8.8 ± 1.1	**15.3 ± 3.4**	12.8 ± 3.9	**12.3 ± 0.9**

The data represent means ± SE of five independent samples. Samples significantly different from the control (P≤0.05, Students *t*-test) are in bold.

Table 2: Weights and sizes of ripe tomato fruits in the WT control and αcp-FBP-transgenic lines.

Lines	Average	
	Weight of Fruit (g)	Size of Fruit (cm)
WT	54.2 ± 1.2 (n=146)	4.8 ± 0.03 (n=146)
#19	**46.4 ± 2.1** (n=42)	**4.4 ± 0.08** (n=42)
#33	**45.8 ± 1.8** (n=52)	**4.4 ± 0.07** (n=52)
#34	**43.5 ± 1.7** (n=62)	**4.3 ± 0.06** (n=62)

The data are means ± SE, number of sample is in parentheses. Significant differences (P≤ 0,05), Student's *t*-test are in bold

A

B

C

Figure 13: Some 65 DAF old fruits from αcp-FBP-transgenic lines (bottom) in comparison with a control fruit (above).(A) Transgenic line #19. (B) Transgenic line #33 (C) Transgenic line #34.

5.3.6 Number of flower, fruit per plant, fruit set and number of days to 50% flowering.

Data of this trait are presented in Table 3. No significant differences were found between the control and the transgenic lines with respect to both number of flower per plant and number of days from planting to 50% flowering, while two transgenic lines (#19 and #33) showed significant reductions in comparison with the control with respect both number of fruit per plant and fruit set. No significant differences were found between the control and line #34 with respect to either number of flower per plant or fruit set.

Table 3: Number of flowers, fruits, fruit set and number of days to 50% flowering in the WT control and αcp-FBP-transgenic lines.

Lines	Average			
	No. of flower/plant	No. of fruit/plant	Fruit set %	No. of days to 50% flowering
WT	42.9 ± 1.7	26.3 ± 1.2	61.0 ± 0.5	51.4 ± 0.5
#19	46.0 ± 4.9	**21.4 ± 1.8**	**47.1 ± 1.6**	50.2 ± 0.4
#33	42.8 ± 2.0	**21.2 ± 1.7**	**49.2 ± 2.2**	50.0 ± 0.6
#34	44.4 ± 4.7	26.8 ± 2.6	60.6 ± 1.1	49.8 ± 0.7

Data represent the mean of fifteen independent measurements ± SE in WT and five independent measurements ± SE in the transgenic lines. Significant differences ($P \leq 0,05$, Student's *t*-test) are in bold.

5.4 Discussion and conclusion

In this study I have described the production of transgenic tomato plants repressed in cp-FBPase activity in the fruit. A fruit specific promoter was used as constitutive reduction of this enzyme represses photosynthesis in leaves and lead to stunted growth of the whole plant (**Kossmann *et al*., 1994**). If plants had been produced in this study using a constitutive promoter it would not have been possible to separate the effect in fruit metabolism of reduced photosynthesis in the leaves from that in fruits. The transgenic plants appeared phenotypically normal, indicating that the antisense effect was indeed restricted to the fruits, a view confirmed by measurement of FBPase activity, which was unchanged in leaves of the transgenic lines. Although unchanged in the leaves, the total FBPase activity was reduced in green fruits. This is a measure of both cp-FBPase and cy-FBPase simultaneously. The reduction in comparison with the total activity was more than 50% in the most inhibited lines

(#19 and #33), which is a smaller reduction in activity than was found in leaves of potato plants where the cp-FBPase was inhibited (up to 85% inhibition; **Kossmann *et al.*, 1994**). This might indicate either that the cy-FBPase contributes a greater proportion of the total FBPase activity in tomato fruits than in potato leaves, or that the fruit specific promoter used in this study does not inhibit the cp-FBPase as strongly as the constitutive promoter used in the experiments of **Kossmann *et al.*** (**1994**). I feel that the former explanation is more likely as immunoblot experiments using an antibody that recognizes cp-FBPase indicated that it was almost completely eliminated in lines #19 and #33, and greatly reduced in lines #34. This indicates that the residual FBPase activity, at least in lines #19 and #33, comes almost entirely from the cy-FBPase. As expected the reduction in FBPase activity was found only in younger fruits (45 DAF or younger). It is known that the cp-FBPase is present in green, but not red, fruits and, therefore, that in red fruits all FBPase activity comes solely from the cy-FBPase (**Büker *et al.*, 1998**). As it was the cp-FBPase isoform that was being repressed, differences in activity should have been noted only in younger fruits.

As was stated above, cp-FBPase activity has previously been repressed in transgenic potato plants and much data has been collected on alterations in metabolism in leaves of those plants. Repression of cp-FBPase in leaves led to decreases in the concentrations of soluble sugars and in starch contents (**Kossmann *et al.*, 1994**). Such drastic differences were not found in the fruits of the transgenic tomato plants repressed in cp-FBPase and the differences that were noted were qualitatively different to those found in potato leaves. Glucose and fructose concentrations, for example, were not decreased in comparison with the control, but rather increased in green fruits. These alterations are at precisely the developmental stage when cp-FBPase would be expected to have the greatest influence indicating that it is indeed a reduction in activity of this enzyme that leads to the increase. This probably indicates that the fruits from the transgenic plants are relying more on imported sucrose than the WT control fruits. Sucrose is degraded very quickly upon import into fruits by either invertase or sucrose synthase. This is demonstrated by the very low concentration of sucrose in relation to either glucose or fructose found in the fruits both in the present study and in previous ones (**Klann *et al.*, 1996**; **Schaffer and Petreikov, 1997**). Although it is not clear in tomato fruits which enzyme has the greatest influence on sucrose degradation, both appear to effect fruit metabolism. Repression of invertase leads to fruits that accumulate sucrose (**Klann *et al.*, 1996**), whilst inhibition of sucrose synthase has been reported to decrease fruit set and sucrose import, but did not alter soluble sugar levels (**D'Aoust *et al.*, 1999**). Invertase degrades

sucrose to glucose and fructose, whilst sucrose synthase produces UDP-glucose and fructose. Increases in concentrations of glucose and fructose in fruits of the transgenic lines indicates, therefore, either that more sucrose has been imported and degraded, or that the glucose and fructose produced are not being utilized as quickly. As the transgenic fruits are repressed in cp-FBPase, and it is known that repression of this enzyme leads to repression of photosynthesis (**Kossmann *et al.*, 1994**), it is likely that the transgenic fruits will have to rely more on imported sucrose for growth than the WT control fruits. The levels of PEP and pyruvate were not altered in the transgenic lines, which indicates that glycolysis has not been down-regulated and, therefore, that utilization of glucose and fructose has also not been reduced. This indicates, therefore, that the increased concentrations of fructose and glucose are due to increased import of sucrose.

In one transgenic line (#19) at both 30 and 35 DAF there was a significantly greatly increased sucrose concentration in comparison with the WT control, and the other transgenic lines. I have no explanation for this, and it was not noted in any other line at any other point, indicating that it was due to random variation. Other than those points, there were no significant differences in sucrose concentrations in the transgenic lines in comparison with the WT control fruits. These lack of differences in soluble sugar concentrations between the transgenics and the WT control are presumably because the fruit can compensate for any reduction in sugar production due to a fruit specific repression in photosynthesis by importing more soluble sugars.

I also measured the concentrations of some metabolites, which might be affected by reductions in cy-FBPase (Table 1). To our knowledge these data represent the first documented measurement of these metabolites in tomato fruit. The levels of most measured metabolites are lower than those observed in leaves or tubers from the closely related potato plant (see for example **Westram *et al.,* 2002; Lytovchenko *et al.,* 2002**) most probably because of the high water content of this tissue. In addition, the relative 3-PGA concentration with respect to the other metabolites is much lower in the WT fruit tissue than that observed in potato leaves **(Lytovchenko *et al.,* 2002)** suggesting that photosynthesis in the tissue studied here is less efficient. Although the levels of hexose-P were unchanged, the hexose-P to 3-PGA ratio - which is indicative of the rate of inter-conversion between Fru-1,6-P_2 and Fru-6-P **(Fernie *et al.,* 2001)** - is moderately decreased in all lines (significantly in the case of #33 and #19). This indicates that the flux through the chloroplastic FBPase is indeed inhibited *in vivo*. Furthermore, evaluation of the metabolic profile of these plants strongly hints that

photosynthesis was repressed by phosphate limitation – the levels of inorganic phosphate (Pi) are somewhat lower in the transgenics (significantly in the strongest line), and most importantly the P-ester to Pi ratio increases. Such changes are characteristic of phosphate limitation of photosyntheis **(Lytovchenko *et al.,* 2002; Leegood and Furbank, 1986)** and indeed resemble those observed on the inhibition of the cytosolic isoform of this enzyme in potato **(Zrenner *et al.,* 1996).** The glycolytic metabolites, PEP and pyruvate were unaltered in the transgenics whilst there was a slight decrease in the levels of Pi. This was only significant, however, in the strongest transgenic line. The ratio of total P-ester to Pi shows a trend of increasing with decreasing cp-FBPase activity, as does the ratio of 3-PGA to Pi.

None of the metabolites downstream of the FBPase reaction (Glc-6-P, Fru-6-P and Glc-1-P) were greatly altered in the fruits of the transgenic plants. Glc-1-P was reduced, but only in the fruits from one line (#19). These data are similar to those reported by **Kossmann *et al.* (1994)** who argued that hexose-P are present mainly in the cytosol and, therefore, are less likely to be influenced by alterations in plastid metabolism. 3-PGA concentrations, however, were significantly increased in two out of the three transgenic lines. This is in contrast to what was found in potato leaves repressed in cp-FBPase where 3-PGA was reduced in concentration **(Kossmann *et al.,* 1994)**. In that study they did find, however, that glyceraldehyde 3-phosphate (G3P), the precursor of which is 3-PGA, was increased in leaves with reduced cp-FBPase. It was argued that photosynthetically active tissues with reduced cp-FBPase should contain less ribulose 1,5-bisphosphate (Ru 1,5-$P_{2)}$ which is the substrate for carboxylation. They argued further that it would be reasonable to assume that under those circumstances G3P would accumulate in preference to 3-PGA as G3P is involved in the regeneration phase of the Calvin cycle while 3-PGA is the primary product of carboxylation. Although these arguments hold true for leaves, where photosynthesis is essential for growth, they do not necessarily hold true for fruits, which have a second source of energy. As the fruit must not rely on its own photosynthate the regeneration phase of the Calvin cycle is not of such importance and, therefore, it is reasonable to assume that reductions in cp-FBPase would result in increases in 3-PGA.

The starch content in two out of three of the transgenic lines was not consistently altered in comparison with the WT control. One line (#34) did produce less starch than the WT control, but this was the line which was least inhibited in FBPase activity indicating that the reduction is not related to the reduction in cp-FBPase activity. Reduction in cp-FBPase activity in potatoes did lead to a reduction in starch accumulation in leaves **(Kossmann *et al.*, 1994)**.

This differences seen in the fruits of the transgenic lines here in comparison with the situation in potato leaves probably due to a major difference between starch production in leaves and fruits. Tomato fruit chloroplasts contain both an active glucose phosphate and a triose phosphate transporter **(Büker *et al.,* 1998)**. Chloroplasts in leaves are not thought to contain a glucose phosphate transporter as, leaves do not appear to accumulate transcript that codes for this transporter **(Kammerer *et al.,* 1998)**. Repression of cp-FBPase activity should reduce flux of carbon produced by photosynthesis into starch. As no consistent reductions in starch were found in the transgenic lines it appears that fruit plastids can compensate for any reduction in flux from photosynthesis, presumably by importing glucose phosphate formed by cytoplasmic sucrose degradation. It appears that AGPase activity was reduced in the transgenic lines by up to 60 %. This is something that would be expected to reduce starch contents, but in the next chapter I will demonstrate that AGPase has to be repressed to a greater extent than was found in the transgenic lines before starch accumulation is affected. In addition the ratio of 3PGA to Pi in the pericarp of the transformants is elevated in the transgenic tomato lines (Table 1), which would be expected to stimulate AGPase activity and might compensate for any reductions in activity. In addition PGM activity was reduced in the transgenic lines at early stages of development. PGM exists as two isoforms, one in the cytoplasm and one in the plastid each of which contributes approximately 50 % of the total activity. It is known that the plastidial isoform is essential for starch biosynthesis **(Hanson *et al,* 1988; Harrison *et al.,* 1998; Tauberger *et al.,* 2000)**, but it is not clear from my measurement which isoform is reduced in activity.

No significant differences were found between the WT control and all of transgenic lines with respect to either number of flower per plant or number of days from planting to 50% flowering. Two transgenic lines (#19 and #33), however, showed significant reductions in comparison with the WT control in respect to both number of fruit per plant and fruit set. These are the two most strongly inhibited lines indicating that this reduction in fruit set is indeed due to the reduction in FBPase activity. It is difficult to understand, however, how cp-FBPase could influence fruit set. It might be possible that its repression leads to reduced pollen viability or to alteration in ovule development. To my knowledge it is not known whether the B33 promoter confers expression in these tissues. If it did then this could be a feasible explanation. It has been demonstrated previously, for example, that reductions in citrate synthase activity in potato leads to disintegration of the ovary (**Landschütze *et al.,* 1995**), indicating that alterations in metabolism can indeed influence fertility. A second

possibility is that very young fruit abort for some unknown reason due to repression of cp-FBPase affecting carbohydrate metabolism within the fruit. These possibilities are something that could be investigated in the future.

One final question that I wished to address was how much carbon was supplied to the fruit through photosynthesis in the fruit itself. I have demonstrated above that cp-FBPase is repressed in the fruits of the transgenic plants, and it is known that this leads to reductions in photosynthesis **(Kossmann *et al.*, 1994)**. In addition, the metabolic analysis that I have carried out (discussed above) indicates that photosynthesis is indeed repressed in the fruits of these transgenic plants by phosphate limitation. The most likely explanation, therefore, for any effect on yield would be that it is caused by repression of photosynthesis in the fruits. Repression of cp-FBPase led to a reduction in both the weights and sizes of ripe fruits in all three transgenic lines (Fig. 13, Table 2). There are three possibilities as to how this could occur. The first is that the reduction in cp-FBPase leads to a reduction in sink strength in the fruits and, thus, to decreased import of carbon from the leaves. This seems unlikely, as it would be expected that reducing the amount of carbon produced by photosynthesis in the fruit would actually increase demand for carbon from the fruit. The second possibility is that in the transgenic plants there is less carbon fixed in the leaves and, thus, less exported to the fruits. This again appears unlikely as there was no effect of the transformation on FBPase activity in the leaves, indicating that the repression was fruit specific and, thus, that the only direct effects would be found in the fruits. The third, and most likely explanation is that the reduction in fruit weight represents the carbon produced within the fruit by photosynthesis. This explanation is supported as the reduction in average fruit weight was between 15-20% in the three lines. This is very similar to the estimate of the contribution of photosynthesis to production of carbon skeletons in green tomato fruits of **Tanaka *et al.* (1974)**.

From the data presented in this chapter, it can be concluded that: (A) cp-FBPase activity is almost completely eliminated in the two most strongly inhibited lines (#19 and #33). (B) Repression of cp-FBPase in fruits leads to some alterations in concentrations of soluble sugars in young fruits. (C) The metabolic profile in fruits of the transgenic lines strongly indicates that photosynthesis is inhibited *in* vivo. (D) Fruit weight and size is reduced in the transgenic lines suggesting a significant contribution of fruit photosynthesis in the provision of carbon and energy required to support fruit expansion. (G) Repression of cp-FBPase affected fruit set, but the reason for this remains unclear.

6 Functional Analysis of ADP-glucose Pyrophosphorylase in Tomato Fruit

6.1 Introduction

ADP-glucose pyrophosphorylase (AGPase) catalyses the first reaction on the committed pathway of starch biosynthesis. In higher plants, AGPase is a heterotetramer consisting of two large and two small sub-units which are 54 and 51 kDa in size respectively (see **Preiss, 1991**). It is known that it is essential for starch production as reductions in its activity in mutant and transgenic plants leads to reductions in starch contents (**Tsai and Nelson 1966, Dickinson and Preiss 1969; Lin *et al.*, 1988; Smith *et al.*, 1989; Müller-Röber *et al.*, 1992**).

