Biochemistry and molecular control of starch synthesis in tomato fruit

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List of abbreviations

3-PGA	3-phosphoglyceric acid
ADP	adenosine diphosphate
ADP-Glc	ADP-glucose
AGPase	ADP-Glc pyrophosphorylase
ATP	adenosine triphosphate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cv	cultivar
DAA	days after anthesis
dd	double distilled water
DIECA	diethyldithiocarbamic acid
DTT	1, 4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FK	fructokinase
Fru-6-P	fructose 6-phosphate
FW	fresh weight
Glc-1-P	glucose 1-phosphate
Glc-1,6 bis-P	glucose 1,6- bis phosphate
Glc-6-P DH	glucose 6-phosphate dehydrogenase
Glc-6-P	glucose 6-phosphate
GK	glucokinase
Hepes	N-(2-hydroxyethyl)piperazine-N'-2-ethane sulfonic acid
HK	hexokinase
HPLC	high pressure liquid chromatography
I	iodine
Inv	invertase
IStS	insoluble starch synthase
K_2HPO_4	potassium hydrogen phosphate
KI	potassium iodide
KCl	potassium chloride
kDa	kilo Dalton
Km	Michaelis constant
KOH	potassium hydroxide
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
MW	molecular weight marker
NAD^+	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	sodium hydrogen phosphate
NaH_2PO_4	sodium hydrogen phosphate
$NaHCO_3$	sodium bicarbonate
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
$(NH_4)_2SO_4$	ammonium sulfate

$(NH_4)H_2PO_4$	ammonium hydrogen phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PGI	phosphoglucose isomerase
PGM	phosphoglucomutase
Pi	inorganic phosphate
PMSF	phenylmethylsulfonyl fluoride
PPi	pyrophosphate
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
SE	standard error
SPS	sucrose phosphate synthase
SStS	soluble starch synthase
SuSy	sucrose synthase
Tris-HCl	tris(hydroxymethyl)aminomethane
TSS	total soluble solids
UDP	uridine diphosphate
UDP-Glc	UDP glucose
UGPase	UDP-Glc pyrophosphorylase
UTP	uridine triphosphate
v/v	volume per volume
w/v	weight per volume
WT	wild type

1. Abstract

Starch accumulates transiently in the young tomato fruits and can contribute approximately 20% to the dry weight of the fruit tissue. The starch degradation in the developing tomato fruit leads to the accumulation of soluble sugars in the ripening fruit, which serve as the main determinant of fruit quality. The goal of the present work was the analysis of the sucrose-to-starch metabolic pathways and the molecular control of starch synthesis in developing tomato fruit, in order to shed light on strategies of increasing fruit starch content.

In a preliminary study comparing the activities of eleven enzymes of the sucrose to starch metabolic pathway, four enzymes showed a coordinated pattern of developmental loss of activity correlated with the cessation of starch synthesis. Two of these enzymes, fructokinase (FK) and ADP-Glc pyrophosphorylase (AGPase) were suggested to be potentially limiting the flux of starch synthesis in tomato fruit starch accumulation. Accordingly, the focus of the research was directed towards these two enzymatic reactions.

Three fructokinase isozymes (FKI, FKII, FKIII), including a novel FKIII, and two hexokinase isozymes (HK1, HK2) from developing tomato fruit were chromatographically separated, partially purified and kinetically characterized. The three FK isozymes could be distinguished from one another with respect to affinity to fructose, Mg^{+2} and nucleosides. However, results from genetically transformed tomato plants with modified FK activities indicated that this enzymatic reaction is not directly limiting starch synthesis in tomato fruit.

AGPase catalyzes the synthesis of ADP-Glc and functions as a limiting enzyme in starch synthesis. The enzyme functions as a heterotetramer consisting of

two large subunits (encoded by three *AgpL* genes, *AgpL*1, *AgpL*2 and *AgpL*3) and two small subunits (encoded by a single *AgpS1* gene). A set of near isogenic tomato plants was developed differing in the origin of the *AgpL1* allele, derived from an interspecific cross between the cultivated *S. lycopersicum* L. (formerly *L. esculentum* Mill., source of the $AgpL1^{E}$ allele) and wild species tomato *S. habrochaites* S. Knapp and D.M. Spooner (formerly *L. hirsutum* Humb. and Bonpl., source of the $AgpL1^{H}$ allele). The fruit carrying the $AgpL1^{H}$ allele is characterized by increased AGPase activity and increased immature fruit starch content, as well as higher total soluble solids (TSS) in the mature fruit, following by the breakdown of the transient starch. The introgression harboring the *AgpL1* locus mapped to the distal portion of chromosome 1, was delimited to ca. 1 cM and does not include other previously reported QTLs for TSS.

In order to determine the cause of the increased enzyme activity due to the $AgpL1^{H}$ allele, we compared the expression of all subunits (L1, L2, L3 and S1) in developing tomato fruit in the two near isogenic lines. The $AgpL1^{H}$ allele in the high starch line was expressed for a prolonged period of fruit development, compared to the $AgpL1^{E}$ allele in the normal starch line. This correlates with increases in AGPase activity, AGPase subunit protein level and starch accumulation patterns during fruit development of the high starch line.

The results suggest that the increased activity of AGPase in the $AgpL1^{H}$ tomatoes is related to the increased expression of the regulatory large subunit and subsequent maintenance of the active heterotetramer.

Comparative analysis of partially purified enzymes from both genotypes did not show differences in biochemical characteristics. Both enzymes are thermoactivated at 56°C with activity increasing up to 90%, whereas the AGPase-$L1^{H}$ protein appears to be slightly more thermostable. Post-translational redox activation of AGPase is

similar in the high and standard starch lines. No significant differences were observed in the kinetic properties of the partially purified AGPase enzymes from the two genotypes, i.e., substrate affinity or sensitivity to 3- PGA/PPi regulation. Furthermore, no significant differences between the metabolite concentrations in the green fruit of the two genotypes were observed.

Analysis of full length sequences of the two alleles of *AgpL1* gene did not indicate significant differences: the two alleles, $AgpL1^{E}$ and $AgpL1^{H}$ are 4500bp and 4497bp long, respectively and both consist of 15 exons with 97% homology between the coding regions, and 14 introns. A number of small differences (SNPs and indels) were found between the two sequences that were specific for $AgpL1^{H}$. Sequencing of 2000pb long putative promoter region showed 86% homology between the two genotypes.

The results of this research indicate that the increased activity of AGPase in tomato fruits harboring the wild tomato *L. hirsutum AgpL1* introgression is due to the increased expression and protein levels of the regulatory large subunit, which subsequently leads to an extended stability and activity of the functional holoenzyme. It serves as an example of intra-molecular heterosis in which the alleles for the individual subunits of the enzyme tetramer complement each other in a heterotic manner.

2. Introduction

2.1 The role of carbohydrates in tomato fruit quality

Tomato is a widespread cultivated annual vegetable crop of great popularity. The fruit taste and quality are strongly influenced by the total soluble sugar and acid content in the mature fruit. The principal mobile assimilate imported by the tomato fruit is sucrose (Walker and Ho, 1977; Yelle *et al.*, 1988) which is directly stored, used for growth and respiration, or converted to alternative storage compounds, such as starch and fats. In general, sugars accumulate in the cultivated tomato fruit in the form of soluble fructose and glucose, in nearly equimolar amounts (Yelle *et al.*, 1988; Young *et al.*, 1993). In standard tomato cultivars, sucrose concentration in the fruit decreases sharply from 1-3% fresh weight at fruit set to less than 0.1%, and remains at low levels until ripening (Ho and Hewitt 1986). In addition to the soluble sugars, the tomato fruit also accumulates starch during the early stages of development in a transient manner and this stored starch is later broken down to soluble sugars (Davies and Hobson, 1981; Yelle *et al.*, 1988; Young *et al.*, 1993).

Starch is the predominant carbohydrate reserve in many plants. Starch found in the chloroplasts of leaves and other photosynthetically competent cells is termed "transitory starch". Long-term storage of starch occurs in amyloplasts, specialized starch-containing plastids, which are conspicuously evident in non-photosynthetic harvestable storage organs such as tubers, roots, seeds and fruits, including tomato fruit (Visser and Jacobsen, 1993). Starch is composed of two types of glucan chains, amylose (an essentially linear polymer of glucosyl residues linked via α-1,4 glucosidic linkage) and amylopectin (a branched α-1,4:α-1,6 D-glucan polymer with about 5% α-1,6 glucosidic linkages) (Preiss and Sivak, 1996). Transitory starch is composed

almost entirely of branched amylopectin, whereas storage starch contains significant amount of linear amylose (~11 to 37%, depending on the species) in addition to amylopectin (Müller-Röber and Kobmann, 1994). Patterns of starch accumulation are specific to the species and are related to the particular pattern of differentiation of the organ (Preiss, 1996; Okita *et a*l., 1998; Slattery, Kavakli et al., 2000).

Starch transiently accumulates during the early stages of tomato fruit development at levels of about 10% of the dry weight in 1-week-old fruit increasing to a peak concentration of approximately 20% before the mature green stage (Fig.1). This

Fig. 1 Three stages of tomato fruit development (cv F144). Stage 1 lasts approximately 12 days after anthesis, stage 2 lasts approximately 20 days after anthesis and prior to the breaker stage. Stage 3 is the red ripe fruit. Freehand cross-sections were stained for starch with I/KI. (Schaffer *et al.*, 1997a)

transient starch is completely degraded during ripening, contributing to the soluble sugar pool, which comprises about 50% of the total solids in the ripe fruit (Ho, 1983). In addition to serving as a carbohydrate reservoir, this accumulation of starch in the early stage of cell enlargement maintains an optimal osmotic pressure in the fruit tissue and provides extra capacity for storing the imported assimilates. Starch has a negligible osmotic pressure and in this way plants are able to store large reserves of D-glucose without disturbing the cellular water relations (Preiss and Sivak, 1996).

The level of transient starch in the green fruit is positively correlated with the soluble sugar level in the ripe fruit, as suggested by a survey of a number of tomato cultivars (Dinar, 1981). Therefore, there is a possibility to enhance tomato fruit sugar content via a strategy of increasing the transient starch concentration in the immature fruit (Schaffer , 1999).

The main purpose of the present work was to analyze the biochemistry of the sucrose-to-starch metabolic pathways and the molecular control of starch synthesis in developing tomato fruit in order to shed light on the strategy of increasing transient starch content.

2.2 Pathways of starch synthesis

The synthesis of starch is achieved through the co-ordinated interactions of a suite of starch biosynthetic and degradative enzymes (Fig. 2). Details of the key components in this pathways have been adequately reviewed (Tetlow IJ 2004), dealing with starch biosynthesis in higher plants and algae (Ball and Morell, 2003), in cereal endosperms (James *et al*., 2003), and starch degradation in leaves (Smith *et al*., 2003). The pathway as it relates to the research project will be briefly described.