Until recently it was thought that the AGPase enzyme was located solely in the plastid in all plant species. Evidence over the past decade, however, has indicated that in cereal endosperm there is a second isoform present in the cytosol also, which contributes the majority of the total activity, at least in barley, maize, wheat and rice (**Thorbjørnsen *et al*, 1996a, Denyer *et al.* 1996; Sikka *et al.*, 2001; Burton *et al.*, 2002**). As starch is manufactured within the plastid this would mean that in cereal endosperm any ADP-glucose produced in the cytosol would have to be imported into the amyloplast. It is thought that this is performed by a protein named Brittle-1. Mutations in the gene coding for this protein in maize lead to a decreased starch content (**Sullivan *et al.*, 1991**) and increased ADP-glucose concentrations (**Shannon *et al.*, 1996**), both of which would be expected if the protein has this function. In addition, it has been localized in the amyloplast membrane (**Cao *et al.*, 1995, Sullivan and Kaneko, 1995**) and analysis of its primary protein sequence indicates that it is a sugar-nucleotide transporter. There is still some controversy about whether a similar system is present in tomato fruits. One immunolocalisation study indicated that AGPase was present both inside and outside the plastid in tomato pericarp (**Chen *et al.*, 1998**), however, its activity was found only in the plastid fraction upon sub-cellular fractionation (**Beckles *et al.*, 2001a**).

Starch accumulates transiently in tomato fruits being present when they are immature, but not when they are ripe. Its role in tomato fruit metabolism is not understood, but it has been proposed that carbohydrate metabolism in this organ is controlled by a number of different futile cycles, one of which involves continuous synthesis and degradation of starch (**Nguyen-Quoc and Foyer, 2001**). In that paper it was proposed that repression of AGPase in tomato fruits would be a good method to examine any such futile cycle.

6.2 Aim of the work

The aim of the work described in this chapter is to repress the activity of AGPase in tomato plants and study the effect on fruit metabolism. It would be hoped that these data would help to answer the question as to whether AGPase is situated both inside and outside the plastid in tomato fruit, and to help to understand the role, if any, of a futile cycle of starch degradation and synthesis in carbohydrate metabolism in tomato fruits.

6.3 Results

6.3.1 Recovery of plants with reduced AGPase activity in the pericarp of tomato fruit

The pericarp of 25 days after flowering (DAF) old tomato fruits were analysed for AGPase activity in forty transgenic lines. Three lines (#2, #7 and #11) showed reductions in AGPase activity and were chosen for further study (see Material and Methods). The transgenic plants themselves were phenotypically identical to the untransformed control (Fig. 14).

AGPase activity was studied throughout fruit development in the pericarp of these plants. There was a significant reduction in AGPase activity in all three transgenic lines throughout fruit developing in comparison with the WT control (Fig. 15A). Initially the reduction in activity of line #7 was 90%, and in the other two lines 70%. The activity in the control decreased during the ripening process, but the activity in all transgenic lines relatively constant during the ripening process. AGPase activities were significantly reduced in comparison with the WT control until 55 DAF in transgenic line #2, and at every time point studied in the other two lines.

Immunoblots using an antibody raised against the a sub-unit of maize AGPase **(Müller-Röber, et al., 1992)**, but which recognizes the tomato protein also indicated that it was completely eliminated in 25 DAF fruit of the lines #7 and greatly reduced in the lines #11 and #2 (Fig. 15B).

6.3.2 Starch and soluble sugar contents in the pericarp of the WT and transgenic lines

Starch and soluble sugar contents were determined in the pericarp between 25-70 DAF. There was net degradation of starch over the ripening period (Fig. 16A). In the WT control the concentration was approximately 18µmol hexose (g FW)$^{-1}$ at 25 DAF, and was 0.3µmol hexose (g FW)$^{-1}$ at the final sample point. The starch contents in lines #2 and #11 were not significantly different in comparison with the control over the entire sampling period, but

those of line #7 were significantly reduced until 50 DAF, after which there were no differences.

Figure 14: Aerial parts of plants in both WT control and α-AGP-transgenic lines after 13 weeks growth in the glasshouse. From left to right: WT control, transgenic line #2, transgenic line #7, transgenic line #11 and transgenic line #11. The α-AGP plants are phenotypically identical to the untransformed WT control.

Both glucose and fructose concentrations increased slightly over time in both the control and the transgenic lines. Glucose concentrations were significantly increased in the WT control in comparison with the three transgenic lines, but only between 55 and 70 DAF. At these time points the concentrations in the WT control fruits increased from approximately 70 to 110 µmol (g FW)$^{-1}$, while in the transgenic fruits they increased from approximately 60 to 90 µmol hexose (g FW)$^{-1}$ (Fig. 16B). There were no significant differences in fructose concentrations between the transgenic lines and the WT control (Fig. 16C).
Sucrose concentrations stayed between 2-4 µmol (g FW)$^{-1}$ in all lines from the first sampling point until 60 DAF, after this point they increased to between 6-8 µmol (g FW)$^{-1}$. The only significant differences in sucrose concentrations between the transgenic lines and the WT

control were at 65 and 70 DAF (Fig. 16D). At these time points there was a significantly greater concentration of sucrose in the pericarp from control than the transgenics.

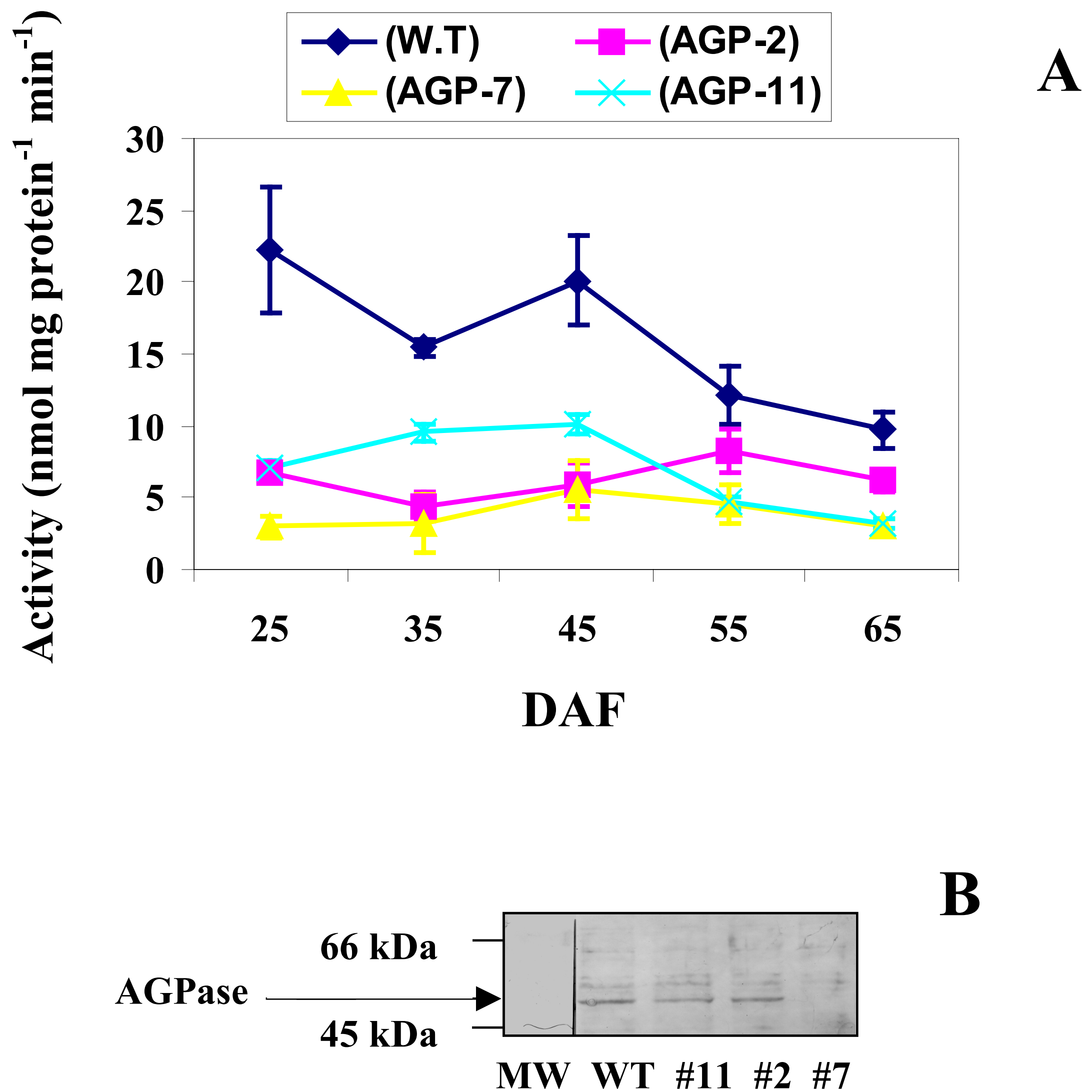


Figure 15: AGPase activity during developmental stage (A) and Western blot analysis in green (25 DAF) (B) in the pericarp of WT control and α-AGP-transgenic lines. Total soluble fruit protein (25µg) was subjected to SDS-PAGE on a 10% (w/v) gel.

6.3.3 Changes in activities in enzymes involved in conversion of sucrose to starch

Sucrose synthase (SuSy) activity approximately 100nmol min^{-1} (mg protein)$^{-1}$ in the pericarp of the control plants at 25 DAF. This decreased to about 25nmol min^{-1} (mg protein)$^{-1}$ at 45 DAF, after which it remained relatively constant. There were no significant differences in

SuSy activity between the transgenic lines and the WT control except at 45 DAF in line #7 where the activity was significantly greater than in the WT control (Fig 17A).

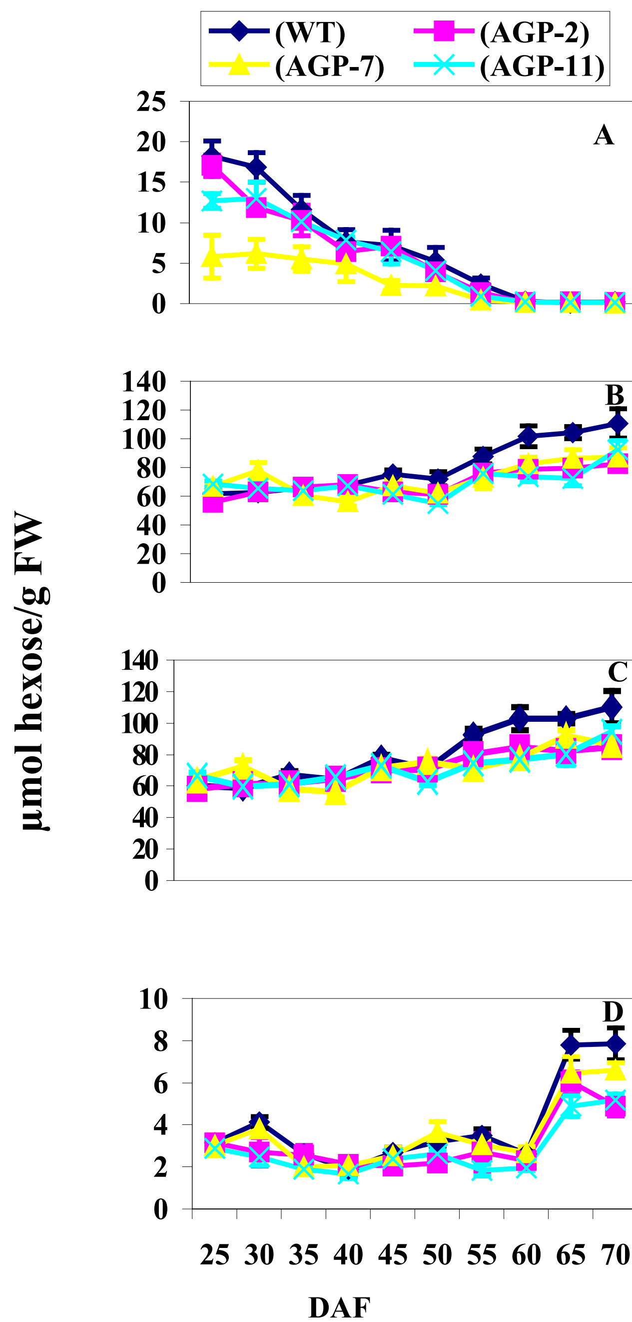


Figure 16: Starch and soluble sugar contents in the pericarp of the WT control and α-AGP-transgenic lines.(A) Starch. (B) Glucose. (C) Fructose. (D) Sucrose. Data represent the mean of five independent measurements + SE in both WT control and transgenic line #7, but four independent measurements + SE in transgenic line #2 and transgenic line #11.

The activity of UDP-glucose pyrophosphorylase (UGPase) increased from approximately 500nmol min^{-1} (mg protein)$^{-1}$ in the WT control at 25 DAF to 1000nmol min^{-1} (mg protein)$^{-1}$ at 55 DAF, before decreasing to 530nmol min^{-1} (mg protein)$^{-1}$ at 65DAF. Some significant differences in activities were noted in the transgenic lines. Activities were significantly reduced in line #2 at 35 DAF and in line #7 at 65 DAF, however the activitiy was significantly increased in line #11 at 45 DAF (Fig 17B).

Phosphoglucomutase (PGM) activity increased from 416nmol min^{-1} (mg protein)$^{-1}$ to 732nmol min^{-1} (mg protein)$^{-1}$ in pericarp from the control between 25 and 45 DAF. It then decreased to 306nmol min^{-1} (mg protein)$^{-1}$ at 65 DAF. The activity in all the transgenic lines was significantly reduced in comparison with the control at 35DAF and was also significantly reduced in line #2 at 45DAF (Fig 17C).

Fructose-1,6-bisphosphatase (FBPase) activity decreased from 12nmol min^{-1} (mg protein)$^{-1}$ at 25 DAF to 4nmol min^{-1} (mg protein)$^{-1}$ at 65 DAF in the WT control. The activities in the transgenic lines were not significantly altered, except at 65 DAF in line #7 when a significant reduction in activity was found (Fig 17D).

6.3.4 Concentration of metabolic intermediates in the pericarp of the WT control and transgenic lines

The concentration of some metabolites were measured in trichloroacetic acid extracts from 30 DAF fruits. The metabolites determined were Glc-6-P, Glc-1-P, Fru-6-P, 3-PGA, PEP, pyruvate and Pi. The data are representing in Table 4. Few significant differences were found between the transgenic lines and the WT control. Both PEP and 3-PGA were significantly reduced ($P \leq 0.01$, $P \leq 0.05$ respectively) in line #7. Pyruvate concentrations were also significantly reduced ($P \leq 0.05$) in lines #2 and #11. No significant differences were found between the transgenic lines and the control for total hexose-P, the hexose-P to 3-PGA ratio and the total phosphateester to Pi ratio, however the 3-PGA to Pi ratio was significantly reduced ($P \leq 0.01$) in line #7.

6.3.5 Analysis of fruit yield

Fruit were harvested, and their weights and sizes determined after 65 DAF. Some fruits of one transgenic line (#7) can be seen in comparison with the WT control in Fig. 18. As can be seen in Table 5 in one transgenic line (#7) the average weights and sizes of harvested fruits were

significantly (P≤ 0.01) reduced in comparison with the WT control. This reduction was 22% and 10% of respectively the weights and sizes of the WT control fruits.

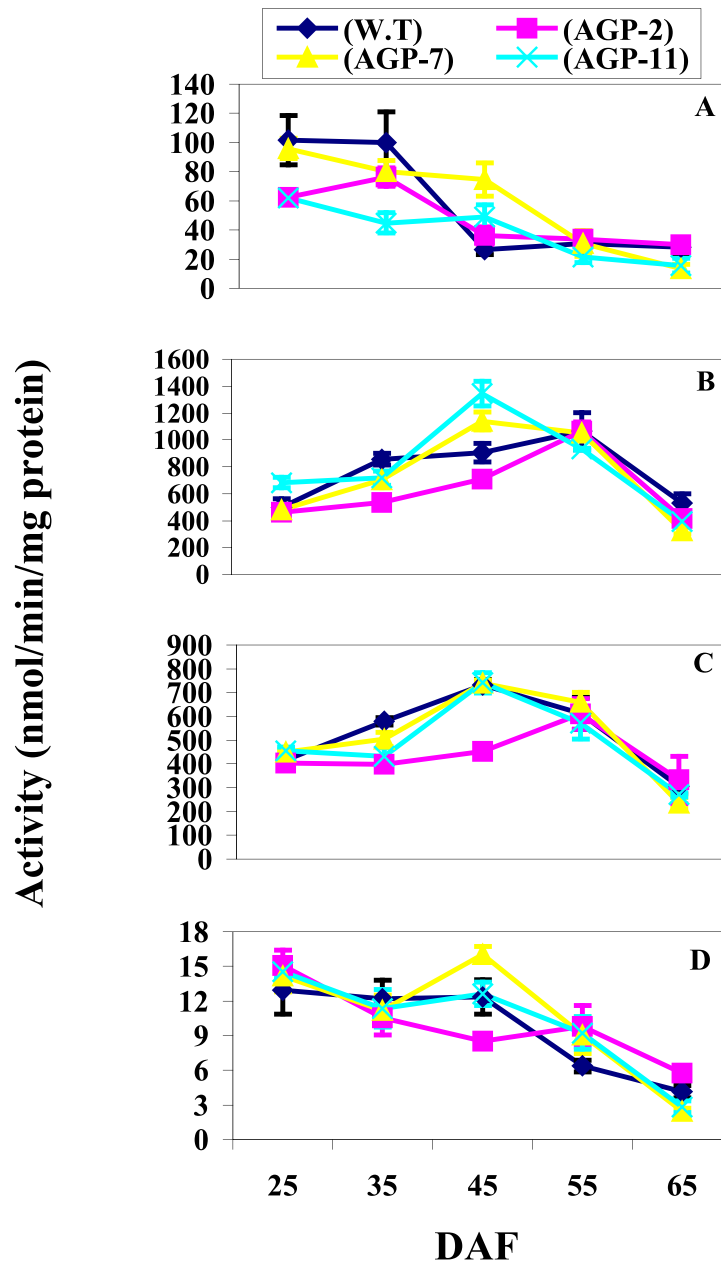


Figure 17: Activities of enzymes involved in the conversion of sucrose to starch in pericarp of the WT control and α-AGP transgenic lines of fruit of the tomato cultivar Moneymaker. (A) SuSy. (B) UGPase. (C) PGM. (D) FBPase. Data represent the mean of five independent measurements + SE in both WT control and transgenic line #7 and four independent measurements + SE in transgenic line #2 and transgenic line #11.

Table 4: Metabolite concentrations in the pericarp of 30 DAF old WT control and α-AGP-transgenic lines.

Conc. nmol.(g FW)$^{-1}$	Lines			
	WT	#2	#7	#11
G6P	67.6 ± 5.5	64.9 ± 3.9	65.9 ± 3.0	56.1 ± 1.8
G1P	9.3 ± 0.7	8.5 ± 0.4	7.9 ± 0.3	8.6 ± 0.1
F6P	22.0 ± 2.1	21.2 ± 2.3	21.0 ± 1.1	18.1 ± 1.2
Total Hexose-P	98.8 ± 7.8	94.6 ± 6.0	94.8 ± 4.1	83.8 ± 2.6
3-PGA	17.4 ± 0.6	16.4 ± 1.7	**13.8 ± 0.8**	18.7 ± 0.5
PEP	5.3 ± 0.6	4.1 ± 0.5	**2.4 ± 0.4**	5.0 ± 0.4
Pyruvate	6.3 ± 0.9	**3.4 ± 0.2**	4.1 ± 0.5	**3.5 ± 0.4**
Pi	1.1 ± 0.01	1.2 ± 0.11	1.2 ± 0.11	1.2 ± 0.05
Ratio				
Hexose-P/3-PGA	5.1 ± 0.29	7.0 ± 0.92	6.7 ± 0.20	4.4 ± 0.05
P-ester/Pi	106.0 ± 7.8	93.2 ± 7.5	90.2 ± 4.6	88.9 ± 6.0
3-PGA/Pi	15.8 ± 0.6	14.1 ± 2.1	**11.3 ± 0.4**	16.2 ± 1.2

The data represent means + SE of five independent samples. Significant differences (P≤ 0.05 and P≤ 0.01, Student's t-test) are in bold.

Table 5: Weights and sizes of ripe tomato fruits in the WT control and α-AGP-transgenic lines.

Lines	Average	
	Weight of Fruit (g)	Size of Fruit (cm)
WT	56.7 ± 2.0 (n=53)	4.8 ± 0.07 (n=53)
#2	59.3 ± 2.7 (n=44)	4.9 ± 0.08 (n=44)
#7	**44.7 ± 1.6** (n=46)	**4.3 ± 0.06** (n=46)
#11	55.6 ± 1.9 (n=44)	4.8 ± 0.07 (n=44)

Data are means ± SE, number of sample is in parentheses. Significant differences (P≤ 0,01), Student's *t*-test are in bold.

Figure 18: Some 65 DAF old fruits from α-AGP-transgenic line #7 (bottom) in comparison with the WT control fruit (above).

6.3.6 Number of flowers, fruits per plant, fruit set and number of days to 50% flowering

Data of these traits are presented in Table 6. The only significant difference in the transgenic lines was that line #7 took longer to reach 50% of its flowers than the control.

Table 6: Number of flowers, fruits, fruit set and number of days to 50% flowering in the WT control and the transgenic lines.

Lines	Average			
	No. of flower/plant	No. of fruit/plant	Fruit set %	No. of days to 50% flowering
WT	50.8 ± 4.7	42.2 ± 3.4	83.6 ± 3.8	54.0 ± 0.5
#2	49.0 ± 3.6	39.0 ± 3.7	79.2 ± 2.3	55.5 ± 1.2
#7	45.4 ± 3.4	34.2 ± 1.9	76.6 ± 6.0	**59.8 ± 1.3**
#11	56.3 ± 3.0	48.5 ± 1.9	86.4 ± 1.3	56.5 ± 1.0

Data represent the mean of five independent measurements ± SE in the WT control and in transgenic line #7, but four independent measurements ± SE in both transgenic lines #2 and #11. Significant (P≤ 0,01, Student's *t*-test) differences from the control are in bold.

6.4 Discussion and conclusion

In this study I have described the production of transgenic tomato plants repressed in AGPase activity using a constitutive promoter. The transgenic plants appeared phenotypically normal, indicating that repression of AGPase activity has no effect on growth of the plants under the conditions used. In addition the activities of other enzymes involved in the conversion of sucrose to starch were not consistently altered indiacting that reductions in AGPase activity did not lead to pleiotropic effects on fruit metabolism.

AGPase activity was demonstrated to be reduced throughout fruit development in the pericarp of these plants, and the degree of reduction correlated with reductions in amount of AGPase protein as determined by immunoblots. This reduction in activity ranged between 70 to 90% of the WT control activity in the different transgenic lines at 25 DAF, with line #7 being the most repressed. AGPase activity in the WT control decreased during fruit ripening, as did starch contents in the pericarp. This is interesting as AGPase activity has often been associated with starch accumulation both in tomato (**Yelle *et al.*, 1988**) and other plants (**Okita, 1992; Preiss, 1988, 1991**). It has indeed been suggested that it may catalyse a rate-limiting step in starch accumulation (**Stark *et al.*, 1992**), although this still remains a controversial idea. My data indicates that AGPase activity is in excess in tomato fruits as repression of its activity by 70% did not greatly alter starch accumulation. It was only in the one line (#7) where activity was reduced by 90 % that an effect on starch accumulation was found. The starch content of that line was only approximately 25 % that of the WT control. This is similar to the data found in the study of **Müller-Röber *et al.* (1992)**, who repressed AGPase activity in potatoes using the same construct as in this study. They found that decreased starch contents were only found when AGPase activity was reduced by more than 50 %. This indicates that in tomato fruit AGPase does not control the amount of starch that accumulates. If that were the case it would be expected that a straight-line relationship would be found between its activity and starch content. Although there is obviously some influence of AGPase on starch content (as demonstrated by line #7), the reductions in activities in lines #2 and #11 were not mirrored by reductions in its accumulation. This indicates that other enzymes also influence the rate of its synthesis. In *Arabidopsis* and potato it has been demonstrated that plastidial PGM – which catalyses the step prior to AGPase – can influence the rate of starch synthesis (**Neuhaus and Stitt, 1990; Fernie *et al.*, 2001**), and it might be that this is the case in tomato also.

In a previous study AGPase was repressed in potato (**Müller-Röber *et al*., 1992**), which led to plants producing tubers containing only a small amount of starch, but high levels of soluble sugars, mainly sucrose and glucose. Such drastic differences were not found in the fruits of the transgenic tomato plants in this study. As was said above, starch contents were reduced in only one of the transgenic tomato lines, but this was not accompanied by increases in soluble sugar concentrations. The only differences noted in respect to this was that there were slight decreases in glucose and sucrose concentrations at the final two sampling points, a time when the fruits are ripe. These were precisely the points where it would not be expected that AGPase would have an influence as both its activity, and starch contents in the pericarp are extremely low. In addition the same reductions in glucose and sucrose were noted in the two transgenic lines where starch was not reduced (#2 and #11). Soluble sugar concentrations are often quite variable, and I feel that it is most likely that these small differences are due to the small number of probes taken (four or five) rather than due to some difference caused by the transgene.

The lack of increase in soluble sugars demonstrates that starch is not used as a major carbon reserve in tomatoes. This is again different to the situation in a potato tuber where the starch content is generally between 25-50 times greater than the maximum amount found in the pericarp in this study. Tomato fruits obviously store more carbon in the form of soluble sugars than starch. In this study in green fruits – the time point when starch contents are maximal and sugar contents minimal - there was approximately six fold more carbon present as glucose, sucrose and fructose than starch. It is, therefore, not surprising that reductions in starch in line #7 did not greatly influence sugar contents as it makes up such a small proportion of the total metabolisable carbon in the fruit.

One aim of this study was to use a genetic approach to examine whether AGPase is present solely in the plastid in tomato fruit. The reason why that is possible is that it is known that the construct I used represses a plastidial isoform of AGPase. This is demonstrated as starch contents are reduced in potato leaves where this isoform has been repressed (**Leidreiter *et al.*, 1995**). Although it is now generally accepted that cereal endosperms contain an extra-plastidic AGPase isoform, it is still assumed that leaves do not as the starch is manufactured from carbon derived directly from photosynthesis, a process that occurs solely in the chloroplast. My data does not rule out the possibility of a cytosolic form of AGPase in tomato fruit as I was only able to repress up to 90 % of the AGPase activity and it is possible that the remaining 10 % of the activity is extra-plastidial, however it does demonstrate that the

majority of the activity is in the plastid. The assumption for this statement is that there is no differential splicing mechanism of the *AGPase* gene in tomato. In wheat and barley it has been demonstrated that the gene coding for the small sub-unit of AGPase is subject to alternative splicing giving rise to two forms of the protein, one of which can be imported into plastids and one which cannot (**Thorbjonsen *et al.*, 1996b; Burton *et al.*, 2002**). This seems unlikely in tomato based on the strong evidence in potato that the *AGPase* small sub-unit gene is not differentially spliced. As potato and tomato are so closely related it is reasonable to assume that is also the case in tomato. The evidence in potato that there is no cytoplasmic AGPase is that when a bacterial form of AGPase was expressed in either the cytoplasm or plastid in potato tubers, increases in starch contents were found only upon expression in the the plastid (**Stark *et al.*, 1992**). If there was a cytosolic form it would be expected that expression in the cytoplasm would lead to increased manufacture of starch also.

In this study I analysed the yield of tomato fruit and found that the both average weights and sizes of fruits in one transgenic line (#7) were significantly ($P \leq 0.01$) reduced in comparison with the WT control. This was the strongest inhibited line, and the only one with a reduced starch content, indicating that this phenotype may well be due to the inhibition of AGPase activity. Unfortunately as this was only found in one line, somaclonal variation cannot be ruled out. The same argument holds true for the delay in flowering noted in line #7. To study whether this was really an effect of strong reductions in AGPase activity, other lines which are equally strongly inhibited would have to be identified.

From the data presented in this chapter it can be concluded that: The line exhibiting the greatest level of AGPase inhibition was characterised by a depressed starch content, significant reduction in fruit yield and a delayed flowering.

7 Analysis of the Function of the GWD protein in Tomato Fruit

7.1 Introduction

Starch is one of the most abundant polymers produced in nature and is synthesized as a storage carbohydrate throughout the plant kingdom. In storage organs it serves as a long-term carbon reserve, whereas in photosynthetically competent tissues it is transiently accumulated to provide both reduced carbon and energy during periods unfavourable for photosynthesis. Starch comprises both linear (amylose) and branched (amylopectin) glucose polymers. Amylopectin from many, but not all plant sources contains phosphate-monoesters that are linked mainly to the C6 and C3 positions of glycosyl residues. The biochemical mechanism of starch phosphorylation has, however, only recently been elucidated. Transgenic potato plants (**Lorberth *et al.*, 1998**) and the sex1 mutant of *Arabidobsis* (**Yu *et al.*, 2001**) are deficient in a starch associated protein, which was provisionally designated as R1, and they synthesise starch with a decreased phosphate content. The purified recombinant R1-protein from potato is able to phosphorylate α-glucans (**Ritte *et al.*, 2002**). It catalyses a dikinase-type of reaction, liberating the γ-phosphate of ATP (resulting in the release of orthophosphate), but using the β-phosphate to phosphorylate glucosyl residues the polyglucan. Because of this the protein has been renamed as GWD (**G**lucan **W**ater **D**ikinase; **Ritte *et al.*, 2003**).

The phosphorylation of starch strongly affects its *in vivo* degradability. This is indicated by the starch-excess phenotype observed in leaves of GWD deficient potato or *Arabidopsis* plants (**Lorberth *et al.*, 1998**; **Yu *et al.*, 2001**). The reasons for this impairment of starch-degradation are, as yet, unknown.

7.2 Aim of the work

The role of starch in tomato fruits is not well understood. It accumulates in young fruits, but afterwards there is net starch degradation leading to it being almost undetectable in ripe fruits. One way of examining this is to alter how much starch accumulates during fruit development. In a previous chapter I described the repression of ADP-glucose pyrophosphorylase (AGPase) in tomato, which leads to reductions in starch accumulation. It would also be interesting, however, to take the reverse approach and study tomato fruits that accumulate starch over a longer time period than normal. As repression of the GWD protein leads to reductions in starch degradation I decided to try and accomplish this by manipulating the amount of GWD protein using genetic engineering techniques

7.3 Results

7.3.1 Recovery of Tomato Plants with Repression of the GWD Protein

As was described in the Material and Methods, immunoblots demonstrated that three transgenic lines (#16, #17 and #20) showed a reduction in GWD protein accumulation (Fig. 20A) and were chosen for further study. The plants in all of these transgenic lines differed phenotypically form the untransformed control (Fig. 19). The leaves senesced much earlier than the control.

Figure 19: Aerial parts of plants in both WT control and α-GWD-transgenic lines after 8 weeks growth in the glasshouse. From left to right: WT control, transgenic line #16, transgenic line #17, transgenic line #20 and transgenic line #20.

To examine at what developmental period the GWD protein is present in tomato fruits, immunoblots was performed using protein extracts from differently aged WT fruits (Fig. 20C) and an antibody raised against the potato GWD protein **(Ritte et al. 2000)**. This showed that it was present at every time point up to 50 DAF, after which it was absent. Immunoblots were also used to demonstrate that the protein was repressed in the pericarp of the transgenic plants

also and it appeared to be completely absent from 25 DAF fruits in all of the transgenic lines (Fig 20B).