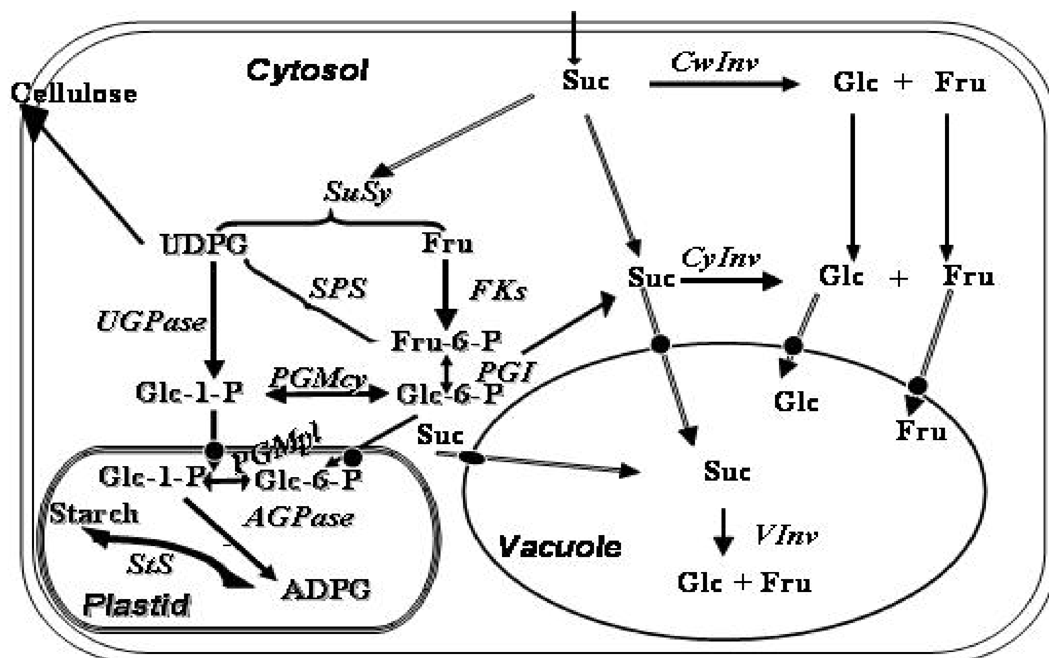

Fig. 2 Pathways of starch accumulation in tomato fruit. Suc, sucrose; Glc, glucose; Fru, fructose; Glc-1-P, glucose 1-phosphate; Fru-6-P, fructose 6-phosphate; Glc-6-P, glucose 6-phosphate; UDPG, UDP-glucose; ADPG, ADP-glucose; *CwInv*, cell wall bound invertase; *CyInv*, cytosolic invertase; *VInv*, vacuolary invertase; *SuSy*, sucrose synthase; *SPS*, sucrose phosphate synthase; *UGPase*, UDP-Glc pyrophosphorylase; *FKs,* fructokinases;

***GK*, glucokinase; *PGI*, phosphoglucose isomerase; *PGMcy*, cytosolic phosphoglucomutase; *PGMpl*, plastidic phosphoglucomutase; *AGPase*, ADP-Glc pyrophosphorylase; *StS*, soluble starch synthase.**

The tomato plant translocates primarily sucrose as assimilate (up to 90% of all assimilates), together with amino and organic acids, Therefore, the accumulation of starch depends on the sucrose to starch metabolic pathway. The amount of sucrose translocated into tomato fruit, as well as the metabolic pathways involved, differ with the developmental stage of the fruit (Walker and Ho, 1977; Ho, 1996; Ruan and Patrick, 1995). The early stage of fruit development is characterized by symplastic sucrose unloading and transport within the fruit, with the initial step in sucrose metabolism is catalyzed by sucrose synthase (SuSy), leading to a transient starch accumulation. After the starch accumulation phase, sucrose unloading takes on an apoplastic pattern, with the initial enzyme of sucrose cleavage is the hydrolytic enzyme invertase (Inv) (Ruan and Patrick, 1995).

In order to characterize the sucrose to starch biosynthetic pathway in developing tomato fruit and to determine developmental and spatial patterns of the enzymes involved, we initially studied the activities of the complete metabolic pathway (11 enzymes) during tomato fruit development (Schaffer and Petreikov, 1997a). We found that the two initial enzymes in the pathway, SuSy and fructokinase (FK), together with the two final enzymes in the pathway, the amyloplastic ADP-Glc pyrophosphorylase (AGPase) and starch synthase, undergo an apparently coordinated regulation of activity (Fig 3).

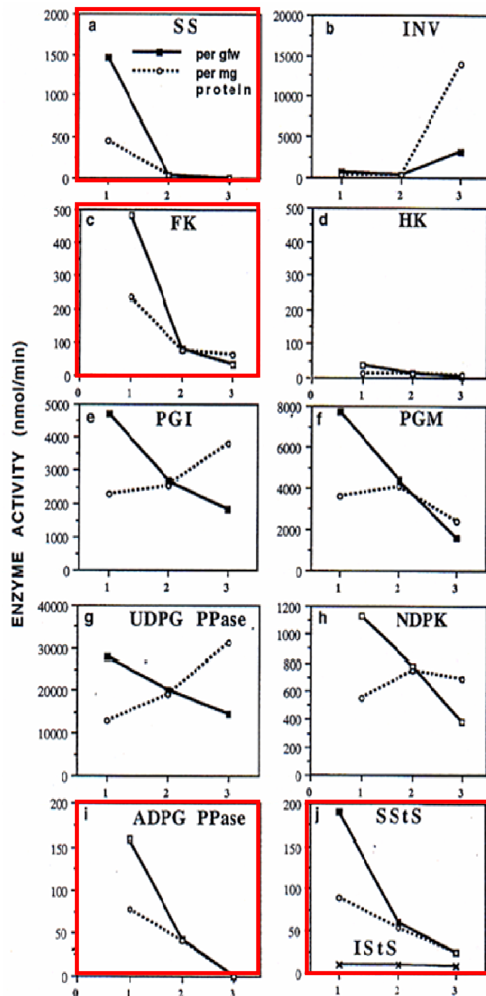

Fig 3. **Enzyme activities in developing fruit pericarp (outer plus inner) of cv F144. The three stages studied correspond to those pictured in Figure 1. Solid lines represent activities expressed on a fresh weight basis; broken lines represent activities expressed on a protein basis. a, Suc synthase (SS); b, acid invertase (INV); c, fructokinase (FK); d, hexokinase (HK); e, PGI; f, PCM; g, UDP-Glc PPase; h, NDP kinase; i, AGPase; j, soluble starch synthase (SStS) and insoluble starch synthase (IStS). IStS is presented only on a fresh weight basis and is represented by the symbol X. gfw, Gram fresh weight (Schaffer and Petreikov, 1997a).**

This was evidenced by the developmental decline in activity of these four enzymes, concomitantly with the decline in starch synthesis. The loss of activity of these enzymes was accompanied by the decrease in the respective enzyme protein level, as measured immunologically (Schaffer and Petreikov, 1997a). Furthermore, the comparison of their *in vitro* maximum activities also pointed to these four enzymes as potentially limiting to starch synthesis. Although we cannot be certain that our observed "maximal" extractable activities actually reflect *in vivo* activities, AGPase and starch synthase activities were closest to the rate of starch accumulation, 20 nmol starch/gfw/min, as calculated from reports in the literature (Ho *et al*., 1983; Ho and Hewitt, 1986). The activities of SuSy and FK were also among the lowest activities

measured in this study, but nevertheless appeared to be in excess of the starch accumulation rate. However, the *in vivo* activities of SuSy and FK may be well below their *in vitro* extractable activities, since both may be significantly inhibited by physiological levels of fructose in tomato fruit. The linkage between SuSy and FK, the two initial enzymes in the sucrose-to-starch pathway, and their possible coordinated control by fructose inhibition can be of great importance (Renz and Stitt, 1993; Sebkova *et al*., 1995; Viola, 1996; Schaffer and Petreikov, 1997a). The substrate inhibition of FK by fructose was described in different plants, including pea seeds (Copeland *et al*. 1984), potato tubers (Gardner *et al*. 1992) and tomato fruit (Martinez-Barajas and Randall, 1996). We showed in an earlier study that the physiological levels of fructose in the young tomato fruit, as determined by methods of compartmental analysis of immature fruit pericarp, reach cytosolic concentrations above 30 mM (Schaffer and Petreikov, 1997b). This concentration is higher than those in other starch synthesizing tissues, such as potato tubers (Morell and Rees, 1986) and pea seeds (Edwards and Rees, 1986), in which fructose concentration is only *ca* 1-2 μmol/gfw. We calculated a significant inhibition (up to 70%) of tomato fruit SuSy and FK by fructose at this physiological concentration of fructose (K*i* 2.4 mM and 2.0 mM, respectively) (Schaffer and Petreikov, 1997b). We also observed that both enzymes are inhibited by apparent physiological levels of Mg^{+2}. Taken together, the inhibitory effects of physiological levels of fructose and Mg^{+2} are potential mechanisms of regulation that can reduce flux of imported sucrose through these two steps to levels approaching in vivo rates of starch synthesis.

The spatial analysis of starch content in immature tomato fruit showed that starch accumulation was partially localized in the inner and radial pericarp and columella, whereas the outer pericarp and seed locule contain little starch (see Fig.1).

The two latter compartments were especially characterised by relatively low activities of AGPase and insoluble starch synthase, whereas activities of SuSy and FK were both high. Thus, the temporal metabolic control is not identical to the spatial control (Schaffer and Petreikov, 1997a) and further suggests a key limiting role of the above enzymes in the pathway.

2.3 The role of starch synthases

One of the four enzymes whose activity declines concomitant with the decline in starch synthesis is starch synthase. In amyloplasts, starch synthases have been identified in soluble (SStS) and starch-granule-associated forms (IStS), both of which utilize ADP-Glucose (ADP-Glc) as a substrate (Visser and Jacobsen, 1993). IStS, but not SStS, was found to have a relatively low activity in the portions of the fruit which contain little starch (Schaffer and Petreikov, 1997a). However, the early literature (Tsai, 1974) indicated that suppression of IStS does not have a significant effect on the total amount of starch produced in the *waxy* (or amylose-free) maize mutant; the lack of IStS, which is associated with amylose synthesis, was compensated by SStS (Tsai, 1974). Accordingly, IStS was deemed not to be an enzyme critical for the differences in starch synthesis in our research.

2.4 The role of sucrose synthase

In general, SuSy is not considered to directly affect starch synthesis. This is most succinctly shown by experiments with transgenic antisense SuSy tomato plants with a reduction in SuSy activity of up to 99% in young tomato fruit. The data show that the loss of SuSy did not affect or only slightly reduced starch accumulation and sugar level in the fruit (Chengappa *et al.*, 1999; D'-Aoust *et al.* 1999). However,

considering the importance of flux through the SuSy step it remains possible that an increase in SuSy could contribute to starch synthesis. Furthermore, the relative rates of synthesis and degradation of starch are controlled by the concentrations of Glc-6-P in the apoplast, which in turn can depend, to some degree, on SuSy activity in the cytosol (Nguen-Quoc and Foyer, 2001).

A novel and controversial mechanism of sucrose-to-starch synthesis was proposed by the research group of Pozueta-Romero (Pozueta-Romero *et al*., 1999). Accordingly, SuSy can be directly involved in ADP-Glc production from sucrose utilizing ATP rather than UTP, thereby directly producing the substrate for starch synthesis. Evidence for such a role was recently presented by this group based on SuSy-overexpressing and SuSy-antisense transgenic potato and *Arabidopsis thaliana* leaves (Munoz *et al*., 2005). Nevertheless, of the enzymes potentially limiting starch synthesis in developing tomato fruit, SuSy does not appear to be a likely candidate, in light of the data of earlier studies (Chengappa *et al*., 1999; D'-Aoust *et al*., 1999).

2.5 The role of fructokinases

Another enzyme that undergoes an apparent coordinated regulation of activity concomitantly with the decline in starch synthesis is FK. The enzyme catalyzes the phosphorylation of fructose, produced from sucrose degradation. The hexose phosphorylation is an essential step involving sugars in to cell metabolism, since non-phosphorylated hexose moieties are relatively inactive metabolically. The hexose phosphorylation is an irreversible metabolic step and the enzymes that catalyze this reaction potentially play an important role in the regulation of sucrose metabolism and sink strength. Fructose is estimated to be about 50% of the carbon provided to a sink as a product of various sucrose degradation reactions (SuSy and Inv). Since SuSy

catalyzes a reversible reaction, the removal of fructose by phosphorylation should shift the equilibrium in to the cleavage direction and enhance the sink strength of the tissue.

Hexose phosphorylating enzymes are functionally classified as either hexokinases (HK) (EC 2.7.1.1), glucokinases (GK) (EC 2.7.1.1) or fructokinases (FK) (EC 2.7.1.4) based on their substrate specificities. HK can phosphorylate a range of hexoses (glucose, fructose, and mannose) although many of the HK enzymes have a preferential affinity to glucose. FK and GK are more specific than HK and favor a particular substrate exclusively that typically more than 50-fold than other sugars (Martinez-Barajas and Randall, 1998; Quick and Schaffer, 1996; Pego and Smeekens, 2000). FKs have been purified and characterized from several plants, and most of studies that had already been reported at the beginning of the research suggested the presence of two FK isoforms.

FK isoforms were separated by ion-exchange chromatography from potato (Gardner *et al.,* 1992), spinach (Schnarrenberger, 1990), barley (Baysforder *et al*., 1989), avocado (Coperland and Tanner, 1988), pea seeds (Coperland *et al.,* 1978), maize (Doehlert, 1989), and *A. thaliana* (Gonzali *et al*., 2001). Two FKs were firstly isolated from young tomato fruit, but they exhibited almost identical kinetic characteristics, and it is not clear from the results published whether these isoforms represent the products of distinct genes (Delhaize and Randall, 1995; Martinez-Barajas and Randall, 1996).