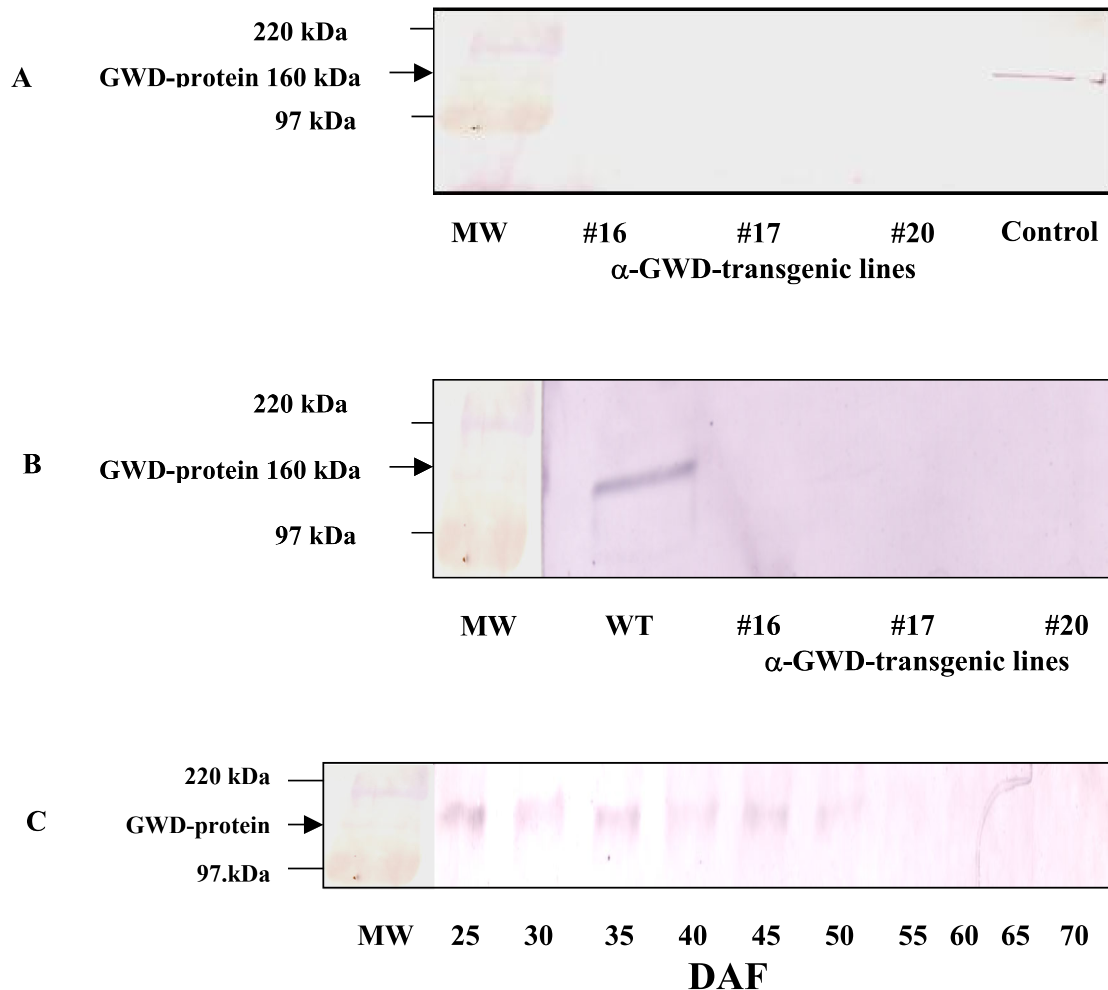


Figure 20: Immunoblot analysis of the GWD protein in (A) leaves of untransformed WT control and three selected transgenic lines [Total soluble leaf protein (15µg) was subjected to SDS-PAGE on an 8% (w/v) gel], **(B) in the pericarp of the WT control and transgenic lines (25 DAF)** [Total soluble fruit (pericarp) protein (30µg) was subjected to SDS-PAGE on an 8% (w/v) gel] **and (C) in the pericarp of the WT control fruits between 25-70 DAF** [Total soluble fruit protein (20µg) was subjected to SDS-PAGE on an 8% (w/v) gel].

7.3.2 Starch and soluble sugar contents in the pericarp of the WT and transgenic lines

Starch and soluble sugar contents were determined in the pericarp between 25-70 DAF. There was net degradation of starch over the ripening period (Fig. 21A). In the control the concentration was approximately 8µmol hexose $(g\ FW)^{-1}$ at 25 DAF, and was less that 0.5µmol hexose $(g\ FW)^{-1}$ at the final sample point. The starch contents in all of transgenic

lines were significantly reduced in comparison with the control until 45 DAF after which there were no differences.

There were no significant differences in glucose concentrations between the transgenic lines and control between 25 and 35 DAF. After that the concentrations were significantly decreased in the three transgenic lines in comparison with the WT. At these time points the concentrations in the transgenic fruits were between 40-50 µmol hexose (g FW)$^{-1}$, while in the control fruits they were between 60-90 µmol (g FW)$^{-1}$ (Fig. 21B).

Fructose concentrations were significantly increased in the three transgenic lines in comparison with the control, but only between 25 and 35 DAF. At these time points the concentrations in the transgenic fruits were between 40-55 µmol hexose (g FW)$^{-1}$, while in the control fruits they were between 15-18 µmol (g FW)$^{-1}$ (Fig. 21C). Between 40-70 DAF, however, the fructose concentrations in the transgenic lines were significantly decreased in comparison with the control. During this period the concentrations in the transgenic fruits ranged between 40-50 µmol hexose (g FW)$^{-1}$, while in the control fruits they were between 60-100 µmol (g FW)$^{-1}$.

Sucrose concentrations in the pericarps of all the transgenic lines were significantly lower than the control at virtually every time point. The exceptions were in line #20 at 30 DAF and line #17 at 65 and 70 DAF where the sucrose concentration was not significantly altered in comparison with the control (Fig. 21D) In the controls the sucrose concentration decreased from about 3µmol hexose (g FW)$^{-1}$ at the first sampling point, to about 1.5µmol hexose (g FW)$^{-1}$ at the final sampling point. Although variable, in the transgenic lines there was a general decrease in sucrose concentrations from approximately 1.5µmol hexose (g FW)$^{-1}$ at 25 DAF to barely detectable levels at the final sample point.

7.3.3 Starch and soluble sugar contents in the leaves of the WT and transgenic lines

The starch and soluble sugar contents were determined also in the leaves of the control and transgenic plants (Fig.22A and B). The all of transgenic lines contained significantly more starch than the control (Sampling was done on fully developed leaves of 8 weeks old plants in the middle of the light period) (Fig.22A). In the control the concentration was 15.1µmol hexose (g FW)$^{-1}$, but in the transgenic lines the concentrations were 26.5, 36,2, and 28,4µmol hexose (g FW)$^{-1}$ for lines #16, #17 and #20 respectively.

There were significantly increased glucose concentrations in leaves of the transgenic lines in comparison with the control (Fig.22B). In the control the glucose concentration was 16.5µmol

hexose (g FW)$^{-1}$, but in all the transgenic lines it was approximately 21.0 µmol hexose (g FW)$^{-1}$. Fructose concentrations conversely were significantly decreased in the three transgenic lines in comparison with the control (Fig.22B). The fructose concentration in the control was 22.2µmol hexose (g FW)$^{-1}$, but in the transgenic lines they were 6.3, 6.6 and 7.0µmol hexose (g FW)$^{-1}$ in lines #16, #17, and #20 respectively. Sucrose concentrations were significantly increased in the three transgenic lines in comparison with WT control (Fig.22B). In the control this was 3.6µmol hexose (g FW)$^{-1}$, however, in the transgenic lines the concentrations were 6.5, 5.3 and 6.9µmol hexose (g FW)$^{-1}$ in lines #16, #17, and #20 respectively

7.3.4 Changes in activities in enzymes involved in conversion of sucrose to starch

Sucrose synthase (SuSy) activity decreased over time in fruits of both the control and transgenic plants. Significant reductions in activities were found in the transgenic lines at 55 DAF (line #17) and 65 DAF (lines #16 and #20; Fig 23A). UDP-glucose pyrophosphorylase (UGPase) activity was high in both the control and transgenic lines, increasing between 25-55 DAF. It then decreased in activity between 55-65 DAF. The pericarp from transgenic lines #17 and #20 contained significantly reduced UGPase activity at 45 DAF else there were no significant differences between the control and transgenic lines (Fig 23B). Phosphoglucomutase (PGM) activity decreased over the developmental period in all the lines. There were significant reductions in activity of PGM in comparison with the control in all the transgenic lines at 25 DAF, in line #17 at 35 and 55 DAF, and in lines #17 and #20 at 65 DAF (Fig. 23C). ADP-glucose pyrophosphorylase (AGPase) activity decreased over time in the control from 39.3 nmol min^{-1} (mg protein)$^{-1}$ at 25 DAF to 19.5 nmol min^{-1} (mg protein)$^{-1}$ at 65 DAF (Fig. 23D). In the pericarp of the transgenic lines there was a significant reduction in AGPase activity at 25 DAF (lines #17 and #20), 35 DAF (line #20), 45 DAF (lines #17 and #20), 55 DAF (line #17) and 65 DAF (line #20). Fructose-1,6-bisphosphatase (FBPase) activity was generally greater in the WT control in comparison with the ά-GWD-transgenic lines. The activity decreased slowly over time period in both WT control and ά-GWD-transgenic lines (Fig. 23E).

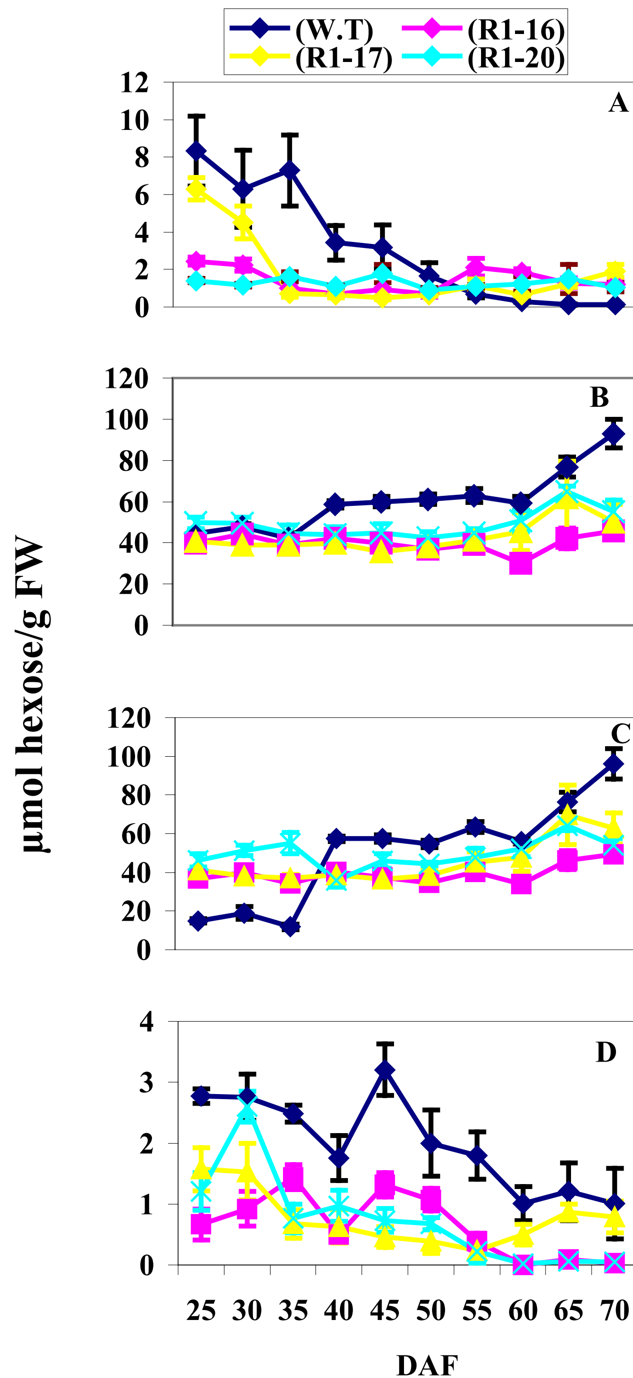


Figure 21: Starch and soluble sugar contents in the pericarp of the WT control and α-GWD-transgenic lines in tomato cultivar Moneymaker during development. (A) Starch. (B) Glucose. (C) Fructose. (D) Sucrose. Data represent the mean of five independent measurements + SE in the WT control and transgenic line #17, but four independent measurements + SE in transgenic lines #16 and #20

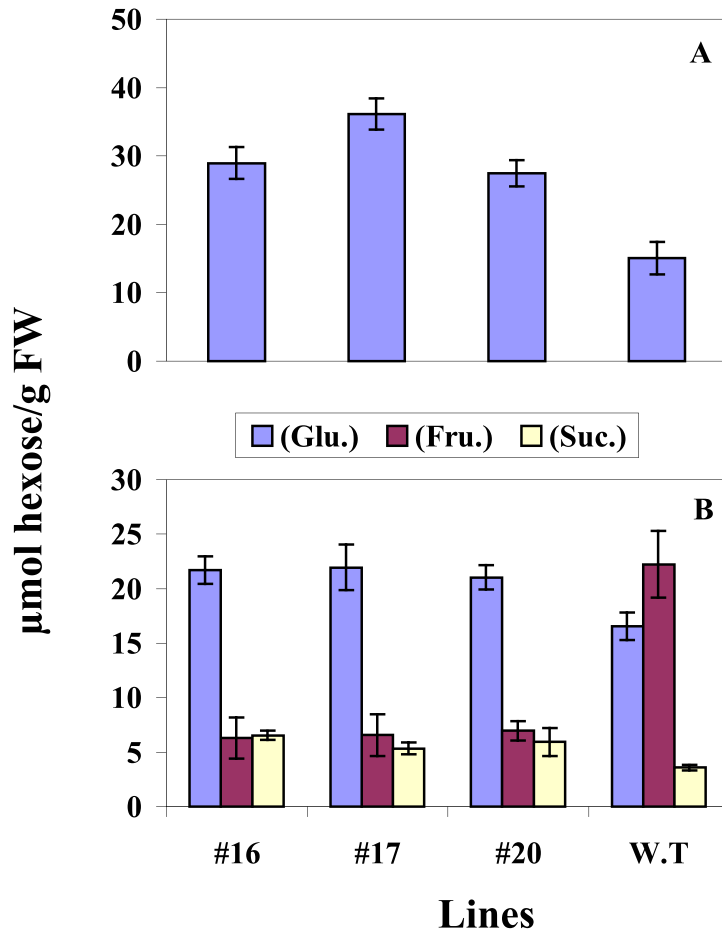


Figure 22: Starch and soluble sugar contents in the leaves of the WT control and transgenic tomato lines lacking the GWD protein. (A) Starch. (B) Soluble sugars. Data represent the mean of five independent measurements + SE in the WT control and transgenic line #17, but four independent measurements + SE in transgenic lines #16 and #20.

7.3.5 Analysis of fruit yield

Fruits were harvested and their weights and sizes determined after 65 DAF. Some fruits of the transgenic line can be seen in comparison with the WT control in Fig. 24. As can be seen in Table 7 both the average weights and sizes of fruits in all of the transgenic lines were significantly ($P \leq 0.01$) reduced in comparison with the control. This reduction was between 24-33% with respect the weights of the control fruits and 12-15% with respect the sizes of the control fruits.