Two genes *LeFRK1* and *LeFRK2* encoding for tomato FK enzymes were cloned and the activity of the expressed proteins was initially characterized in our laboratory (Kanayama *et al*., 1997, 1998). One of them (*LeFRK2*) is expressed primarily in the early stages of fruit development with mRNA localized in starch storing pericarp cells, and the product of the gene expressed in yeast shows inhibition

by high concentrations of fructose. The other gene (*LeFRK1*) is expressed throughout fruit development with mRNA distributed ubiquitously in the pericarp and its gene product is not inhibited by fructose.

During reinvestigation of FK activity in developing tomato fruit, we further separated and characterized three peaks with FK activity and two peaks with HK activity. The third FK enzyme, termed FK3, was established as a novel isozyme (Petreikov *et al*, 2001). Comparing the characteristics of FK isozymes and the products of two previously mentioned FK genes expressed in yeast (*LeFRK1* and *LeFRK2*), we determined that FKI is the gene product of *LeFRK2* gene and FKII is the gene product of *LeFRK1*. Following this study, we showed that *LeFRK2*, the gene encoding the major FK in tomato fruits, is not required for starch biosynthesis in developing fruits based on transgenic antisense FK tomato plants (Dai *et al*., 2002). Accordingly, following our research on FK characterization, the emphasis of the present research project was directed on the enzyme AGPase.

2.6 ADP-Glc pyrophosphorylase, a key enzyme in starch biosynthesis

ADP-Glc pyrophosphorylase (AGPase) activities were earlier reported to closely follow the starch level pattern in the developing tomato fruit (Yelle *et al*., 1988). In our preliminary study it emerged as the key enzyme potentially limiting to starch synthesis, both temporally and spatially (Schaffer and Petreikov, 1997a). AGPase (EC 2.7.7.27) catalyzes the synthesis of ADP-Glc, a soluble precursor and substrate for the starch synthases. In light of the fact that the enzyme plays no known role in plant metabolism other than in the pathway providing activated ADP-Glc for starch synthesis, it was postulated that AGPase functions as a limiting enzyme in starch synthesis and controls starch accumulation (Preiss and Sivak, 1996, 1998).

Reports on AGPase-mutant starchless genotypes (Tsai and Nelson, 1966; Dickinson and Preiss, 1969; Lin *et al*., 1988; Smith *et al*., 1989) and on transgenic plants with reduced AGPase activity and decreased starch synthesis (Lin *et al*., 1988; Müller-Röber *et al*., 1992**;** Geigenberger *et al.,* 1999; Weber *et al*., 2000; Obiadalla Ali, 2003) all point to the key role of this enzyme in limiting and determining starch formation. The first starchless maize mutants studied were *shrunken2 (sh2)* and *brittle2 (bt2)* mutants, which were found to contain 25 to 30% of the wild type starch content and exhibited a significant decrease of 90-95% in AGPase activity (Tsai and Nelson, 1966). Similarly, transgenic potato plants (Müller-Röber *et al*., 1992) and tomato fruit (Obiadalla Ali, 2003) with reduced AGPase activity also showed reduced starch content. In transgenic tomato fruit, 90% repression of AGPase activity resulted in decreased starch levels of up to 25% of wild type content; lower repression levels did not appear to affect the fruit starch content (Obiadalla Ali, 2003). The reverse also appears to be true: potato and tomato fruit over-expressing AGPase showed an increase in starch content (du Jardin *et al*., 1997), although the increase of starch content was in some cases disproportionate to the increase in activity (Stark *et al*., 1992; Sweetlove *et al*., 1996). Enhanced turnover of transitory starch was also reported by expression of up-regulated AGPase in *A. thaliana* **(**Obana *et al***.,** 2006).

Besides the genetically engineered modulation of starch content, we identified a natural genetic variability for increased starch in tomato fruit. This was observed in introgression lines developed from a cross between the cultivated tomato (*Solanum lycopersicum,* formerly *Lycopersicon esculentum;* Peralta *et al*., 2005) and a wild species of tomato (*S. habrochaites,* formerly *L. hirsutum*, LA1777) (Schaffer *et al*., 2000). In the present work the previous nomenclature will be used to designate the plant material. These introgression lines were characterized by increased starch

content as well as increased soluble solids content in the mature fruit (Schaffer *et al.*, 1999). These introgression lines were used as the plant material for our further investigation of the role of AGPase in transient starch synthesis in tomato fruit, since we observed early in the research that the increase in starch content was related to an increase in the activity of the limiting enzyme of starch synthesis, AGPase (Schaffer *et al.*, 2000).

The biochemistry of AGPase has been extensively reviewed (e.g., Preiss and Sivak, 1996; Ballicora *et al.*, 2004). AGPases from plants are heterotetramers comprised of two large subunits and two small subunits. The mature tomato protein has a *ca* 220 kD molecular mass with 50 and 51 kD for the small and large subunits, respectively, indicating a tetrameric structure for tomato fruit AGPase (Chen and Janes, 1997; Park and Chung, 1998). The single AGPase enzyme in the dicot tomato fruit is located in the plastid (Beckles *et al.*, 2001a, b), although a cytosolic location in this dicot also there has been reported (Chen *et al.*, 1998b), similar to that found in monocots.

The small subunit is generally considered to be the catalytic component with both catalytic and regulatory properties, whereas the large subunit functions primarily as an allosteric regulator of the enzyme. In plant tissues, the catalytic activity of AGPase is allosterically regulated by small effector molecules, 3-phosphoglyceric acid (3-PGA) and inorganic phosphate (Pi), whose levels control the rate of ADP-Glc formation and, in turn, starch biosynthesis (Preiss, 1982). The regulatory and kinetic properties of AGPase are not simply due to the large subunit modulating the properties of the small subunit but, rather, are a product of synergistic interaction between the two subunits (Kavakli *et al.*, 2001). Research indicates a common origin of the two subunits, with the progenitor characterized by both catalytic and allosteric

components. The large subunit lost catalytic residues more than 130 million years ago, evolving to have a regulatory role. It is possible to convert the modulatory large subunit into a catalytic one by substituting Lys 44/Arg44 and Thr54/Lys54 in potato tubers (Ballicora *et al*., 2005). In light of this common origin, variants of the subunits have been developed, which show that the large subunit can be further activated (Frueauf *et al*., 2003). Recent research has shown that both subunits of AGPase are involved in the allosteric regulation. Alterations in the large subunit (Kavakli *et al*., 2002) or in the small subunit (Cross *et al*., 2004; Hwang *et al*., 2005) can modify allosteric properties of the whole enzyme.

Post-translational modifications of AGPase were found in photosynthetic and non-photosynthetic tissues from a number of species. The formation of the small subunit dimers via intramolecular disulfide bonding between the two Cys_{12} of the small subunit N-termini results in partial inactivation of the potato enzyme (Tiessen *et al*., 2002). The signaling components leading to redox modulation of AGPase are thought to involve sucrose and glucose. In response to high sucrose levels the dimerization in potato tubers is reduced, resulting in increased AGPase activity and stimulation of starch synthesis (Tiessen *et al*., 2002). Starch synthesis in leaves is controlled by the redox modulation of AGPase in response to light and sugar level (Hendrics *et al*., 2003). This novel mechanism combines with allosteric and transcriptional control to coordinate AGPase activity in a network that allows starch synthesis to respond to a variety of physiological and environmental inputs. Allosteric control by 3-PGA and Pi operates in a time-frame of seconds, post-translational redox-modulation leads to changes in enzyme activity in a time-frame of about 30-60 min, while transcriptional regulation requires days to develop (Geigenberger, 2003).

A sequence comparison of both subunits from different plants and organs reveals that the small subunit is generally more conserved than the large subunit. Plant AGPase large subunits are tissue specific and can be divided into four groups: stem/ tuber, leaf, fruit/ root, and endosperm AGPase (Cross *et al*., 2004). In dicots, in general, a gene family of 3 or 4 members encodes for the large subunit, whereas only a single gene encodes for the catalytic small subunit. In *A. thaliana*, six AGPase-encoding genes were reported, four for the large subunit and two for the small subunit (Crevellin *et al*., 2005). As in potato, tomato contains three genes encoding for the large subunit (Agp*L1*, Agp*L2* and Agp*L3*) and only one for the small subunit (Agp*S1*) (Chen *at al*., 1998a; Park and Chung, 1998). Tomato isoforms have very high identities of 96-98% to the corresponding potato isoforms (Chen *at al*., 1998a). The three tomato large subunit coding regions are highly homologous (63-68%), whereas their 3'-noncoding regions showed below 40% homology (Park and Chung, 1998). Of the tomato large subunit genes, the *AgpL1* is most strongly expressed in developing fruit, followed by the *AgpL2*; the *AgpL3* is expressed in leaves (Chen *at al*., 1998a; Park and Chung, 1998; Li *et al*., 2002). Recent results of Li *et al*., (2002) showed that expression of the *AgpS1* extends for a longer period of tomato fruit development, remaining until 40—45 days after anthesis, as compared to the expression of *AgpL1*, which was observed only until 25 days, similar to the more weakly expressed *AgpL2*. Accordingly, the transcription of the *AgpL1* ceases earlier in development than that of the *AgpS1*, which may have significance with regard to the enzyme activity during fruit development.

2.7 <u>Enzymes involved in starch degradation</u>

There is a large group of enzymes involved in the process of starch degradation, consisting of starch phosphorylase, isoamylases, α-amylase, β-amylase, and disproportionating enzymes or D-enzymes. In tomato fruits, the most important enzyme in starch breakdown is starch phosphorylase, which degrades starch to produce Glc-1-P. The amylase activity is very low and the activities of both starch phosphorylase and amylase change little during fruit development (Yelle *et al*., 1988). While the rate of starch synthesis is variable, the rate of breakdown appears to be relatively constant. The content of accumulated starch decreases when the quantity of newly synthesized starch is lower than that being degraded. The phenomenon of simultaneous starch synthesis and degradation in amyloplasts has been described for other species (Nguyen-Quoc and Foyer, 2001). However, it appears that the synthesis of starch via the synthetic enzyme, AGPase, rather then starch degradative enzymes (amylase and starch phosphorylase) regulate the transient accumulation of starch in cultivated tomato fruit *L. esculentum.* (Robinson *et al*.,1988; Yelle *et al*., 1988).

3. Objectives

The general objective of this work was to analyze the biochemistry and molecular control of starch synthesis in tomato fruit.

The specific objectives were:

1. Separation and characterization of hexose-kinases potentially limiting starch synthesis in tomato fruit.
2. Determining the role of AGPase in the temporal and spatial synthesis of starch in tomato fruit.
3. Exploring the role of the large subunit $AgpL1^{H}$ from the wild tomato *L. hirsutum S.* in contributing to high AGPase activity and elevated transient starch accumulation in tomato fruit.
4. Sequencing and comparative analysis of two AGPase L1 alleles from the wild *L. hirsutum S.* ($AgpL1^{H}$) and cultivated *L. esculentum M.* ($AgpL1^{E}$) tomato.

4. Methodologies

4.1 Plant material

The high starch breeding line used in this study (line 904) was developed from the interspecific cross of *L. hirsutum* (LA1777) and *L. esculentum* and a backcross program to recurrent *L. esculentum* cvs., as described previously (Schaffer *et al*., 1999, 2000), using continuous pedigree selection for total soluble solids (TSS) and soluble sugar content of the mature fruit. In addition, the 904 line, which itself was developed by backcrossing to the recurrent cv. M-82 and was crossed again to the recurrent parent resulted in genetic populations segregating for starch content and AGPase enzyme activity, which we refer to as near isogenic lines. Plants of subsequent generations resulting from these lines (NILs) were grown either in the field during the summer or in a heated greenhouse during the winter, according to standard growing conditions. For the analysis of FK isozymes, plants of tomato cvs. 7844 and F144 were used.

Flowers of similar cluster position were tagged at anthesis and fruitlets sampled at 5-10 days intervals during ripening. The samples were immediately frozen in liquid nitrogen and stored at -80°C.

Chemicals and enzymes were purchased from Sigma, Bio-Rad, and Boehringer Mannheim.

4.2 Sugar and starch assays

Soluble carbohydrates and starch were assayed from approximately 1g fresh weight of tissue as previously described (Schaffer *et al*., 1997; Miron and Schaffer, 1991). Soluble sugars were extracted three times in hot 70% EtOH, concentrated by

evaporation, dissolved in water and separated by HPLC using a Bio-Rad Fast Carbohydrate Column with HPLC-grade H_2O as solvent and refractometric detection, according to the manufacturer's directions. Sucrose, glucose, and fructose were identified by their retention times and quantified according to standards.

Starch content was measured in the insoluble fraction. The insoluble fraction was dried, autoclaved with 6 ml of water for 1 hr, cooled to room temperature. After addition of 0.5 ml of 2 M acetate buffer (pH 4.8), 2.5 ml water, and 1 ml amyloglucosidase (10 mg/ml) for starch digestion, the sample was incubated at $55^{O}C$ overnight. For quantification of released glucose, the Sumner reagent (1.3 M sodium potassium tartarate, 40 mM 3,5-dinitrosalicylic acid, 0.4 N NaOH) (Sumner, 1921) was added to the filtrated soluble fraction in the proportion 1:1, the mixture was boiled for 3min, cooled and the absorbance determined at 550 nm. Glucose was used to produce a calibration curve.

For visualization of starch accumulation, freehand sections of tomato fruit were stained with a 0.3 % I /1.5 % KI solution.

TSS (Brix) values were measured on the extruded fruit juice using an Atago hand held refractometer (Atago, Tokio, Japan)

4.3 <u>Enzyme extraction and assays</u>

4.3.1 Fructokinase (FK) and hexokinase (HK)

a) Extraction

FK (EC 2.7.1.4) and HK (EC 2.7.1.1) activities were extracted as follows: approximately 2.5 g fresh weight of fresh pericarp tissue was homogenized (Kinematica homogenizer, Littau, Switzerland) in 2 volumes of chilled extraction buffer containing 50 mM Hepes-NaOH (pH 7.5), 1 mM $MgCl_2$, 1 mM EDTA, 10 mM

KCI, 2.5 mM DTT, 1% PVPP, 3 mM DIECA, and 1mM PMSF. After centrifugation at 18,000g for 30 min, the supernatant was precipitated with 80% ammonium sulfate and centrifuged for 10 min at 18,000g. The precipitate was resuspended in 1 ml of the extraction buffer and desalted on a Sephadex G-25 column with washing buffer containing 50 mM Hepes-NaOH (pH 7.5), 1 mM EDTA, and 1 mM DTT (buffer W). The desalted extract was used as a first step purified enzyme preparation.

b) Separation on HPLC

For separation of FK and HK isozymes, 25 to 80% ammonium sulfate precipitate was collected, resuspended in extraction buffer, desalted on Sephadex G-25, and filtered through a 0.2 μm cellulose acetate membrane (Schleicher & Schuell, Germany). Enzyme separation was performed on a Shimadzu HPLC system. For the separation of the FK enzymes in young and mature fruit, as well as the yeast-expressed enzymes, the protein extract was applied (flow rate 0.5 ml/min) to a MonoQ column HR 5/5 (Pharmacia Biotech AB, Uppsala, Sweden) pre-equilibrated with buffer containing 20 mM HEPES (pH 7.0) and 2.5 mM DTT. Unbound protein was eluted with the same buffer, followed by a 0 to 0.5 M KCl gradient. Protein was monitored at 280 nm. Fractions of 0.5 ml were collected and both fructose and glucose phosphorylating activities were measured. The fractions containing peak activities of the individual isozymes were bulked for further characterization. For the results presented in Fig. 4, a MonoP column was used under identical elution and buffer conditions described for the MonoQ column. The use of a MonoP column allowed better separation of FKI and HKI isozymes.

c) Protein extraction from yeast.

The yeast-expressed enzymes were extracted as described previously (Kanayama *et al*., 1998). Cells (40 ml) were centrifuged for 5 min at 3,800 g, washed twice with 40 ml water, and resuspended in 1.0 ml of water. Cells (pellet approximately 0.25 ml) were extracted twice with 0.5 ml of extraction buffer (50 mM Hepes pH 7.5, 1 mM EDTA, and 1mM PMSF) by vortexing with 0.25 ml of glass beads. Following vortexing for 90 s, the mixture was centrifuged for 5 min at 12,000*g* at 4°C, and the supernatant was brought to 80% $(NH_4)_2SO_4$ saturation. After centrifugation at 12,000*g* at 4°C, the pellet was resuspended in 0.5 ml of washing buffer (buffer W), desalted on a G-25 Sephadex column, filtered through a 0.2 µm cellulose acetate membrane and used as a first step purified enzyme preparation for subsequent enzymatic analysis. Enzymes were separated on MonoQ ion-exchange chromatography under identical conditions used for the fruit pericarp enzyme extracts.

d) Enzyme-linked assay

FK and HK activities were measured by an enzyme-linked assay, according to a modification of the method of Huber and Akazawa (1985). Two different assays were used for measuring FK activity.

Assay 1 for FK activity was conducted with low fructose and magnesium concentrations and the reaction mixture (1 ml) contained 30 mM HEPES (pH 7.6), 9 mM KCl, 0.5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD, 1 unit PGI (type III), 1 unit NAD-dependent Glc-6-P DH (from *Leuconostoc*) and the reaction was initiated with 1 mM fructose.

Assay 2 for FK activity was conducted with high fructose (10 mM) and Mg^{+2} (3 mM) concentrations. For determining HK activity, glucose (10 mM) served as substrate, $MgCl_2$ levels was 3 mM and PGI was excluded from the reaction mixture.

All the enzymatic reactions were carried out at 37°C and monitored continuously at 340 nm, as previously described (Schaffer and Petreikov, 1997a). Optimum conditions for each isoenzyme were used in enzyme assays for characterization of the three FK isoforms. For FKI assay 1 (low fructose and Mg^{+2}), while for FKII and FKIII, assay 2 (high fructose and Mg^{+2}) was used. ADP inhibition studies were carried out using the same enzyme extract with ADP concentrations ranging up to 5.0 mM. For kinetic studies, the substrates fructose, NTP and $MgCl_2$ were used in the increasing concentrations from 0 to 1mM.

4.3.2 Soluble acid invertase (Inv) and sucrose synthase (SuSy)

Inv (EC 3.2.1.26) and SuSy (EC 2.4.1.13) were assayed in the cleavage direction according to Miron and Schaffer (1991) with slight modifications. Approximately 1 g fresh weight frozen tissue was homogenized in a Kinematica homogenizer in 3 volumes of extraction buffer containing 50 mM Hepes-NaOH (pH 7.5), 0.5 mM Na- EDTA, 2.5 mM DTT, 3 mM DIECA, and 1mM PMSF. After centrifugation at 18,000g for 30 min, supernatants were dialyzed for about 16 h against 25 mM Hepes-NaOH (pH 7.5) and 0.25 mM Na-EDTA and used as a partially purified soluble enzyme preparation.

Inv activity was assayed in a reaction mixture containing 0.3 ml of 0.1 M K_2HPO_4 - 0.1 M citrate buffer (pH 5), 0.1 ml o 0.1 M sucrose, and 0.1 ml of enzyme extract. For SuSy synthase assay the buffer was adjusted to pH 7 and 25 μl of 100 mM UDP was added as a second substrate to the reaction mixture (Schaffer *et al*., 1987).

Following the incubation for 30 min at 37°C, the reaction was stopped by adding 0.5 ml of Sumner reagent and reducing sugars were measured at 550 nm (see starch determination). For control, the enzyme was added after the reaction was stopped by addition of Sumner reagent.

4.3.3 UDP-glucose pyrophosphorylase (UGPase)

For the assay of UGPase (EC 2.7.7.9), 1 g fresh weight of fruit tissue was ground in liquid nitrogen and extracted in 2 ml of chilled extraction buffer containing 50 mM Hepes-NaOH (pH 7.8), 1 mM $MgCL_2$, 1 mM EDTA, 2.5 mM DTT, 3 mM DIECA, and 1mM PMSF. The supernatant after centrifugation (18,000g, 30 min) was used for assaying UGPase activity.

UGPase was measured spectrophotometrically as described by Doehlert *et al.* (1988) with minor modifications via a continuous enzyme-linked assay coupling the pyrophosphate-dependent production of Glc-1-P to Glc-6-P (with PGM) and monitoring the Glc-6-P DH catalyzed production of NADH at 340 nm.. The 1ml of reaction mixture contained 50 mM Hepes-NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM UDP-Glc, 1 mM NAD, 1 unit of Glc-6-P DH, 2 units of PGM, 20 μM Glc-1,6-bisP, and 3 to 5 μl of the enzyme extract. Following 5-min incubation at $37^{O}C$, the reaction was initiated by the addition of 1 mM PPi for a total volume of 1 ml.

4.3.4 Phosphoglucoisomerase (PGI)

PGI (EC 5.3.1.9) was extracted and assayed as described for UGPase, in a 1ml of reaction mixture containing 50 mM Hepes-NaOH (pH 7.8), 5 mM $MgCl_2$, 5 mM Fru-6-P,1 mM NAD, 1 U Glc-6-P DH, and 5 to 10 μl of the enzyme extract.

4.3.5 Phosphoglucomutase (PGM)

PGM (EC 5.4.2.2) was extracted and assayed as described for UGPase, in a 1ml of reaction mixture containing 50 mM Hepes-NaOH (pH 7.8), 5 mM $MgCl_2$, 5 mM Glc-1-P, 20 μM Glc-1,6-bisP, 1 mM NAD, 1 U Glc-6-P DH, and 10 to 20 μl of the enzyme extract.

4.3.6 ADP-Glccose pyrophosphorylase (AGPase)

a) Extraction

AGPase (EC 2.7.7.27) was extracted according to the method of Chen and Janes (1995) with slight modifications. 1 g frozen tissue was ground in liquid nitrogen and quartz sand in 2 ml of chilled extraction buffer containing 50 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 3 mM DIECA, and 1mM PMSF. The supernatant after centrifugation (13,000g, 20 min) was incubated for 4 min at $60^{O}C$, chilled on ice and the supernatant obtained after additional centrifugation was used as the first step enzyme. The heat treatment increased extractable enzyme activity by approximately 10-fold.

b) Separation on HPLC

For kinetic analysis and electrophoresis the first step purified enzyme extract from 4.5 g frozen fruit tissue was filtered through 0.2 μm cellulose acetate filter (Schleicher & Schuell, Germany) and loaded onto a MonoQ column HR 5/5 (Pharmacia Biotech AB, Uppsala, Sweden) for additional purification by HPLC , as described above for FK (see 4.3.1b). A single peak of AGPase activity was eluted at

0.45 M KCl (pH 6.8) with approximately 200-fold purification (13μmol/mg protein/min for AGPase-L1^H and 7.5 μmol/mg protein /min for AGPase-L1^E).

c) Enzyme-linked assay

***Pyrophosphorolytic direction* (AGP-Glc + PPi → ATP + Glc-1-P)**

Assay 1. For the determination of enzyme activity in first step purified enzyme preparation purified enzyme preparation and for monitoring HPLC purification steps, AGPase activity was assayed in the pyrophosphorolytic direction, as previously described (Schaffer and Petreikov, 1997a) with minor modifications. The 0.5 ml of assay mixture contained 50 mM Hepes-NaOH (pH 7.8), 5 mM $MgCl_2$ (buffer A), 1 mM ADP-Glc, 1 mM NAD, 10 mM 3-phosphoglyceric acid (3-PGA), 5μM Glc-1,6-bisP, 10 mM freshly prepared NaF, 1U Glc-6-P DH (from *Leuconostoc*), 2U PGM, and up to 0.1 ml of enzyme extract in of. Following 5 min of incubation at 37°C, the reaction was initiated by the addition of 1 mM PPi. The production of NADH was monitored spectrophotometrically at 340 nm. One unit of pyrophosphorolytic activity was defined as the amount of enzyme which catalyzes the formation of 1 nmol of Glc-1-P/min.