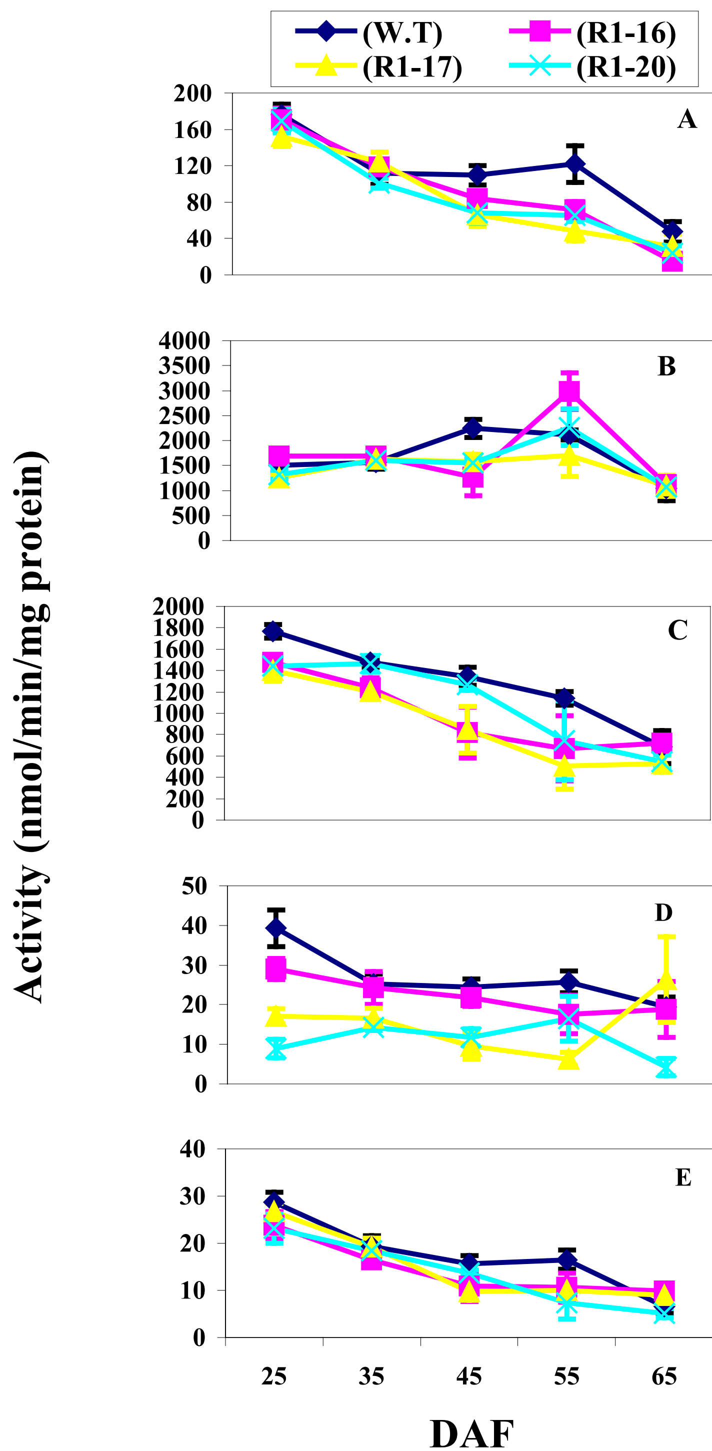


Figure 23: Activities of enzymes involved in the conversion of sucrose to starch in the pericarp of the WT control and α-GWD-transgenic lines of tomato cultivar Moneymaker.(A) SuSy. (B) UGPase. (C) PGM. (D) AGPase. (E) FBPase. Data represent the mean of five independent measurements + SE in WT control and transgenic line #17, but four independent measurements + SE in transgenic lines #16 and #20.

Table 7: Weights and sizes of ripe tomato fruits in WT control and α-GWD-transgenic lines.

Lines	Average	
	Weight of Fruit (g)	**Size of Fruit (cm)**
WT	54.2 ± 1.2 (n=146)	4.8 ± 0.03 (n=146)
#16	**36.1 ± 1.8** (n=21)	**4.1 ± 0.08** (n=21)
#17	**36.2 ± 1.4** (n=31)	**4.1 ± 0.05** (n=31)
#20	**40.9 ± 1.3** (n=27)	**4.2 ± 0.05** (n=27)

Data are means ± standard error, number of samples is in parentheses. Significant differences (P≤ 0,01), Students *t*-test are in bold.

7.3.6 Number of flower, fruit per plant, fruit set and number of days to 50% flowering

Data of this trait are presented in Table 8. As can be seen in Table 8, the both average number of fruit per plant, fruit set and number of days to 50% flowering of all of the transgenic lines were significantly (P≤ 0.01) reduced in comparison with the WT control. This reduction was between 42-46% with respect the number of fruit per plant of the control fruits and 49-53% with respect the fruit set of the control fruits. Transgenic lines (#17) was significantly (P≤ 0.05) increased with respect number of flower per plant in comparison with the WT control, while no different significant were found between WT control and transgenic lines (#16 and #17) with respect number of flower per plant.

Table. 8: Number of flowers, fruit set and number of days to 50% flowering in the WT control and α-GWD-transgenic lines.

Lines	Average			
	No. of flower/plant	**No. of fruit/plant**	**Fruit set %**	**No. of days to 50% flowering**
WT	42.9 ± 1.7	26.3 ± 1.2	61.0 ± 0.5	51.4 ± 0.5
#16	47.5 ± 3.3	**14.8 ± 2.3**	**30.8 ± 3.3**	**54.8 ± 1.3**
#17	**53.2 ± 3.2**	**15.2 ± 1.4**	**28.6 ± 1.8**	**56.2 ± 0.9**
#20	49.5 ± 4.4	**14.0 ± 1.6**	**28.1 ± 1.0**	**57.0 ± 1.4**

Data represent the mean of fifteen independent measurements ± SE in the control, five independent measurements ± SE in line #17 and four independent measurements ± SE in lines #16 and #20. Significant differences (P≤ 0.05 and P≤ 0.01, Student's *t*-test) are in bold.

A

B

C

Figure 24: Some 65 DAF old fruits from α-GWD-transgenic lines (bottom) in comparison with the WT control fruit (above). (A) Transgenic line #16. (B) Transgenic line #17. (C) Transgenic line #20.

7.4 Discussion and conclusions

In this study I have described the production of transgenic tomato plants repressed in the amount of GWD protein using a constitutive promoter. The GWD protein was demonstrated to be present in the pericarp of WT fruits only between 25-50 DAF, which is also the period when starch is present in the pericarp. This is consistent with the known role of the protein as an amylopectin phosphorylating enzyme. The protein was completely lacking in both leaves and pericarp of three transgenic lines chosen for further study.

Potato and *Arabidopsis* plants lacking the GWD protein do not degrade starch in their leaves as quickly as WT plants, leading to a starch-excess phenotype **(Lorberth *et al.*, 1998, Yu *et al.*, 2001)**. This was also found in leaves of the tomato plants lacking GWD protein which contained approximately twice the starch content of the control. Interestingly, however, the starch content in the pericarp was decreased in the transgenic lines rather than increased. The most likely explanation for this comes from studying the phenotype of the transgenic plants. The leaves of these plants senesced extremely early in comparison with the WT control. This is presumably some form of stress response caused by the accumulation of large amounts of starch in the leaves. This response was not noted in potato plants lacking the GWD protein **(Lorberth *et al.*, 1998)**, indicating that tomato responds differently to potato when it is repressed in starch degradation. *Arabidopsis* plants lacking GWD protein do, however, grow significantly worse than controls under certain environmental conditions **(Caspar *et al.*, 1991; Trethewey and ap Rees, 1994b)**. In any case, the senesced leaves should also be inhibited in photosynthesis meaning that they would not be able to produce as much sugar to export to the fruits as the control. I demonstrated in a previous chapter that fruit specific repression of chloroplastic fructose-1,6-bisphosphatase (cp-FBPase) – which leads to fruit specific inhibition of photosynthesis – did not produce significant reductions in starch contents, indicating that fruit starch is mainly the product of sucrose imported from the leaves. As the fruits from the transgenic plants in this study should be receiving less sucrose than the control it is reasonable to assume that this is what causes the reductions in starch contents. In addition I found that the activity of ADP-glucose pyrophosphorylase (AGPase) was reduced in the transgenic fruits. AGPase has often been considered to be an important enzyme in determining starch contents in storage organs, including tomato fruits **(Yelle *et al.*, 1988)**, and decreases in its activity would be expected to lead to reductions in starch contents. The reasons for its reduction in activity are not clear, but it is reasonable to assume that it may have something to do with alterations in soluble sugar concentrations within the pericarp.

Soluble sugar levels have often been considered to be important in regulating transcription of genes, and it has been demonstrated, for example, that *AGPase* transcript is greatly affected by growing *Arabidopsis* leaf discs on different soluble sugars **(Sokolov *et al.*, 1998)**. In the pericarp of the transgenic plants it was found that the concentrations of sucrose, fructose and glucose were lower than in the control at virtually every time-point measured indicating that starch synthesis may indeed be regulated in this way in tomato fruits.

In this study I also analysed the yield of tomato fruits from the lines and found the both average weights and sizes of fruits of all of the transgenic lines were significantly ($P \leq 0.01$) reduced in comparison with the WT control. This reduction was between 24-33% with respect the weights of the WT control fruits and 12-15% with respect the sizes of the WT control fruits. This is again presumably due to the leaves exporting less sugar to the fruits due to the leaf senescence phenotype that the transgenic lines exhibited. If the fruits receive less sugars they would not contain as much carbon as the controls and would not grow so large.

I also found that there was a significant ($P \leq 0.01$) reduction in all of the transgenic lines in comparison with the WT control with respect to both average number of fruit per plant and fruit set. This is despite the increased numbers of flowers in all of the transgenic lines in comparison with the WT control. The factors that control fruit set are not well understood, but it is again reasonable to assume that supply of sugars from the leaves would be important. There would be a selective advantage for the plant if a reduced supply of carbon were distributed between a smaller numbers of fruits than normal. These fruits would be larger and contain more nutrients than they would otherwise have done and would, therefore, have an increased chance of producing viable seeds than smaller fruits containing fewer nutrients.

The problem with all the data presented in this chapter was that there was a completely unexpected and drastic effect on leaf growth and photosynthesis caused by the transformation. This means that it is impossible to separate out phenotypes caused by reduction of the GWD protein in the leaf to those caused by reductions in the fruits. The original aim of this experiment, for example, was to try and delay starch degradation in the fruits through repression of the GWD protein, but this was something that turned out not to be feasible with the fruits accumulating actually less starch in the transgenic lines. In a previous chapter I used a fruit specific promoter to reduce the activity of cp-FBPase and it would be possible to repress the GWD protein also in a fruit specific manner using this promoter. Production of such plants should enable the elucidation of the influence of the GWD protein on fruit metabolism.

From the data presented in this chapter it can be concluded that: (A) The GWD protein is mainly present in green, but not red tomato fruit. (B) Repression of the GWD proteins in tomato plants leads to early senescence of leaves presumably with a concomitant reduction in photosynthesis. (C) The leaf senescence phenotype leads to large alterations of metabolism in both leaves and fruits.

Summary

Carbohydrate metabolism was studied during the development of fruits of the tomato cultivar Micro-Tom. The metabolism of the pericarp and placental tissues was found to be different. Starch being degraded more slowly in the placenta than in the pericarp, while soluble sugars accumulated to a greater extent in the pericarp. The activities of glycolytic enzymes tended to peak at 40 DAF. Two of these, phosphoenolpyruvate phosphatase and pyruvate kinase, showed a dramatic increase in activity just before this peak possibly indicating a role in up-regulating glycolysis to generate ATP for climacteric respiration. The expression of some plastidial transporters was also studied. Both the triose phosphate transporter (TPT) and Glc-6-P transporter was expressed greatest in green fruits, before declining. The expression of the triose phosphate transporter (TPT) was greater than that of Glc-6-P transporter. The ATP/ADP transporter was expressed to a low level throughout fruit development. These changes in transcript profiles are reflective of a switch from partially photosynthetic to fully heterotrophic metabolism. Whilst these characteristics are largely equivalent to those previously observed for normal sized tomato cultivars and as such indicated the suitability of Micro-Tom for studies of carbohydrate metabolism repeated failure to transform this cultivar made it inappropriate for further study.

Activity repression using potato cDNA encoding for the cp-FBPase, AGPase, and the GWD-protein for antisense inhibition studies was therefore performed in normal sized tomatoes of the cultivar Moneymaker. In the case of cp-FBPase, transgenic plants were isolated in which this activity was reduced by more 50% of the WT control in green fruits. Immunoblots indicated that the chloroplastidial isoform was almost completely eliminated in the most strongly inhibited lines. Measurements of metabolite levels in green fruits of the transgenic plants were consistent with an inhibition of photosynthesis, but there was little differences in the levels of metabolites or of other key enzyme activities at other time points. Consistent with the inhibition in photosynthesis the average weight and size of fully ripe fruits were significantly decreased by up to 20% in the transgenic lines. In addition the fruit set in these plants was markedly reduced, however from the present study it was not able to discriminate the reason for this.

In the case of AGPase, transgenic plants were isolated in which this activity was reduced by more 90% of the control in green fruits with immunoblots indicating that the AGPase was almost completely eliminated in the strongly inhibited line. Analysis of metabolites through

development revealed little change in early development but a decreased content of glucose and fructose at latter stages of development. Furthermore, the line exhibiting the greatest level of AGPase inhibition was characterised by a depressed starch content. Phosphorylated intermediates determined in green fruit were also largely unchanged with the exception that 3-PGA and PEP which were significantly decreased in the strongly inhibited line. The AGPase antisense plants were characterised by significant reduction in fruit yield and the strongest line also exhibited a delayed flowering, however, from this study it was not able to explain why this phenomenon appears.

In the case of GWD protein, transgenic plants were selected by immunoblots in leaves which revealed that the GWD protein was almost completely eliminated in all transgenic lines (further experiments confirmed this was also true in the pericarp of the transgenics). Western blot analysis of GWD protein abundance revealed that it was present in green but not red fruit in the WT control. GWD-transgenic plants were phenotypically dramatically different from wild WT control, where, leaves of these plants senesced much earlier than the WT control. Analysis of metabolites through development revealed large change in early development (with respect starch and fructose content) but a decreased content of glucose and fructose at latter stages of development. On the other hand, sucrose concentration was low, and was decreased in GWD transgenic lines through development. Analysis of leaf metabolites revealed that glucose and sucrose and starch concentrations were increased in leaves in the transgenic lines, but fructose concentration was significantly decreased in leaves in the transgenic lines. The average weight and size of fully ripe fruits were high significantly decreased by up to 33% and 15% in all transgenic lines in comparison with the WT control with respect to average of weight and size respectively. Furthermore, the time of flowering was significantly delayed in these lines and the fruit set was dramatically reduced. However, the large changes in leaf metabolism combined with the fact that these are opposite in trend to those observed in the fruit make it hard to dissect the role of GWD protein in the fruit and suggests that the use of a fruit specific promoter have been a better approach by which to address this question.

The role of three enzymes (cp-FBPase, AGPase and GWD protein) are thought to influence the accumulation of starch in early development in tomato fruit were studied using antisense technique under the control of the patatin B33 promoter in the case of cp-FBPase, and the CaMV 35S promoter in the case of AGPase and GWD protein. It appears that repression of cp-FBPase and AGPase in tomato fruits does not influence metabolite levels as greatly as it

does in leaves, possibly because any alterations are buffered by the ability of the fruit to import sugars. On the other hand, the repression of GWD protein in tomato fruits has been strongly affected on metabolite levels.

Zusammenfassung

Während der Entwicklung von Früchten der Tomate (Sorte „Micro-Tom“) wurde der Kohlenhydrat-Stoffwechsel untersucht. Es wurde ein Unterschied zwischen dem Metabolismus im Perikarp und dem des Plazenta-Gewebes gefunden. Stärke wurde in der Plazenta langsamer abgebaut als im Perikarp, während lösliche Zucker im Perikarp stärker akkumulierten. Die Aktivitäten der glykolytischen Enzyme tendierten zu einem Maximum 40 Tage nach der Blüte. Zwei davon, Phosphoenolpyruvat-Phosphatase und Pyruvat-Kinase, zeigten einen starken Anstieg der Aktivität kurz vor diesem Maximum. Diese Tatsache weist möglicherweise auf eine Rolle dieser Enzyme in der Hochregulierung der Glykolyse, um ATP für die klimakterische Respiration zu erzeugen, hin. Weiterhin wurde die Expression einiger plastidärer Transporter untersucht. Sowohl der Triosephosphat-Tranporter (TPT) als auch der Glukose-6-phosphat-Transporter wurde am stärksten in grünen Früchten exprimiert, danach nahm die Expression ab. Der ATP/ADP-Transporter wurde während der Fruchtentwicklung nur schwach exprimiert. Diese Änderungen der Transkriptionsprofile deuten auf einen Wechsel von teilweise photosynthetischem zu vollständig heterotrophem Metabolismus hin. Diese Eigenschaften entsprechen zwar weitgehend den vorher in normalgroßen Tomaten-Sorten beobachteten und schlagen dadurch die Eignung der Sorte „Micro-Tom“ für das Studium des Kohlenhydrat-Stoffwechsels vor; jedoch erwies sich diese Sorte letztendlich als ungeeignet, da mehrere Versuche einer Transformation erfolglos blieben.