Assay 2. For kinetic analysis in the pyrophosphorolytic direction a two-step enzyme linked endpoint assay was used. The Glc-1-P production was measured at 37°C in buffer A containing 1 mM ADP-Glc, 10 mM 3-PGA, 10 mM NaF and 20 μl of enzyme extract in a total volume of 200 μl. The reaction was initiated by addition of 1 mM PPi and stopped after 5 min by boiling for 2 min. After cooling on ice a mixture containing 300 μl buffer A, 1 mM NAD, 10 μM Glc-1,6 bis-P, 1U Glc-6-P DH (from *Leuconostoc*), 1U PGM was added. After 40 min of incubation at 37°C, absorbance of produced NADH was recorded at 340nm. Concentrations from 0 to 1 mM of the

substrates ADP-Glc and PPi and the activator 3-PGA were compared. The amount of Glc-1-P produced was quantified using a calibrating curve of 0-100 nmol of Glc-1-P in 0.5 ml of reaction mixture under the same assay conditions and was defined as an amount of enzyme necessary to produce 1μmol Glc-1-P per min at 30°C.

For the studying the enzyme heat stability assay 2 was used. The reaction mixture including the enzyme was heated at temperatures ranging from 40°C to 72°C for 3 min. After cooling and centrifugation at 4°C (13,000g), enzyme activity was determined as Glc-1-P produced as above. Preliminary studies indicated that activity was proportional to enzyme content and incubation time.

ADP-Glc synthesis direction (ATP + Glc-1-P → ADP-Glc +PPi)

Assay 3. In the synthesis direction enzyme activity was analyzed as described by Tiessen *et al*. (2002) with slight modifications. The assay contained 1.5 mM Glc-1-P, 3 mM ATP, 5 mM 3-PGA and 20 μl of enzyme extract in 200 μl of buffer A. After incubation at 37°C for 10 min the reaction was stopped by boiling for 2 min. For kinetic studies the substrates Glc-1-P and ATP and the activator 3-PGA were used in the concentrations ranging from 0 to 3 mM and from 0 to 1 mM, respectively. The ADP-Glc product was quantified by HPLC using a reverse-phase Adsorbosphere Nucleotide-Nucleoside column (see nucleotide-nucleotide sugar assay below). The Km and $A_{0.5}$ values corresponding to the substrate or activator concentrations, respectively, at which the reaction velocity is half-maximal, were calculated using Lineweaver-Burk plots and regression plot Excel program 2002.

4.3.7 Soluble and insoluble starch synthases (SStS, IStS) (EC 2.4.1.21)

SStS and IStS were extracted according to a modification of the method of Ching *et al.* (1983). Two grams of fresh weight of tomato fruit tissue were ground in

two ml of chilled extraction buffer consisting of 100 mM Hepes-NaOH (pH 7.5), 1 mM EDTA, 5 mM DTT, 10 mM K_2HPO_4, and 1 mM PMSF. After homogenization and centrifugation (18,000g, 40 min), the pellet was washed in the same buffer and centrifuged for 10 min at 18,000g. The supernatant of the wash was added to the initial supernatant and served as the crude soluble enzyme. The remaining pellet was suspended in 2 ml of the extraction buffer and served as the crude insoluble enzyme.

Starch synthase activity was assayed spectrophotometrically by measuring the ADP-Glc dependent production of ADP. For the soluble enzyme, the assay buffer contained, in a total volume of 0.75 ml, 100 mM Tricine (pH 8.6), 25 mM potassium acetate, 2 mM DTT, 2 mM ADP-Glc, 3.75 mg of insoluble amylopectin, and 0.2 ml of enzyme extract. Reactions were carried out for 15 min at 30°C and terminated by boiling for 1 min. Blank reactions in which the enzyme was added immediately prior to boiling were performed. IStS was assayed as above, but without priming with amylopectin. The ADP product was measured via an enzyme-linked assay with pyruvate kinase / lactate dehydrogenase, according to a modification of the method of Jaworek and Welsch (1985). Following centrifugation, 100 ml of the ADP product from the first step reaction was added to 400 ml a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 20 mM KCI, 0.2 mM NADH, 0.8 unit of pyruvate kinase and 1.2 units of lactate dehydrogenase was added and incubated at 37°C for 30 min. Reactions were started with 1 mM PEP, and the NAD production was measured as A_{340}.

4.4 Genotypic determination of the *AgpL1* allele

Genomic DNA (gDNA) was isolated by method of Edwards *et al*. (1991) from young leaves parental and plants of segregating generations. The parental wild species

LA1777 and the *L. esculentum* cultivar M-82 were used as parental plants. The determination of the *AgpL1* genotype in segregating populations was based on a PCR reaction with specific primers for polymorphism found in N-terminal duplication of EKK in *AgpL1*^{H} (*L. esculentum* accession U81033 and *L. hirsutum* accession AF184345). The primers used were: forward-A1PF (5'GAGGGAGTTTGAACAACAATC3'), and reverse-A1PR (5'TAAGCAACCCCAGGTTTAA3') with amplicons of 94bp and 85bp for *AgpL1*^{H} and *AgpL1*^{E}, respectively. Alternatively, based on a 15bp insertion in *AgpL1*^{H} intron 13, primers offering better resolution were designed for genotypic analysis:

Forward - A3PF (5'GATTGCCTCCCTGTTAGCAG3') and

Reverse - A3PR (5'CCTATCTTTGCGTTCTTGTCAA3') with an amplicon 205bp and 190bp for *AgpL1*^{H} and *AgpL1*^{E}, respectively. The PCR reaction consisted of 35 cycles at 94°C for 15 sec, 60°C for 15 sec, and 72°C for 15 sec. Based on polymorphism found in the promoter region of the two *AgpL1* alleles, a couple of primers with good resolution for screening large segregating population of tomato plants were designed. The PCR reaction conditions were as previously mentioned with the following primers:

Forward - AprF (5'CCCTTTAAATTTCTTAGCCYAC3'), and

Reverse - AprR (5'GTTGGAGATTAGCTGACAAT TTC3) with amplicons of 184bp and 266bp for *AgpL1*^{H} and *AgpL1*^{E} respectively.

4.5 Determination of introgression size

The primers for the genetic map markers used to define the introgression size were designed based on published database sequences (www.sgn.cornell.edu):

Marker		Primer	T ann ^{O}C	Product,bp
TG161	161F	5'CTCGCATTACTGGAGATGAT3'	58	1300
	161R	5'AAAGGCTTACATCATTAAGATTC3'	60	
TG237	237F	5'ATTGGCTTGTTATGAAGTTATA3'	57	1500
	237R	5'GTCGCCTGAAACCTGAGAG3'	61	
TG255	255F	5'GCTGCGATATACACAACAGG3'	60	238
	255R	5'GAAGAGTCCTAACAACATAGAT3'	60	
TG158	158F	5'CCCCTAAGACTCTCCAGATT3'	60	188
	158R	5'CTCCGAGGAAAGAGGATAGA3'	60	
TG269	269F	5'TCTCCCCAACATAAACAGCA3'	60	329
	269R	5'AAGGCAAAACCATGTGATAC3'	59	
TG 90	90F	5'GTAGACCATCTTTGATCGTAACTAAT3'	70	esc-400
	90R	5'GGACTACGTATACTTGGGCCTGTT3'	72	hir-200

Three additional markers were obtained by transformation of relevant plasmids pGEM4Z (RFLP Stock of tomato at the Plant Genome Center, Weizmann Institute, Israel) into competent cells (JM109, Promega, USA) and sequencing of the purified product (AccuPrep Plasmid Extraction Kit, BioNeer, Korea, Hy-Labs) with T7 and SP6 primers. Primers developed for these markers were as follows:

Marker		Primer	T ann ^{O}C	Product, bp
TG267	267F	5'CGCCCCTCTTAGCAAAGGT3'	62	1000
	267R	5'GAGAGTTAACTGTTCGTATCCA3'	62	
TG389	389F	5'GCATGCTAACATTTATGCATC3'	58	480
	389R	5'GCAGACTTACAGATCCAATG3'	58	
TG159	159F	5'GCCATAACACAGATTCCACAG3'	62	420
	159R	5'GGAACAGAATGTCATCCAATG3'	60	

Primers were synthesised by IDT, Inc., USA. Polymorphisms for each of the markers were identified by sequencing the PCR product. Sequencing was carried out at The Center for Genomic Technologies, The Hebrew University of Jerusalem and DBS Company, Israel.

4.6 <u>Genomic sequencing</u>

A complete gDNA sequencing of the AGPase large subunit (*AgpL1*) in the parental plants and in the segregating genotypes was performed. DNA was isolated by using genomic extraction kit G-spin IIp for plants (iNtron Biotechnology, Korea). Primers were designed according to the published gene sequences: *L. esculentum* accession U81033, *L. hirsutum* accession AF184345 and promoter regions of tomato *L. esculentum* accession AY858853 and potato *S. tuberosum* accession X75017.

4.7 <u>Quantitative real-time PCR</u>

RNA was isolated from three individual tomato fruits using EZ- RNA Total RNA Isolation Kit (Biological Industries, Israel), according to the manufacturer's instructions. Reverse transcription reactions and real-time PCR on the GeneAmp 5700 Sequence Detection System (PE Biosystems) were performed as described in Miron *et al*. (2002).

The specific primers were designed using Primer Express software, Version 2.0 (Applied Biosystems) base on published sequences:

AgpL1 (U81033) Forward - 5'GATTATATGGAGTTGGTGCAGAACC 3',

Reverse - 5'ACCAGCCCAAAATCTGATGCT 3';

AgpL2 (U81034) Forward - 5'GTCGGGCCAAACACTAAGATACA 3',

Reverse - 5'TCAGCTTCTTCAACACCTTGCTT 3';

AgpL3 (U85497) Forward - 5' AACCTTGCACTCACTGAACATCC 3',

Reverse - 5'TTATCAATCGCTGATGGAGGTAAGT 3';

AgpS1 (L41126) Forward - 5'AAAATGCTTGATGCCGATGTC3',

Reverse - 5'TGATCTGAGCCCAACCACG3'.

Actin (U 60482) was used for normalizing the expression data with the primers:

Forward - 5'CACCATTGGGTCTGAGCGAT3',

Reverse - 5'GGGCGACAACCTTGATCTTC3'. Each specific amplicon in all reactions had only one dissociation peak and calibration curves for all genes had $R^2 = 0.94-0.99$.

The program used for the real-time PCR was as follows: 10 min at $95^{O}C$, 40 cycles of 15 s at $95^{O}C$, 30 s at $60^{O}C$, and 30 s at $72^{O}C$. Small products of the amplification reactions of approx 100 bp amplicons were analyzed by dissociation curve for existence of only one peak and sequenced to ascertain the PCR product. The specific gene expression was calculated relative to the expression of actin according to the equation $2^{-(Ct\ sample - Ct\ actin)}$, where Ct is the threshold cycle of the specific gene and actin.

4.8 Electrophoresis and immunoblotting

Protein of partially purified AGPase, after either heat treatment of the crude extract or MonoQ chromatography, was concentrated by acetone precipitation. The pellet was diluted in the 1×sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, and 0.1% bromophenol blue) up to 2 μg/μl protein concentration, incubated in ice for 1 hour, boiled for 3 min, centrifuged and subjected to SDS-PAGE in a Bio-Rad Mini-Electrophoresis System (10% or 15% 1 mm acrylamide gel according to Laemmli (1970)). Precision Plus Protein Standards (Bio-Rad laboratories, Inc) were used as markers for estimating the size of separated proteins. The separation was carried out at RT in running buffer (25 mM Tris-HCl, pH 8.35; 189 mM Glycine, 0.10% SDS) as follows: 80 V for 10 min, 120 V up to the sample is concentrated on the lower gel edge, 200 V up to required separation.

Proteins in gel were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 in fixative (40% methanol, 10% acetic acid) for 1 hr and destaining overnight in destaining solution (20% methanol, 10% acetic acid).

For protein transfer the gel and the cellulose nitrate (E) Protran BA 83 membrane (Schleicher & Schuell, Germany) were pre-incubated in chilled transfer buffer (25 mM Tris-HCl, pH 8.35; 192 mM Glycine, 20% v/v methanol) for 30min, and the transfer process was carried out at 100 V for 1hr in chilled buffer. After protein transfer, the membrane was washed 3x10 min in wash buffer (1xPBS, 0.1% Tween 20, 0.02% sodium azide) and pre-incubated in block solution (wash buffer with 0.2% casein (I-Block, Applied Biosystems, USA) for 30 min for blocking most nonspecific binding sites for immunoglobulins. The phosphate buffer 10xPBS, used for preparation of wash and block solutions consists of 0.58 M Na_2HPO_4, 0.17 M NaH_2PO_4, 0.68 M NaCl (pH 7.35). The membrane was incubated with rabbit antibodies raised against the specific AGPase large and small subunits from potato tubers in dilution of 1:5,000 in block solution at RT for 3 hr or overnight at $4^{O}C$. The specific antibodies were kindly supplied by TW Okita. After incubation with an alkaline phosphatase-conjugated anti-rabbit antibodies for 2 hr at RT, bands were visualized in the dark using 5-bromo-4-chloro-3 indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) (Promega, Madison WI, USA). The staining buffer consisted of 16 µl NBT and 10 µl BCIP in 10 ml of 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5).

For the determination of post-translational redox modification, the proteins from green tomato fruits and from small potato tuber (11gr) were extracted, according to Tiessen *et al.* (2002). Frozen plant material was extracted directly with 1×sample buffer in a ratio 1mg tissue:5 µl sample buffer. After centrifugation of the sample for

30 s, the supernatant was boiled immediately for 5 to 10 min. To a part of the sample, 4 mM DTT was added (+DTT), whereas the rest of the sample was used without DTT (-DTT). Electrophoresis and immunoblotting were carried out as above. The intensity of the bands was estimated by the use of TotalLab v.2.00 software program.

4.9 Metabolite analysis

4.9.1 Metabolite extraction

Glc-6-P, Glc-1-P, 3-PGA, ATP and ADP-Glc were extracted from 0.5 g of frozen whole fruit tissue in liquid nitrogen by grinding in 1ml of cold 5% perchloric acid, followed by incubation on ice for 1 hr. The pellet after centrifugation at 13,000 g for 5 min at $4^{O}C$ was washed with 0.125 ml dd water and the combined supernatant neutralized with 3 M KOH. After 10min incubation on ice and centrifugation, the Glc-1-P, Glc-6-P and 3-PGA were immediately assayed. For sugar-nucleotide determination the extraction was performed on 1.5 g tissue, the neutralized supernatant was lyophilized, re-dissolved in 0.5 ml HPLC grade water and insoluble residues were removed by centrifugation at 13,000g for 10min at $4^{O}C$. To assess the recovery from the tissue, 125 nmol of each metabolite was added to the perchloric acid during extraction at the grinding stage.

4.9.2 Glc-P assay

Glc-6-P and Glc-1-P were measured in a coupled enzyme assay in 0.5 ml of 50 mM HEPES (pH 7.8) containing 1 mM $MgCl_2$, 7 mM EDTA, 1 mM NAD, 5 μM Glc-1,6 bis-P, 100 μl of metabolite extract and initiated with 0.5 U Glc-6-P DH (from *Leuconostoc*). After 10min incubation at $37^{O}C$, the absorbance was monitored spectrophotometrically at 340nm for Glc-6-P determination. 1U of PGM was added to

the reaction mixture for measuring Glc-1-P concentration and the absorbance was monitored after an additional 20min of incubation. The amount of Glc-P produced was quantified from a standard calibration curve (r^2=0.998) of 0-50nmol Glc-6-P/Glc-1-P in 0.5ml of reaction mixture under the same assay conditions.

4.9.3 3-PGA assay

100 µl of metabolite extract was added to a final volume of 0.5 ml consisting of 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM ATP, 20 µM NADH (in 5% $NaHCO_3$). The reaction was started by 10 U 3-phosphoglyceric phosphokinase and 10 U glyceraldehyde-3-phosphate DH and monitored spectrophotometrically at 340 nm for 10 min at 37°C. The amount of 3-PGA produced was quantified from a standard calibration curve (r^2=0.999) of 0-50 nmol 3-PGA in 0.5 ml of reaction mixture under the same assay conditions.

4.9.4 Nucleotide-nucleotide sugar assay

The nucleotide ATP and nucleotide sugar ADP-Glc were separated and quantified by HPLC using the reverse-phase Adsorbosphere Nucleotide-Nucleoside C18column (7µm, 7.5 x 4.6mm, Alltech Associates, USA). The mobile phase contained 40mM $NH_4H_2PO_4$ and 31mM triethylamine, pH 7.4. The same mobile phase was used for column pre-equilibration and for the metabolites separation, 2 ml/min flow, at RT for 50min. The identification of each metabolite was carried out by comparing the retention times with commercial standards and the quantity was determined by comparing the sample peak areas to standard peak calibration curve areas. The absorption was monitored at 254nm by UV detector (Jasco UV-975, Japan).

4.10 <u>Protein estimation</u>

The Bio-Rad protein assay using BSA as a standard was applied to estimate the protein concentration according to the method of Bradford (1976).

4.11 <u>Statistical calculations</u>

The estimation of standard errors and statistical significant were calculated using Excel software 2002 and JMP 5 Statistical Discovery software.

5. Results

5.1 Characterization of the native and yeast-expressed tomato fruit fructokinase enzymes

5.1.1 Fructokinase isoenzymes during fruit development

5.1.1.1 *Separation on HPLC-ion exchange column*

Hexose phosphorylation activity from young tomato fruit was separated by HPLC-ion exchange chromatography (Fig. 4). Three peaks of activity were observed with fructose as substrate, as well as two additional peaks of activity with glucose (termed HK1 and HK2). The three fructose phosphorylating peaks (termed FKI, FKII and FKIII, according to the order of elution) were detected using two different assays. One assay contained low concentrations of fructose (1.0 mM) and Mg^{+2} (0.5 mM), in the presence of which the FKI enzyme showed maximal activity. The second assay contained high fructose (10 mM) and high Mg^{+2} (3 mM), which gave maximal activities for FKII. The FKIII enzyme, which eluted only under high ionic concentration, showed approximately equal activities with both assays. The two glucose phosphorylating peaks (HK1 and HK2) were assayed with high glucose (10 mM) and high Mg^{+2} (3 mM) concentrations. Both HK1 and HK2 peaks were highly glucose specific, and had minor activity with fructose as a substrate; the HK enzymes were not inhibited by elevated concentrations of glucose or $MgCl_2$ (Fig. 5).

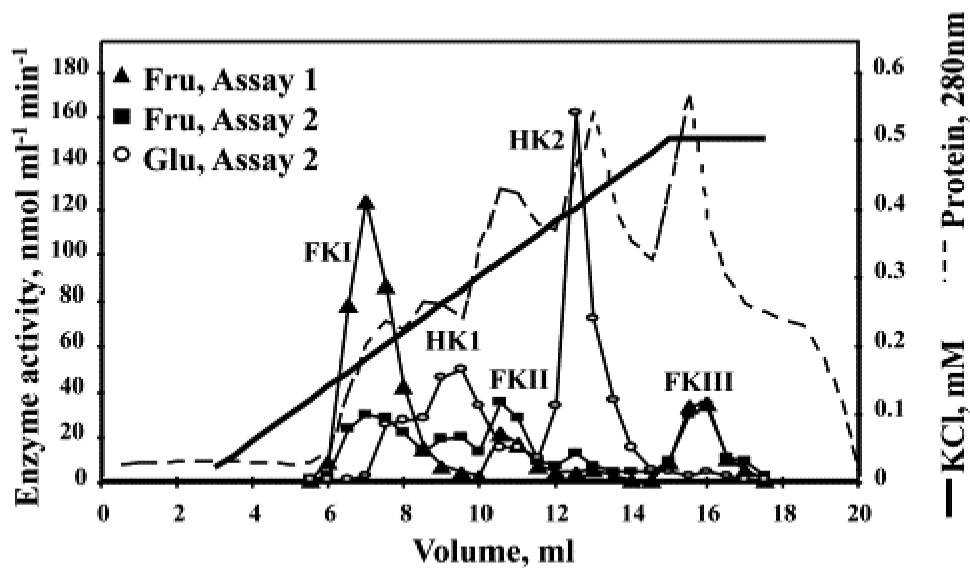

Fig. 4. HPLC-ion exchange (MonoP) separation of hexose kinase activity in immature tomato fruit pericarp. Assay 1 contained low fructose (1.0 mM) and low Mg^{+2} (0.5 mM). Assay 2 contained high fructose or glucose (10 mM) and high Mg^{+2} (3 mM).

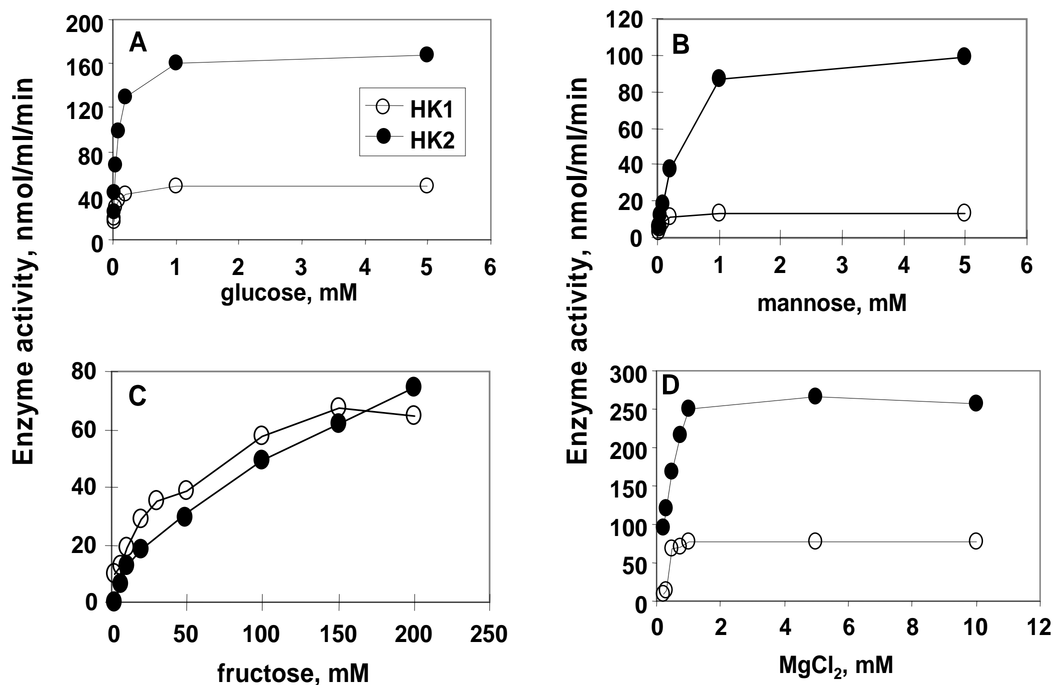

Fig. 5. Effect of glucose (A), mannose (B), fructose (C) and $MgCl_2$ (D) concentrations on the activity of the two HK isoforms from immature tomato fruit pericarp. Hexose kinase activity of HK1, ● (black circles); and HK2, ○ (white circles). Note the different scale of fructose concentrations. HK1 isozyme with fructose as substrate was extracted from transgenic tomato plants expressing antisense FKI, kindly provided by Dr. Nir Dai, in order to assure that measured activity with fructose as substrate was not due to FKI residual activity.

5.1.1.2 ***Activity during development***

The total FK activity in first step purified enzyme preparation extracts of immature (15 DAA) and ripe fruit pericarp is presented in Table 1. Recovery experiments indicated that differences in extractable activity between the different stages of fruit development were not an artifact caused by inhibitory substances in the mature fruit. Similar recovery of a known amount of purified FKI (from the MonoQ fractions, as described further on) was obtained from both fruit developmental stages. Activity by both assay systems was higher in the young fruit and sharply declined in the more mature fruit. At both stages of fruit development, activity was higher in the low fructose/low Mg^{+2} assay system, indicating that even in the mature fruit the activity is inhibited by excess of fructose and Mg^{+2}.

Table 1. Fructokinase activity in immature and mature tomato fruit pericarp
Assay 1 contained low fructose (1.0 mM) and low Mg^{+2} (0.5 mM).
Assay 2 contained high fructose (10 mM) and high Mg^{+2} (3 mM).
Data are averages ±SE of 4 (immature) and 7 (mature) separate extractions from individual fruit.

Maturity stage	Fructokinase activity (nmol/gfw/min)	
	Assay 1	Assay 2
Immature	184 ± 25	85 ± 8
Mature	63 ± 8	27 ± 1

A comparison of the chromatograms of the pericarp fructokinase enzymes from immature (15 DAA) and ripe fruit stages (Fig. 6) indicates that the three peaks of fructose phosphorylation activity were observed in both stages. The enzyme first eluted (FKI) remained the dominant peak of activity even in the ripe fruit. The FKII enzyme was present in the ripe fruit, but generally was the least active of the three FKs.