Stattdessen wurde die Sorte „Moneymaker“ mit normalgroßen Früchten für Untersuchungen zur Repression der Aktivität von plastidärer FBPase, AGPase und GWD-Protein mittels Antisense-Inhibition verwendet. Im Falle der plastidären FBPase wurden transgene Pflanzen isoliert, in denen diese Aktivität in grünen Früchten um mehr als 50% im Vergleich zur Wildtyp-Kontrolle reduziert war. Ein Immunoblot zeigte, daß die plastidäre Isoform in den am stärksten inhibierten Linien fast nicht mehr vorhanden war. Die Messungen verschiedener Metaboliten-Konzentrationen in grünen Früchten der transgenen Pflanzen waren zwar im Einklang mit einer Inhibierung der Photosynthese, aber es konnten kaum Unterschiede der Metaboliten-Konzentrationen oder der Aktivitäten von Schlüssel-Enzymen für andere Zeitpunkte in der Fruchtentwicklung gefunden werden. Entsprechend der Inhibierung der Photosynthese war das durchschnittliche Gewicht und die Größe vollreifer Früchte in den transgenen Linien signifikant (um bis zu 20%) kleiner als im Wildtyp.

Desweiteren war die Fruchtanlage in diesen Pflanzen deutlich reduziert, es war jedoch in der vorliegenden Studie nicht möglich, einen Grund für diese Reduktion zu finden.

Im Falle der AGPase wurden transgene Pflanzen isoliert, in denen diese Aktivität in grünen Früchten um mehr als 90% im Vergleich zur Wildtyp-Kontrolle reduziert war. Auch hier zeigte ein Immunoblot, daß die plastidäre Isoform in der am stärksten inhibierten Linie fast nicht mehr vorhanden war. Die Analyse der Metaboliten während der Entwicklung der Frucht zeigte nur geringe Änderungen in der frühen Entwicklung, jedoch eine geringere Glukose- und Fructose-Konzentration in späteren Stadien der Entwicklung. Desweiteren zeigte die Linie mit der stärksten AGPase-Inhibition einen verminderten Stärkegehalt. Phosphorylierte Zwischenprodukte in grünen Früchten waren auch weitgehend unverändert, mit Ausnahme von 3-PGA und PEP, die in der am stärksten inhibierten Linie deutlich abnahmen. Die AGPase-Antisense Pflanzen zeigten eine erhebliche Abnahme der Fruchtausbeute, und die stärkste Linie wurde ausserdem durch ein verspätetes Blühen charakterisiert. Es war jedoch in dieser Studie nicht möglich herauszufinden, warum es zu diesen Phänomenen kommt.

Im Falle des GWD Proteins wurden transgene Pflanzen durch Immunoblots mit Blättern isoliert. Die Blots zeigten, daß das GWD Protein in allen transgenen Linien fast vollständig verschwunden war (weitere Experimente zeigten, daß dies auch im Perikarp der transgenen Früchte der Fall war). Western-Blot-Analysen der Verbreitung des GWD Proteins zeigten, daß dieses in grünen, nicht aber in roten Früchten des Wildtyp vorkommt. Die transgenen GWD Protein Pflanzen zeigten eine drastische phänotypische Veränderung im Vergleich zum Wildtyp. Die Blätter dieser Pflanzen wiesen eine extrem frühe Seneszenz auf. Eine Analyse der Metaboliten während der Entwicklung zeigte große Veränderungen in den frühen Entwicklungsstadien der Frucht (bezüglich Stärke- und Fructosegehalt), aber einen verringerten Gehalt an Glukose und Fruktose in späteren Entwicklungsstadien. Auf der anderen Seite war die Saccharosekonzentration gering, und nahm in den GWD Pflanzen während der Entwicklung ab. Eine Analyse der Metaboliten im Blatt brachte hervor, daß die Glukose-, Saccharose- und Stärkekonzentration in den transgenen Pflanzen im Vergleich zum Wildtyp erhöht war, die Fruktose-Konzentration hingegen war in Blättern deutlich geringer in den transgenen Linien. Gewicht und Größe der vollreifen Früchte waren im Durchschnitt um bis zu 33% bzw. 15% im Vergleich zu der Wildtyp-Kontrolle erhöht. Desweiteren war der Blütezeitpunkt in diesen Linien deutlich verspätet und die Fruchtmenge war sehr stark reduziert. Die starken Änderungen des Metabolismus im Blatt zusammen mit der Tatsache,

daß diese einen gegenläufigen Trend zu denen der Frucht aufweisen, erschweren allerdings den Schluß auf eine Rolle des GWD Proteins in der Frucht. Der Gebrauch eines fruchtspezifischen Promoters wäre ein besserer Ansatz gewesen, diese Frage zu untersuchen.

Es besteht die Hypothese, daß die Rolle dieser drei Enzyme (plastidäre FBPase, AGPase und GWD Protein) eine Beeinflussung der Stärke-Akkumulation in der frühen Entwicklung der Tomaten-Frucht ist. Diese Hypothese wurde durch Antisense-Technik mit der plastidären FBPase (unter der Kontrolle des B33 Promoters), sowie mit der AGPase und dem GWD Protein (beide unter der Kontrolle des CaMV 35S-Promoters) untersucht. Die Repression von plastidärer FBPase oder AGPase in der Frucht der Tomate scheint die Metaboliten-Konzentrationen nicht so stark wie in den Blättern beobachtet zu beeinflussen. Der Grund hierfür ist wahrscheinlich, daß jede Veränderung durch die Fähigkeit der Frucht, Zucker zu importieren, abgepuffert wird. Auf der anderen Seite hatte die Repression des GWD Proteins in der Frucht der Tomate starke Effekte auf die Metaboliten-Konzentrationen.

8 Literature Cited

ap Rees, T (1995) Where do plants make ADPglucose? In: sucrose, Metabolism, Biochemistry, Physiology and Molecular Biology, American Society of Plant Physiologists H.G. Pontis, G.L. Salerno, E.J. Echeverria E.J., (eds), Rockville, M.D., pp 143-155.

ap Rees, T. (1980) Integration of pathways of synthesis and degradation of hexose phosphates. In: The biochemistry of plants. J.Preiss (ed.). Academic Press Incorporated, San Diego. 3, pp 1-42.

Appeldorn, N.J.G., deBruijn, S.M., KootGronsveld, E.A.M., Visser, R.G.F., Vreugdenhil, D. and vanderPlas, L.H.W. (1999) Developmental changes in enzymes involved in the conversion of hexose phosphate and its subsequent metabolites during early tuberization of potato. Plant Cell and Environment, *22*, 1085-1096.

Ballicora, M.A., Laughlin, M.J., Fu, Y.B., Okita, T.W., Barry, G.F. and Preiss, J. (1995) Adenosine 5´-diphosphate-glucose pyrophosphorylase from potato tuber. Significance of the N terminus of the small subunit for catalytic properties and heat stability. Plant Physiology, *109*, 245-252.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) In-planta Agrobacterium-mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. Comptes Rendusde L' Academie des Sciences Serie III-Sciences de la vie-Life Sciences, *316,* 1194-1199.

Beck, E. and Ziegler, P. (1989) Biosynthesis and degradation of starch in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology, *40*, 95-117.

Beckles, D., ap Rees, T. and Smith, A.M. (2001a) A cytosolic ADPglucose pyrophosphorylase is a feature of graminaceous endosperms, but not of other starch storing organs. Plant Physiology, *125*, 818-827.

Beckles, D., Graig, J. and Smith, A.M. (2001b) ADPglucose pyrophosphorylase is located in the plastid in developing tomato fruit. Plant Physiology, *126*, 261-266.

Bhattacharrya, M.K., Smith, A.M., Ellis, T.H.N., Hedley, C. and Martin, C. (1990) The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch branching enzyme. Cell, *60,* 115-121.

Boorer, K.J., Loo, D.D.F., Formmer, W.B. and Wright, E.M. (1996) Transport mechanism of the cloned potato H+/sucrose cotransporter StSUT1. Journal of Biological Chemistry, *272*, 25139-25144.

Bradford, M.M (1976) Rapid and quantitative method for the quantitation of microgram quantities of protein utilizing the principle of protein-dey binding. Analytical Biochemistry, *72,* 248-252.

Bryce, J.H. and Thornton, J.M. (1996) Respiration and growth Metabolism; In: Photoassimilate Distribution in plants and Crops, Source-Sink Relationships (Zamski, E. and Schaffer, A.A., eds.), pp.43-61; Marcel Dekker Inc., New York

Büker, M., Schünemann, D. and Borchert, S. (1998) Enzymic porperties and capacities of developing tomato (Lycopersicon esculentum L.) fruit plastids. Journal of Experimental Botany, *49*, 681-691.

Bullock, W.O., Fernandez, J.M. and Short, J.M. (1987) XL1-blue: a high efficiency plasmid transforming recA Escherichia coli with β-galactosidase selection. Biotechniques, *5*, 376-378.

Burrell, M.M., Mooney, P.J., Blundy, M., Carter, D., Wilson, F., Green, J., Blundy, K.S. and ap Rees, T. (1994) Genitic manipulation of 6-phosphofructokinase in potato tubers. Planta, *194*, 95-101.

Burton, R.A., Bewley, J.D., Smith, A.M., Bhattacharyya, M.K., Tatke, H., Ring, S., Bull, V., Hamilton, W.D.P. and Martin, C. (1995) Starch-branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. Plant Journal, *7,* 3-15.

Burton, R.A., Johnson, P.E., Beckles, D.M., Fincher, G.B., Jenner, H.L., Naldrett, M.J. and Denyer, K. (2002) Characterization of the genes encoding the cytosolic and plastidial forms of ADPglucose pyrophosphorylase in wheat endosperm. Plant Physiology, *130*, 1464-1475.

Cao, H.P., Sullivan, T.D., Boyer, C.D. and Shannon, J.C. (1995) Bt1, a structural gene for the major 39-44 kDa amyloplast membrane polypeptides. Phisiologia Plantarum, *95*, 176-186.

Caspar, T., Lin, T.-P., Monroe, J., Benbow, L., Preiss, J. and Somerville, C.R. (1991) Mutants of Arabidopsis with altered regulation of starch degradation. Plant Physiology, *95*, 1181-1188.

Chen, B.Y., Wang, Y. and Jane, H.W. (1998) ADPglucose pyrophosphorylase is localized to both the cytoplasm and plastids in developing pericarp of tomato fruit. Plant Physiology, *116*, 101-106.

Chengappa, S., Guilleroux, M., Phillips, W., and Shields, R. (1999) Transgenic tomato plants with decreased sucrose synthase are unaltered in starch and sugar accumulation in the fruit. Plant Molecular Biology, *40*, 213-221.

Critchley, J.H., Zeeman, S.C., Takaha, T., Smith, A.M. and Smith, S.M. (2001) A critical role for disproportionating enzymes in starch breakdown is revealed by a knock-out mutation in Arabidopsis. Plant Journal, *26*, 89-100.

D'Aoust, M-A., Yelle, S., and Nguyen-Quoc, B. (1999) Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and sucrose unloading capacity of young fruit. Plant Cell, *11*, 2407-2418.

Damon, S., Hewitt, J., Nieder, M. and Bennett, A.B. (1988) Sink metabolism in tomato fruit. II. Phloem unloading and sugar uptake. Plant Physiology, *87*, 731-736.

Deblaere, R., Bytebier, B., Degreve, H., Deboeck, F., Schell, J., van Montagu, M., and Leemans, J. (1985) Efficient octopine Ti-plasmid derived vectors for Agrobacterium-mediated gene transfer to plants. Nucleic Acids Research, *13*, 4777-4788.

DemnitzKing, A., Ho, L.C. and Baker, D.A. (1997) Activity of sucrose hydrolysing enzymes and sugar accumulation during tomato fruit development. Plant Growth Regulation, *22*, 193-201.

Denyer, K., Dunlap, F., Thorbjonsen, T., Keeling, P. and Smith, A.M. (1996) The major isoform of ADPglucose pyrophosphorylase in maize endosperm is extraplastidial. Plant Physiology, *112*, 779-785.

Denyer, K., Johnson, P., Zeeman, S. and Smith, A.M. (2001) The control of amylose synthesis. Journal of Plant Physiology, *158*, 479-487.

Dickinson, D.B. and Preiss, J. (1969) Presence of ADPglucose pyrophosphorylasein shrunken-2 and brittle-2 mutants of maize endosperm. Plant Physiology, *44*, 1058-1062.

Dietze, J., Blau, A. and Willmitzer, L. (1995) Agrobacterium-mediated transformation of potato (Solanum tuberosum). In: Gene Transfer to plants (Potrykus, I. and Spangenberg, G., eds.), pp.24-29; Springer-Verlag, Berlin.

Duff, S.M.G., Lefebvre, D.D. and Plaxton, W.C. (1989) Purification and characterisation of a phosphoenolpyruvate phosphatase from Brassica nigra suspension cells. Plant Physiology, *90*, 734-741.

Entwistle, G. and ap Rees, T. (1990) Lack of fructose 1,6-bisphosphatase in a range of higher plants that store starch. Biochemical Journal, *271*, 467-472.

Fernie, A.R., Roscher, A., Ratcliffe, R.G. and Kruger, N.J. (2001) Fructose 2,6-bisphosphate activates pyrophosphate: fructose 6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. Planta, *212*, 250-263.

Frommer, W.B. and Sonnewald, U. (1995) Molecular analysis of carbon partitioning in Solanaceous species. Journal of Experimental Botany, *46*, 587-607.

Geigenberger, P., Geiger, M. and Stitt, M. (1998) High-temperature perturbation of starch synthesis by decreased levels of glycerate-3-phosphate in growing potato tubers. Plant Physiology, *117*, 1307-1316.

Geigenberger, P., Reimholz, R., Geiger, M., Merlo, L., Canale, V. and Stitt, M. (1997) Regulation of sucrose and starch metabolism in potato tubers in responce to short term water deficit. Planta, *201*, 502-518.

Gerhardt, R., Stitt, M. and Heldt, H.W. (1987) Subcellular metabolite levels in spinach leaves: Regulation of sucrose synthesis during diurnal alteration in photosynthetic partitioning. Plant Physiology, *83*, 399-407.

Hagelin, K., Rodriguez-Suarez, R., Katzen, F., Wolosiuk, R.A., Baldi, P.C., Giambartolomej, G.H., Fossati, C.A. and Dyer, T. (1996) Interspecies cross-reactivity of a monoclonal antibody directed against wheat chloroplast fructose-1,6-bisphosphatase. Cellular and Molecular Biology, *42*, 673-682.

Hanahan, D. (1983) Studies on transformation of Escherichia coli with plasmids. Journal of Experimental Botany, *166*, 557-580.

Hanson, K.R. and Mchale, N.A. (1988) A starchless mutant of Nicotiana-Sylvestris containing a modified plastid phosphoglucomutase. Plant Pysiology, *88*, 838-844.

Harrisson, C.L., Hedley, C.L. and Wang, T.L. (1998) Evidence that the rug3 locus of pea encodes plastidial phosphoglucomutase confirms that the imported substrate for starch synthesis in pea amyloplasts is glucose 6-phosphate. Plant Journal, *13*, 753-762.

Hatzfeld, W.-D. and Stitt, M. (1990) A study of the rate of recycling of triose phosphates in heterotrophic Chernopodium rubrum cells, potato tubers, and maize endosperm. Planta, *180*, 198-204.

Heldt, H.W., and Flügge, U.I. (1987) Subcellular transport of metabolites in a plant cell. In The Biochemistry of Plants. P.K. Stumpf and E.E. Conn (eds.). Academic Press Incorporated, San Diego. 12, pp 49-85.