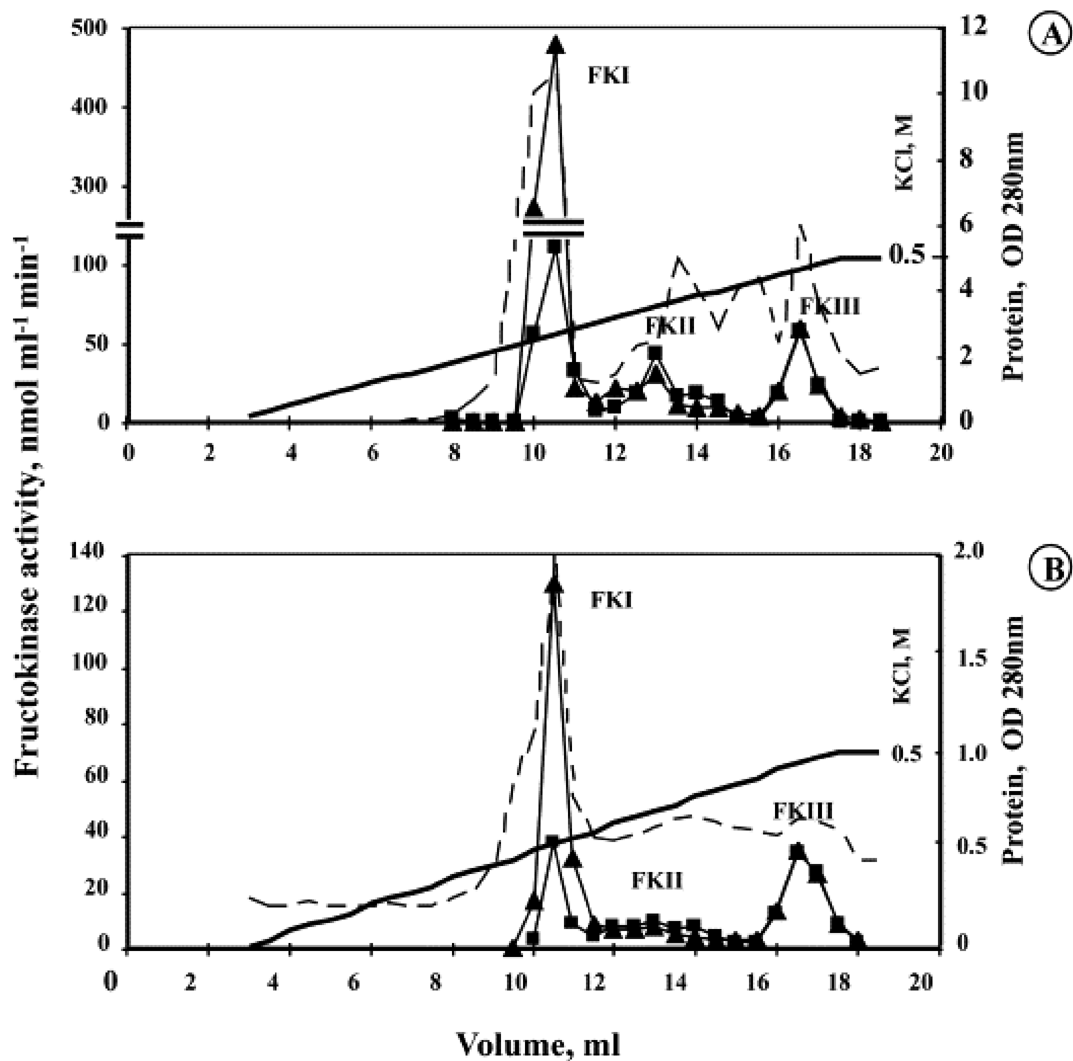

Fig. 6. HPLC-ion exchange (MonoQ) separation of fructokinase activity in immature (A) and mature (B) tomato fruit pericarp. Assays are as in Figure 4. Note the different y scales in the two chromatograms.

5.1.2 Characterization of the three tomato fruit FK enzymes and comparison with the two yeast-expressed *LeFRK* (*LeFRK-Y*) gene products

In order to determine the relationships between the native FK enzymes and the products of the two *LeFRK* genes, enzyme extracts from mutant yeast lacking endogenous hexose kinase activity and expressing each of the two described *LeFRK* genes were separated on MonoQ ion chromatography under the same conditions as the

tomato extract. The yeast mutants lacking endogenous hexose kinase activity and expressing each of the two described *LeFRK* genes were kindly provided by Dr. Nir Dai. The partially purified native FK enzymes and the yeast-expressed enzymes were partially characterized and compared as described below. The gene encoding the third FK enzyme, FKIII, had not yet been cloned at the time that this research was carried out. It was cloned subsequently and expressed in yeast, as well, and was conclusively found to encode FKIII. These results were not presented in this thesis but were reported later in German *et al*., 2004.

5.1.2.1 *Hexose specificity and inhibition by fru*ctose

The three native fruit enzymes were specific for fructose, with no discernible activity with glucose or mannose. Of separated enzymes, FKII had the lowest affinity for the substrate (Fig. 7a). Both yeast-expressed enzymes were also specific for fructose, with no discernible activity with glucose or mannose. The *LeFRK1*-Y enzyme has an affinity pattern for fructose simular to that of FKII (Fig. 7b). Both FKI and FKIII from tomato fruit showed the characteristic substrate inhibition by high levels of fructose, while FKII showed Michaelis-Menten kinetics with fructose concentrations up to 10 mM (Fig. 7a). The yeast-expressed *LeFRK1*-Y showed similar Michaelis-Menten kinetics with fructose concentrations up to 10 mM, while *LeFRK2*-Y showed the characteristic substrate inhibition by high levels of fructose (Fig. 7b).

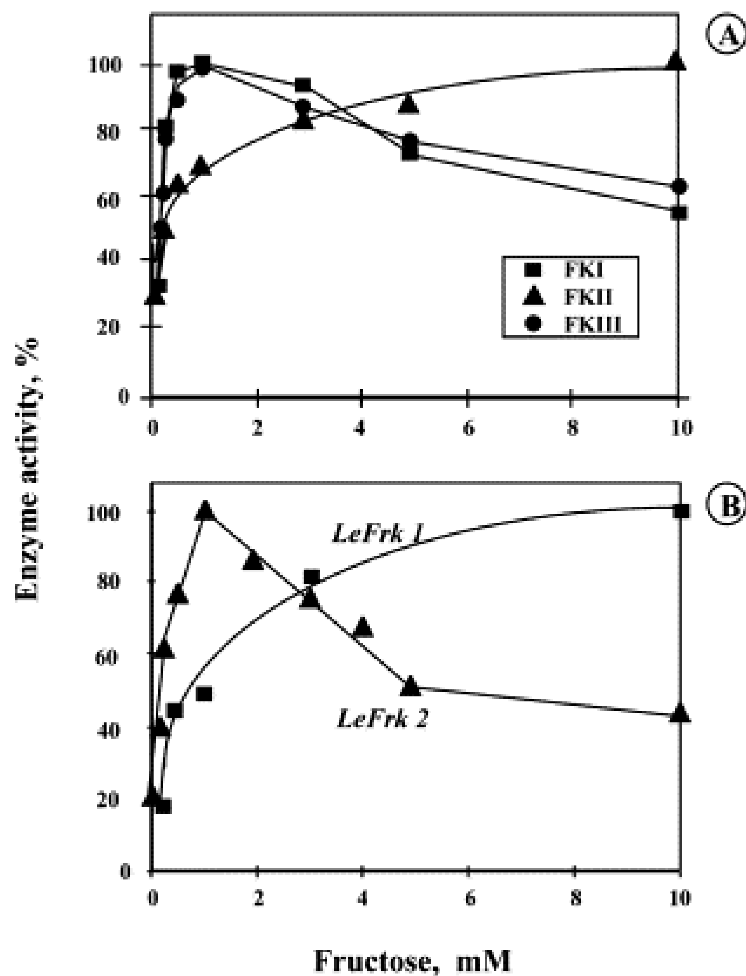

Fig. 7. Effect of fructose concentrations on the activity of the three FK isoforms from immature fruit pericarp (A) and from yeast-expressed *LeFRK* genes (B). Data are expressed as percentages of maximal activity for each enzyme in order to emphasize the similar and distinct patterns of activity. Maximal activities in nmol g^{-1} fw min^{-1} were: FK1, 710; FKII, 38; FKIII, 71; *LeFRK1-Y,* 148; *LeFRK2-Y,* 238.

5.1.2.2 *Inhibition by* Mg^{+2}

Of the tomato fruit enzymes, only FKI showed the characteristic of inhibition by Mg^{+2} concentrations above 1 mM (Fig. 8a). FKII showed no inhibition by Mg^{+2}, while FKIII showed little, if any, inhibition. Of the yeast-expressed enzymes, *LeFRK2*-Y was inhibited by Mg^{+2} concentrations above 1 mM, while *LeFRK1*-Y was not (Fig. 8b).

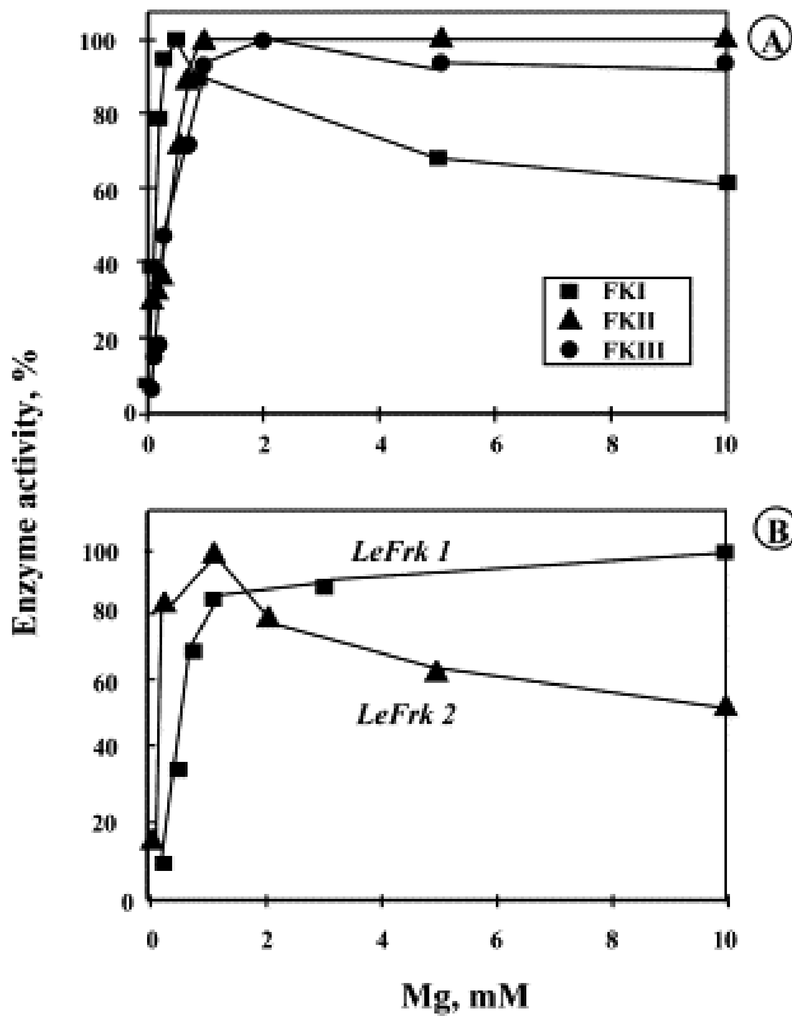

Fig. 8. Effect of Mg^{+2} concentration on the activity of the three fructokinase isoforms from immature fruit pericarp (A) and from yeast-expressed *LeFRK* genes (B). Data are expressed as percentages of maximal activity for each enzyme. Maximal activities in nmol ml^{-1} min^{-1} were: FK1, 840; FKII, 52; FKIII, 161; *LeFRK1-Y,* 150; *LeFRK2-Y,* 226.

5.1.2.3 *Nucleotide specificity*

The three fruit enzymes responded differently to the four nucleotide substrates examined (Fig. 9 a–c). FKI had highest activity with ATP and GTP at concentrations up to 0.5 mM. However, above 0.5 mM this enzyme was inhibited by GTP and CTP. FKII and FKIII showed the highest activity with ATP, with no indication of inhibition by high GTP or CTP concentrations.