Heldt, H.W., Chong, J.C., Maronde, D., Herold, A., Stankovic, Z.S., Walker, D., Kraminer, A., Kirk, M.R. and Heber, U. (1977) Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated compounds. Plant Physiology, *59*, 1146-1155.

Hill, L.M. and Smith, A.L. (1991) Evidence that glucose 6-phosphate is imported as substrate for starch synthesis by the plastids of developing pea embryos. Planta, *185,* 91-96.

Hille, J., Koorneef, M., Ramanna, M.S. and Zable, P. (1989) Tomato: a crop species amenable to improvement by cellular and molecular methods. Euphytica, *42*, 1-23.

Ho, L.C. (1996) Tomato. In: Photoassimilate distribution in plants and crops, source-sink relationships. (Zamski E, Shaffer AA, eds.), pp. 709-728. New York: Marcel Dekker, Inc.

Ho, L.C. and Hewitt, J.D. (1986) Fruit development. In: Atherton, J.G., Rudich, J., eds. The tomato crop. London: Chapman and Hall, pp 201-239.

Höfgen, R. and Willmitzer, L. (1990) Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (Solanum tuberosum). Plant Science, *66*, 221-230.

Holtman, W.L., Heistek, J.C., Mattern, K.A., Bakhuizen, R. and Douma, A.C. (1994) β-oxidation of fatty acids is liked to the glyoxylate cycle in the aleurone but not in the embryo of germinating barley. Plant Science, *99*, 43-53.

Hopkins, W.G. (1995) Cellular Respiration: Retrieving the energy in Photoassimilates; In: Introduction to plant physiology (Hopkins, W.G., de.), pp 235-254; John Wiley and Sons, Inc., USA

Huber, S.C. and Huber, J.L.A. (1992) Role of sucrose-phosphate synthase in sucrose metabolism in leaves. Plant Physiology, *99*, 1275-1278.

Hughes, D.W. and Galau, G. (1988) Preparation of RNA from cotton leaves and pollen. Plant Molecular Biology Report, *6*, 253-257.

Iglesias, A.A., Barry, G.F., Meyer, C., Bloksberg, L., Nakata, P.A., Greene, T., Laughlin, M.J., Okita, T.W., Kishore, G.M. and Preiss, J. (1993) Expression of the potato tuber ADPglucose pyrophosphorylase in Escherichia coli. Journal of Biological Chemistry, *268*, 1081-1086.

Journet, E.P. and Douce, R. (1985) Enzymic capacities of purified cauliflower bud plastids for lipid synthesis and carbohydrate metabolism. Plant Physiology, *79*, 458-467.

Kammerer, B., Fischer, K., Hilpert, B., Schubert, S., Gutensohn, M., Weber, A. Flugge, U.I. (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: The glucose 6-phosphate phosphate antiporter. Plant Cell, *10*, 105-117.

Keeling, P.L., Wood, J.R., Tyson, R.W. and Bridges, I.G. (1988) Starch Biosynthesis in developing wheat grain: Evidence against the direct involvement of triose phosphates in the metabolic pathway. Plant Physiology, *87*, 311-319.

Khyse-Andersen, J. (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. Journal of Biochemical Biophysical Methods, *10*, 203-209.

Klann, E.M., Hall, B. and Bennett, A.B. (1996) Antisense acid invertase (TIV1) gene alters soluble sugar composition and size in transgenic tomato fruit. Plant Physiology, *112*, 1321-1330.

Knee, M. (1995) Do tomatoes on the plant behave as climacteric fruits? Physiologia Plantarum, *95*, 211-216.

Koncz, C. and Schell, J. (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Molecular and General Genetics, *204*, 383-396.

Koßmann, J. and Lloyd, J.R. (2000) Understanding and influencing starch biochemistry. Critical Reviews in Plant Science, *19*, 171-226.

Koßmann, J., Müller-Röber, B., Dyer, T.A., Raines, C.A., Sonnewald, U. and Willmitzer, L. (1992) Cloning and expression analysis of the plastidic fructose 1,6-bisphosphatase coding sequence from potato- Circumstantial evidence for the import of hexoses into chloroplasts. Planta, *188*, 7-12.

Koßmann, J., Sonnewald, U. and Willmitzer, L. (1994) Reduction of the chloroplastic fructose 1,6-bisphosphatase in transgenic potato plants impairs photosynthesis and plant growth. Plant Journal, *6*, 637-650.

Koßmann, J., Visser, R.G.F., Müller-Röber, B., Willmitzer, L. and Sonnewald, U. (1991) Cloning and expression analysis of a potato cDNA that encodes branching enzyme: Evidence for co-expression of starch biosynthetic genes. Molecular and General Genetics, *230*, 39-44.

Kruger, N.J. and Beevers, H. (1984) Effect of fructose 2,6-bisphosphate on the kinetic properties of cytoplasmic fructose 1,6-bisphosphate in germinating castor bean endosperm. Plant Physiology, *76*, 49-54.

Krukeberg, A., Neuhaus, H., Feil, R., Gottleib, L. and Stitt, M. (1989) Decreased activity mutants of PGI in the cytosol and chloroplast of Clarkia xantiana: I Impact on mass action ratios and the flux to sucrose and starch and estimation of the flux control coefficients and elasticity coefficients. Biochemical Journal, *261*, 457-467.

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature, *227*, 680-685.

Lalonde, S., Boles, E., Hellmann, H., Barkler, L., Patrick, J.W., Frommer, W.B. and Ward, J.M. (1999) The dual function of sugar carriers: transport and sugar sensing. Plant Cell, *11*, 707-726.

Landschütze, V., Willmitzer, L. and Müller-Röber, B. (1995) Inhibition of flower formation by antisense repression of metochondrial citrate synthase in transgenic potato plants leads to a specific disintegration of the ovary tissues of flowers. EMBO Journal, *14*, 660-666.

Leegood, R.C and Furbank R.T. (1986) Stimulation of photosynthesis by 2% oxygen at low temperatures is restored by phosphate. Planta, *168*, 84-93.

Leegood, R.C. (1996) Primary Photosynthate production: Physiology and Metabolism; In: Photoassimilate Distribution in plants and Crops, Source-Sink Relationships (Zamski, E. and Schaffer, A.A., eds.), pp.21-41; Marcel Dekker Inc., New York

Lehrach, H., Diamond, D., Wozney, J.M. and Boedtker, H. (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical re-examination. Biochemistry, *16,* 4743-4751.

Leidreiter, K., Heineke, D., Heldt, H.W., Müller-Röber, B., Sonnewald, U. and Willmitzer, L. (1995) Leaf-specific antisense inhibition of starch biosynthesis in transgenic potato plants leads to an increase in photoassimilate export from source leaves during the light period. Plant and Cell Physiology, *36*, 615-624.

Leutwiler, L.S., Hough-Evans, B.R. and Meyerowitz, E.M. (1984) The DNA of Arabidopsis thaliana. Molecular and General Genetics, *194*, 15-23.

Lin, T.P., Caspar, T., Somerville, C. and Preiss, J. (1988a) Isolation and characterisation of a starchless mutant of Arabidopsis thaliana (L.) Henyh lacking ADPglucose pyrophosphorylase activity. Plant Physiology, *86*, 1131-1135.

Lin, T.P., Caspar, T., Somerville, C. and Preiss, J. (1988b) A starch deficient mutant of Arabidopsis thaliana with low ADPglucose pyrophosphorylase activity lacks one of two subunit of the enzyme. Plant Physiology, *88*, 1175-1181.

Lloyd, J.R., Springer, F., Buleon, A., Müller-Röber, B., Willmitzer, L. and Kossmann J. (1999) The influence of alterations in ADPglucose pyrophosphorylase activities on starch structure and composition in potato tubers. Planta, *209*, 230-238.

Lorberth, R., Ritte, G., Willmitzer, L. and Kossmann J. (1998) Inhibition of a starch-granule-bound protein leads to modifide starch and repression of cold sweetening. Nature Biotechnology, *16*, 473-477.

Lytovchenko, A., Bienerich, K, Willmitzer, L. And Fernie, A.R. (2002) Carbon assimilation and metabolism in potato leaves deficient in plastidial phosphoglucomutase. Planta, *215*: 802-811.

Macdonald, F.D. and ap Rees, T (1983) Enzymic properties of amyloplasts from suspension cultures of soybean. Biochimica et Biophysica Acta, *755*, 81-89.

McCormick, S., Niedmeyer, J., Fry, J., Barnason, A., Horsch, K. and Fraley, R. (1986) Leaf disc transformation of cultivated tomato (L. esculentum) using Agrobacterium tumefaciens. Plant Cell Reports, *5*, 81-84.

Meissner, R., Jacobsen, Y., Melamed, S., Levyatuv, S., Shalev, G., Ashri, A., Elkind, Y. and Levy, A. (1997) A new model system for tomato genetics. Plant Journal, *12*, 1465-1472.

Miller, J.F., Dower, W.J. and Tompkins, L.S. (1988) High-voltage electroporation of bacteria: Genetic transformation of Campylobacter jejuni with plasmid DNA. Proceeding of the National Academy of Sciences USA, *85*, 856-860.

Müller-Röber, B., Koßmann, J., Hannah, L.C., Willmitzer, L. and Sonnewald U (1990) One of two different ADPglucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. Molecular and General Genetics, *224*, 136-146.

Müller-Röber, B., Sonnewald, U. and Willmitzer, L. (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO Journal, *11*, 1229-1238.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobaco tissue cultures. Physiologia Plantarum, *15*, 473-497.

N'tchobo, H. (1998) Sucrose unloading in tomato fruits. II. Subcellular distribution of acid invertase and possible roles in sucrose turnover and hexose storage in tomato ftuit. PhD thesis, Laval University, pp 87-118.

Naeem, M., Tetlow, I. And Emes, M.J. (1997) Starch synthesis in amyloplasts purified from developing potato tubers. Plant Journal, *11*, 1095-1103.

Neuhaus, H. E., Kruckeberg, A.L., Feil, R. and Stitt, M. (1889) Reduced-activity mutants of phosphoglucose isomerase in the cytosol and chloroplast of Clarkia xanthiana. Planta, *178*, 110-122.

Neuhaus, H.E. and Stitt, M. (1990) Control analysis of photosynthate partitioning: impact of reduced activity of ADP-glucose pyrophosphorylase or plasti phosphoglucomutase on the fluxes to starch and sucrose in Arabidopsis thaliana (L.) Heynh. Planta, *182*, 445-454.

Neuhause, H.E. and Stitt, M. (1989) Perturbation of photosynthesis in spinach leaf discs by low concentrations of methyl viologen. Influence of increased thylakoid energisation on ATP synthesis, electron transport, energy dissipation, light activation of the Calvin cycle enzymes, and control of starch and sucrose synthesis. Planta, *179*, 51-60.

Nguyen-Quoc, B. and Foyer, C.H. (2001) A role for " futile cycles" involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. Journal of Experimental Botany, *52*, 881-889.

Nguyen-Quoc, B., N'tchobo, H., Foyer, C.H. and Yelle, S. (1999) Overexpression of sucrose phosphate synthase increase sucrose unloading in transformed tomato fruit. Journal of Experimental Botany, *50*, 785-791.

Ohyama, A., Ito, H., Sato, T., Nishimura, S., Imai, T. and Hirai, M. (1995) Suppression of acid invertase activity by antisense RNA modifies the composition of tomato fruits. Plant and Cell Physiology, *36*, 369-376.

Okita, T.W. (1992) Is there an alternative for starch synthesis? Plant Physiology, *100*, 560-564.

Patrick, J.W. (1990) Sieve element unloading: cellular pathway, mechanism and control. Physiologia Plantarum, *78*, 298-308.

Plaxton, W.C. (1990) Glycolysis. In Methods in Plant Biochemistry: Enzymes of Primary Metabolism. (ed. P.J. Lea). Academic Press Incorporated, San Diego, 3, pp 145-173.

Pozeuta-Romero, J., Ardita, F. and Akazawa, T. (1991a) ADPglucose transport by the chloroplast adenylate transporter is linked to starch biosynthesis. Plant Physiology, *97*, 1565-1572.

Pozeuta-Romero, J., Frehner, M., Viale, A.M. and Akazawa, T. (1991b) Direct transport of ADPglucose is linked to starch biosynthesis in amyloplasts. Proceeding of the National Academy of Sciences USA, *88*, 5769-5773.

Preiss, J. (1988) Biosynthesis of starch and its regulation. In: The Biochemistry of plants. Preiss, J. (ed) Academic Press, San Diego, 14, pp 181-254.

Preiss, J. (1991) Biology and molecular biology of starch synthesis and its regulation. In: Miflin, J., ed. Oxford surveys of plant molecular and cell biology, 7. New York: Academic Press, pp 59-114.

Raleigh, E.A., Lech, K. and Brent, R. (1989) Current protocols in molecular biology (eds.Ausubel, F.M. et al.,) Publishing Associates and Wiley Interscience, New York, USA.

Renz, A. Merlo, L. and Stitt, M. (1993) Partial purification from potato tubers of three fructokinases and three hexokinases which show differing organ and developmental specificity. Planta, *190*, 156-165.

Rick, C.M. and Yoder, J.I. (1988) Classical and molecular genetics of tomato: highlights and perspective. Annual Review Genettics, *22*, 281-300.

Riesmeier, J.W., Willmitzer, L. and Frommer, W. (1994) Evidence for a role of the sucrose transporter in phloem loading and assimilate partitioning. EMBO Journal, *13*, 1-7.

Ritte, G., Lloyd, J.R., Eckermann, N., Rottmann, A., Kossmann, J. and Steup, M. (2002) The starch related R1 protein is an α-glucan water dikinase. Proceedings of the National Academy Science USA, *99*, 7166-7171.

Ritte, G., Lorberth, R. and Steup, M. (2000a) Reversible binding of the starch related R1 protein to the surface of transitory starch granules. Plant Journal, *21*, 387-391.

Ritte, G., Steup, M., Kossmann, J. and Lloyd, J.R. (2003) Determination of the starch phosphorylating enzyme activity in plant extracts. Planta, *216*, 798-801.

Robinson, N.L., Hewitt, J.D. and Bennett, A.B. (1988) Sink metabolism in tomato fruit. I. Developmental changes in carbohydrate metabolising enzymes. Plant Physiology, *87*, 727-730.

Rocha-Sosa, M., Sonnewald, U., Frommer, W., Stratmann, M., Schell, J. and Willmitzer, L. (1989) Both developmental and metabolic signals activate the promotor of a class I patatin gene. EMBO Journal, *8*, 23-29.

Rodriguez-Lopez, P., Baroja-Fernandez, E., Zandueta-Criado, A. and Pozueta-Romero, J. (2000) Adenosine diphosphate glucose pyrophosphorylase: A plastidial phosphodiesterase that prevents starch biosynthesis. Proceedings of the National Academy Science USA, *97*, 8705-8710.

Ruan, Y.L. and Patrick, J. W. (1995) The cellular pathway of post-phloem sugar transport in developing tomato fruit. Planta, *196*, 434-444.

Saltveit, M.E. (1993) Internal carbon dioxide and ethylene levels in ripening tomato fruit attached to or detached from the plant. Physiologia Plantarum, *89*, 204-210.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual; 2. edition, Cold Spring Harbor Laboratory Press , New York, USA.

Schaffer, A.A. and Petreikov, M. (1997a) Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. Plant Physiology, *113*, 739-746.

Scheidig, A., Frohlich, A., Schulze, S., Lloyd, J.R. and Kossmann, J. (2002) Downergulation of a chloroplast-targeted beta-amylase leads to a starch-excess phenotype in leaves. Plant Journal, *30*, 581-591.

Schott, K., Borchert, S., Müller-Röber, B. and Heldt, H.W. (1995) Transport of inorganic phosphates and C3 sugar and C6 sugar phosphates across the envelope membranes of potato tuber amyloplasts. Planta, *196*, 647-652.

Schünemann, D, Schott, K, Borschert, S. and Heldet, D. (1996) Evidance for the expression of the triose-phosphate phosphate translocator in green and non-green tissues of tomato and potato. Plant Molecular Biology, *31*, 101-111.

Scott, P., Lange, A.J., Pilkis, S.J. and Kruger, N.J. (1995) Carbon metabolism in leaves of transgenic tobacco (Nicotiana tabacum L.) containing elevated fructose-2,6-bisphosphate levels. The Plant Journal, **7**, 461-469.

Sehnke, P.C., Chung, H.-J. and Ferl, R.J. (2001) Regulation of starch accumulation by granule associated plant 14-3-3 proteins. Proceedings of the National Academy Science USA, *98*, 765-770.

Shannon, J.C., Pien, F.M. and Liu, K.C. (1996) Nucleotides and nucleotide sugars in developing maize endosperm. Plant Physiology, *110*, 835-843.

Shannon, J.C., Pien, F.M., Cao, H. and Liu, K.C. (1998) Brittle-1, an adenylate translocator, facilitates transfer of extra-plastidial synthesized ADPglucose into amyloplasts of maize endosperms. Plant Physiology, *117*, 1253-1252.

Sharkey, T.D. and Vanderveer P.J. (1989) Stromal phosphate concentration is low during feedback limited photosynthesis. Plant Physiology, *91*, 679-684.

Shellie, K.C. and Saltveit, M.E. (1993) The lack of a respiratory rise in muskmelon fruit ripening on the plant challenges the definition of climacteric behaviour. Journal of Experimental Botany, *44*, 1403-1406.

Sikka, V.K., Choi, S.B., Kavakli, I.H., Sakulsingharoj, C., Gupta, S., Ito, H. and Okita, T.W. (2001) Subcellular compartmentation and allosteric regulation of the rice endosperm ADPglucose pyrophosphorylase. Plant Science, *161*, 461-468.

Smith, A.M. (1988) Major differences in isoforms of starch-branching enzyme between developing embryos of round and wrinkled-seeded peas (Pisium sativum L.). Planta, *175*, 270-279.

Smith, A.M., Bettey, M. and Bedford, I.D. (1989) Evidence that the rb locus alters the starch content of developing pea embryos through an effect on ADPglucose pyrophosphorylase. Plant Physiology, *89*, 1279-1284.

Smith, A.M., Denyer, K. and Martin, C. (1997) The synthesis of the starch granule. Annual Review of Plant Physiology and Plant Molecular Biology, *48*, 65-87.

Smith, S.B., Taylor, M.A., Burch, L.R. and Davies H.V. (1993) Primary structure and characterisation of a cDNA clone of fructokinase from potato (Solanum tuberosum L cv Record). Plant Physiology, *102*, 1043-1043.

Sokolov, L.N., Dejardin, A. and Kleczkowski, L.A (1998) Sugars and light/dark exposure trigger differential regulation of ADP-glucose pyrophosphorylase genes in Arabidopsis thaliana (thale cress). Biochemical Journal, *336*, 681-687.

Sonnewald, U. and Willmitzer, L. (1992) Molecular Approaches to Sink-Source Interactions. Plant Physiology, *99*, 1267-1270.

Sowokinos, J.R. and Preiss, J. (1982) Pyrophosphorylases in Solanum tuberosum .III. Purification, physical, and catalytic properties of ADP-glucose pyrophosphorylase in potatoes. Plant Physiology, *69*, 1459-1466.

Stark, D.M., Timerman, K.P., Barry, G.F., Preiss, J. and Kishore, G.M. (1992) Role of ADP glucose pyrophosphorylase in regulating starch levels in plant tissues. Science, *258,* 287-292.

Steup, M., Robenek, H. and Melkonian, M. (1983) In –vitro degradation of starch granules isolated from spinach chloroplasts. Planta, *15,* 428-436.

Stitt, M. and Heldt, H.W. (1981) Simultaneous synthesis and degradation of starch in spinach chloroplasts in the light. Biochimistry Biophyics Acta, *638,* 1-11.

Stitt, M. (1990) Fructose 2,6-bisphosphate as a regulatory molecule in plants. Annual Review of Plant Physiology and Plant Molecular Biology, *41,* 153-185.

Stitt, M. (1997) The flux of carbon between the chloroplast and the cytoplasm. In Plant Metabolism. 2. edition, Dennis, D.T., Turpin, D.H., Lefebvre, D.D., Layzell, D.B (eds). Longman, London, pp 382-400.

Stitt, M., Herzog, B. and Heldt, H.W. (1984b) Control of photosynthetic sucrose synthesis by fructose 2,6 bisphosphate. I Coordination of carbon dioxide fixation and sucrose synthesis. Plant Physiology, *75,* 548-553.

Stitt, M., Kurzel, B. and Heldt, H.W. (1984c) Control of photosynthetic sucrose synthesis by fructose 2,6 bisphosphate. II Partitioning between sucrose and starch. Plant Physiology, *75,* 554-560.

Stitt, M., Lilley, R.McC., Gerhardt, R. and Heldt, H.W. (1989) Metabolite levels in Specific Cells and Subcellular Compartements of plant Leaves. In: Methods Enzymology, *174,* 518-552.

Strand, A., Zrenner, R., Trevanion, S., Stitt, M., Gustafsson, P. and Gardestrom, P. (2000) Decreased expression of two key enzymes in the sucrose biosynthesis pathway, cytosolic fructose 1,6-bisphospharase and sucrose phosphate synthase, has remarkably different consequences for photosynthetic carbon metabolism in transgenic Arabidopsis thaliana. Plant Journal, *23*, 759-770.

Sullivan, T.D. and Kaneko, Y. (1995) The maize brittle1 gene encodes amyloplast membrane polypeptides. Planta, *196,* 477-484.

Sullivan, T.D., Stretlow, L.I., Illingworth, C.A., Phillips, R.L. and Nelson, O.E. (1991) Analysis of maize Brittle 1 alleles and a defective suppressor mutator inducible allele. Plant Cell, *3*, 1337-1348.

Sweetlove, L.J., Burrel, M.M. and ap Rees, T. (1996) Starch metabolism in tubers of transgenic potato (Solanum tuberosum) with increased ADP glucose pyrophosphorylase. Biochemical Journal, *320,* 493-498.

Takaha, T., Critchley, J., Okada, S. and Smith, S.M. (1998) Normal starch content and composition in tubers of antisense potato plants lacking D-enzyme (4-alpha-glucotransferase). Planta*, 205,* 445-451.

Takamiya, S. and Fukui, T. (1978) Purification and multiple forms of phosphoglucomutase from potato tubers. Plant and Cell Physiology, *19*, 759-768.

Tanaka, A., Fujita, K. and Kikuchi, K. (1974) Nutrio-physiological studies on the tomato plant. III. Photosynthetic rate on individual leaves in relation to dry matter production of plants. Soil Science and Plant Nutrition*, 20*, 173-183.

Tauberger, E., Fernie, A.R., Emmermann, M., Renz, A., Kossmann, J., Willmitzer, L. and Trethewey, R.N. (2000) Antisense inhibition of plastidial phosphoglucomutase provides compelling evidence that potato tuber amyloplasts import carbon from the cytosol in the form of glucose-6-phosphate. Plant Journal, *23,* 43-53.

Tegeder, M., Wang, X.D., Frommer, W.B., Offler, E.O. and Patrick, J.W. (1999) Sucrose transport into developing seeds of Pisium sativum. Plant Journal, *18*, 151-161.

Tetlow, I.J., Blissett, K.J. and Emes, M.J. (1994) Starch synthesis and carbohydrate oxidation in amyloplasts from developing wheat endosperm. Planta, *194,* 454-460.

Thorbjornsen, T., Villard, P., Denyer, K., Olsen, O.A. and Smith, A.M. (1996b) Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplast in barley endosperm. Plant Journal, *10,* 243-250.

Tjaden, J., Möhlmann, T., Kampfenkel, K., Henrichs, G. and Neuhaus, H.E. (1998) Altered plastidic ATP/ADP-transporter activity influences potato (Solanum tuberosum L.) tuber morphology, yield and composition of tuber starch. The plant Journal, *16*, 531-540.

Trethewey, R.N. and ap Rees, T. (1994a) The role of the hexose transporter in the chloroplast of Arabidopsis thaliana. Planta, *195,*168-174.

Trethewey, R.N. and ap Rees, T. (1994b) A mutant of Arabidopsis lacking the ability to transport glucose across the chloroplast envelope. Biochemical Journal, *301*, 449-454.

Trethewey, R.N., Geigenberger, P., Riedel, K., Hajirezaei, M.R., Sonnewald, U., Stitt, M., Riesmeier, J.W. and Willmitzer, L. (1998) Combined expression of glucokinase and invertase in potato tubers leads to a dramatic reduction in starch accumulation and a stimulation of glycolysis. Plant Journal, *15 (1),* 109-118.

Trethewey, R.N. and Smith, A.M. (2000) Starch metabolism in leaves. In: Photosynthesis: Physiology and Metabolism. (Leegood, R., Sharkey, T.D. and Van Caemmerer, S., eds.), Kluwer Academic Publisher, pp 205-231.

Tsai, C.Y. and Nelson, O.E. (1966) Starch-deficient mutant lacking adenosine diphosphate glucose pyrophosphorylase activity. Science, *151,* 341-343.

Tyson, R.H. and ap Rees, T. (1988) Starch synthesis by isolated amyloplasts from wheat endosperm. Planta, *175,* 33-38.

Veramendi, J., Roessner, U. and Renz, A. (1999) Antisense repression of hexokinase 1 leads to an over accumulation of starch in leaves of transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. Plant Physiology, *121,* 123-133.

Veramendi, J., Roessner, U. and Renz, A. (1999) Antisense repression of hexokinase 1 leads to an over accumulation of starch in leaves of transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. Plant Physiology, *121***,** 123-133.

Vervliet, G., Holster, M., Teuchey, H., van Montagu, M. and Schell, J. (1975) Characterisation of different plaque-forming and defective temperate phages in Agrobacterium strains. Journal of General Virology, *26*, 33-48.

Viola, R., Davis, H.V. and Chudeck, A.R. (1991) Pathway of starch and sucrose biosynthesis in developing tuber of potato (Solanum tuberosum L.) and seed of faba been (Vicia faba L.): elucidation by 13C-nuclear-magnetic-resonance spectroscopy. Planta, *183,* 202-208.

Viola, R., Roberts, A.G., Haupt, S., Gazzani, S., Hancock, R.D., Marmirroli, N., Machray, G.C. and Oparka K.J. (2001) Tuberisation in potato involves a switch from apoplastic to symplastic phloem loading. Plant Cell, *13,* 385-398.

Walker, A.J. and Ho, L.C. (1977) Carbon translocation in the tomato: carbon import and fruit growth. Annals of Botany, *41,* 813-823.

Westram, A., Lloyd, JR, Roessner, U, Riesmeier, J and Kossmann, J. (2002) Increases of 3-phosphoglyceric acid in potato plants through antisense reduction of cytoplasmic phosohoglycerate mutase impairs photosynthesis and growth, but does not increase starch contents. Plant Cell Environment, *25,* 1133-1143.

Wischmann, B., Nielsen, T.H. and Moller, B.L. (1999) In vitro biosynthesis of phosphorylated starch in intact potato amyloplasts. Plant Physiology, *119,* 455-462.

Yelle S., Hewitt J.D., Robinson N.R., Damon S. and Bennett A.B. (1988) Sink metabolism in tomato fruit. III. Analysis of carbohydrate assimilation in a wild species. Plant Physiology, *87,* 737-740.

Yu, T.-S., Kofler, H., Häusler, R.E., Hille, D., Flügge, U.-I., Zeeman, S.C., Smith, A.M., Kossmann, J., Lloyd, J.R., Ritte, G., Steup, M., Lue, W.-L., Chen, J. and Weber, A. (2001) The Arabidopsis sex1mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. Plant Cell, *13*, 1907-1918.

Zeeman, S.C., Northrop, F., Smith, A.M. and ap Rees, T. (1998) A starch-accumulating mutant of Arabidopsis thaliana deficient in a starch hydrolysing enzyme. Plant Journal, *15*, 357-365.

Zrenner, R, Willmitzer, L. and Sonnewald, U. (1993) Analysis of the expression of potato uridinephosphate glucose pyrophosphorylase and its inhibition by antisense RNA. Planta, *190,* 247-252.

Zrenner, R., Krause, K.P., Apel, P. and Sonnewald, U. (1996b) Reduction of the cytosolic fructose 1,6-bisphosphatase in transgenic potato plants limits photosynthetic sucrose biosynthesis with no impact on plant growth and tuber yield. Plant Journal, *9*, 671-681.

Zrenner, R., Schüler, K. and Sonnewald, U. (1996a) Soluble acidic invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers. Planta, *198*, 246-252.

Acknowledgements

I am greatly indebted to Prof. Dr. Lothar Willmitzer for his valuable advice and all the interesting discussions throughout the course of my work.

I would particularly like to thank Prof. Dr Frank Pohlheim for helping to organise this dissertation at the branch vegetable, faculty of Agriculture and Horticulture, Humboldt-University of Berlin.

I would like to express my greatest appreciation to Dr. Jens Kossmann for giving me the opportunity for this PhD project.

I would like to express my greatest appreciation to Dr. James Lloyd for supervising my work and for the advice encouragement that he has given me throughout my PhD work.

I am particularly also indebted to Dr. Alisdair Fernie for his continuity supervising my work, for his advice encouragement and assistance that he has given me throughout my PhD work.

I would like to thank Dr. Christine Raines (University of Essex) for the gift of the antiserum recognising cp-FBPase.

I am very grateful to Romy Baran for performing the plant transformation and for her attention to my plants in tissue culture. I am also very grateful to all our gardeners and Dr. Karin Köhl for taking care of my plants in the glasshouse.

I would like to acknowledge Josef Bergstein for photographic work. I would also like to acknowledge Eckard Simmat and Wilfried Grauholz for computer work.

My special thank for Bjoern Junker and Anna Lytovchenko for their helping me during my PhD work. I thank also my lab mates Ilse, Andrea, Bion, Fernando, Eva, Ute, Adreano, Claudia, Lilia, Ania, Fransosska for their assistance and all the fun we had in the last three years.

CURRICULUM VITAE

Civil Status:

Full Name	Hazem Abd El-Rahman Obiadalla Ali
Place / Date of Birth	Sohag, Egypt,14 September 1969
Nationality	Egyptian
Marital Status	Married, 2 children
Office Address	Max-Planck-Institute of Molecular Plant Physiology, Golm, Germany.
Present Employment	Ph.D. student at Max-Planck-Institute of Molecular Plant Physiology.
Previous Employment	Assistant Lecturer in Faculty of Agriculture in Sohag, South Valley University, Egypt.

Academic Qualifications:

1975-1981	Primary school: Sohag Egypt
1981-1984	Preparatory school: Sohag Egypt
1984-1987	Secondary school: Sohag Egypt
1987-1991	Bachelor of Agriculture Science (B. Sc.) (Vegetables), Faculty of Agriculture, Assiut University, Egypt.
1992-1993	Military service
1993-1996	Master of Agriculture Science (M. Sc.) (Vegetables). Faculty of Agriculture, Assiut University, Egypt.
1996-1999	Assistant Lecturer in Sohag , South Vally University
1999-2003	Ph.D. student at Max-Planck-Institute of Molecular Plant Physiology.

Publication:

(1) M.Sc. thesis: Effect of inbreeding and outbreeding on Mungbean crop (*Vigna radiata* L.), Hazem Abd El-Rahman Obiadalla, Vegetables, Faculty of Agriculture, Assiut University, Egypt.

(2) Obiadalla-Ali, H., Fernie, A.R., Kossmann, J. and Lloyd, J.R (2003) Developmental Analysis of Carbohydrate Metabolism in Tomato (*Lycopersicon esculentum* cv. Micro-Tom) Fruits. Submitted to *Physiologia Plantarum*.

(3) Obiadalla-Ali, H., Fernie, A.R., Kossmann, J. and Lloyd, J.R (2003)

Inhibition of chloroplastic fructose 1,6-bisphosphatase in tomato fruits leads to surprisingly small changes in carbohydrate metabolism and decreases fruit size, Submitted to *Plant Physiology*.