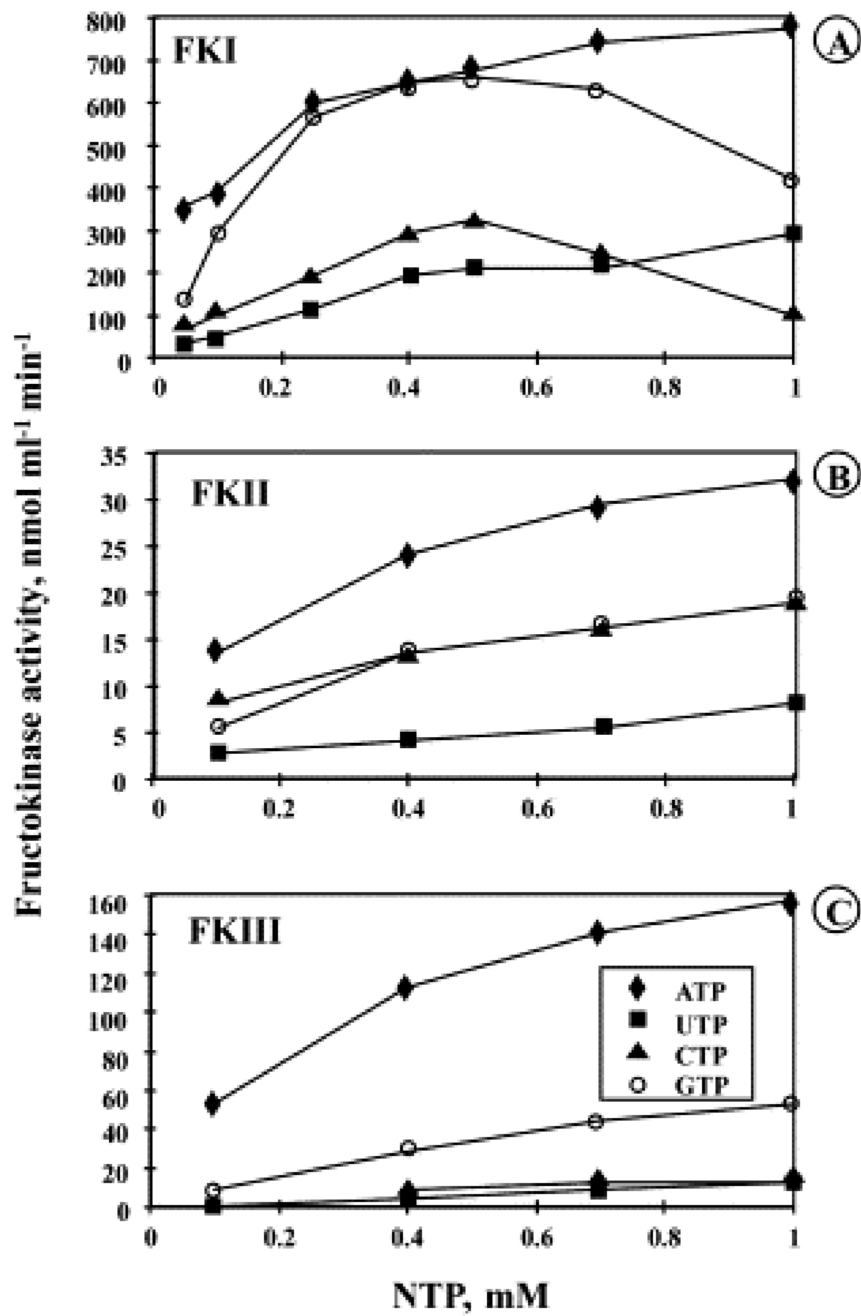

Fig. 9. Effect of nucleotide concentrations on the activity of the three FK isoforms from immature tomato fruit pericarp. (A) FKI; (B) FKII; (C) FKIII.

All three enzymes also showed product inhibition by ADP similar to that shown for FKI (Fig. 10 and Table 2).

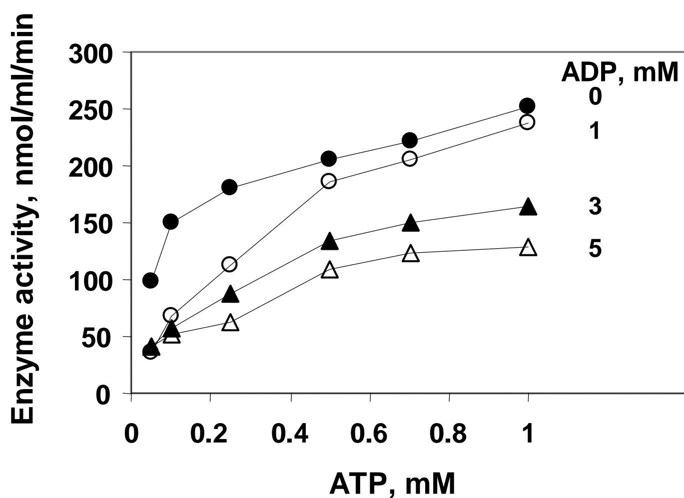

Fig. 10 Effect of ADP concentrations on the activity of FKI from immature tomato fruit pericarp. The increasing ADP concentrations in the assay mixture from 0 to 1, 3, and 5 mM are designated by (●) closed circles, (○) open circles, (▲) closed triangles, and (∆) open triangles respectively. The product inhibition was measured by a coupled enzyme assay for each curve with elevated ATP concentrations and a constant ADP concentration in the reaction mixture.

The two yeast-expressed enzymes also responded differently to the four nucleotide substrates (Fig. 11a and b). *LeFRK2*-Y responded similarly to the tomato fruit FKI, showing highest activity with ATP and GTP at concentrations up to 0.5 mM and inhibition by GTP and CTP at concentrations above 0.5 mM. *LeFRK1*-Y showed preferential activity with ATP alone. Both enzymes also showed product inhibition by ADP.

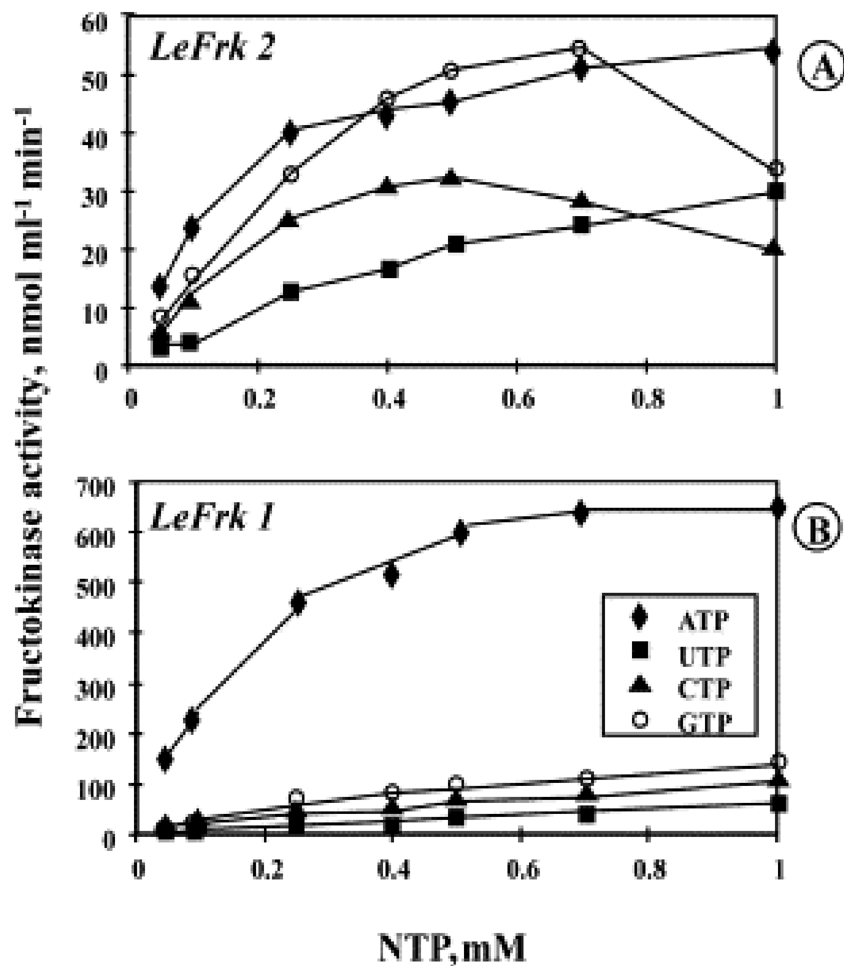

Fig. 11. Effect of nucleotide concentrations on the activity of the two yeast expressed FK enzymes. (A) *LeFRKI*; (B) *LeFRKII*.

5.1.2.4. *Co-elution of yeast-expressed and tomato fruit fructokinase*

Each of the *LeFRK-Y* gene products were chromatographed individually, and also together with the partially purified enzyme extract from tomato pericarp, in order to determine whether they co-elute with any of the tomato enzymes. *LeFRK2*-Y co-eluted with the tomato FKI under the same conditions used for the separation of the tomato fruit enzymes on HPLC. *LeFRK1*-Y eluted slightly after FKII, co-eluting with the second tomato HK enzyme, HK2 (for HK2 location during HPLC separation see

Fig. 4). Kinetic characteristics of tomato fruit FK isozymes are summarized in the table 2.

Table 2. Kinetic characteristics of partially purified FK isozymes in immature tomato fruit pericarp. Data are averages of at least two replications from two individual fruit. The Km values were calculated from Lineweaver-Burk plot of corresponding substrate saturation curves of FK isozymes activities presented above.

	FKI	**FKII**	**FKIII**
Substrate		**Km, mM**	
Fructose	0.33	0.16	0.27
Fructose inhibition	+	-	+
Mg	0.34	0.83	4.66
Mg inhibition	+	-	-
ATP	0.075	0.14	0.18
CTP	0.50	0.14	0.50
GTP	0.50	0.38	2.10
UTP	0.50	3.44	3.00
ADP inhibition, $I_{0.5}$, mM	2.0	3.6	0.6

In conclusion, three fructokinase isozymes (FKI, FKII, FKIII) were separated from both immature and ripe tomato fruit pericarp. All three isozymes were specific for fructose with undetectable activity towards glucose or mannose. The three isozymes could be distinguished from one another with respect to response to fructose, Mg and nucleotide donor concentrations and this allowed the comparison of the fruit enzymes with the gene products of the two known yeast-cloned tomato fructokinase genes, *LeFRK1* and *LeFRK2*. FKI was characterized by both substrate (fructose), as well as Mg^{+2}, inhibition; FKII was inhibited by neither fructose nor Mg^{+2}; and FKIII was inhibited by fructose but not by Mg^{+2}. ATP was the preferred nucleotide donor for

all three FKs. FKI showed inhibition by CTP and GTP above 1 mM. All three FKs showed competitive inhibition by ADP. During the maturation of the tomato fruit total activity of all three FKs decreased dramatically, but all were still active in the ripe fruit. The results indicate that FKI is the gene product of *LeFRK2* and FKII is probably the gene product of *LeFRK1*. The results of the present study show that the FKI remains the major native FK in the ripe fruit. It does not undergo any particular loss of activity, compared to the other fructokinases and its suggested specific role in transient starch synthesis is not certain.

Results from a parallel study carried out during my research (Dai *et al*., 2002; German *et al*., 2003) on transgenic tomato plants expressing the *LeFRK* genes and the recent report on transgenic potato plants with antisense inhibition of StFK1, the predominant fructokinase isoform in the tuber (Davies *et al*., 2005), indicate that FKs are not important for the control of starch synthesis. Accordingly, the emphasis of the present research project was directed on the contribution of the AGPase enzyme to starch synthesis in tomato fruit.

5.2 ADP-Glclucose pyrophosphorylase activity and starch accumulation in immature tomato fruit: the effect of a *Lycopersicon hirsutum*-derived introgression encoding for the large subunit

According to our preliminary study AGPase was the only enzyme that appeared to limit starch synthesis in both the temporal and spatial studies of fruit development (Schaffer and Petreikov, 1997a). A determinant, M-82 like shaped breeding line of tomato (line 904) was developed from an initial interspecific cross of *Lycopersicon esculentum* cultivar, M-82, with the wild *Lycopersicon hirsutum* tomato, followed by backcrossing with the *Lycopersicon esculentum* parent. This breeding line was characterized by a high level of starch in the immature fruit and a concomitantly increased soluble sugar level in the mature fruit. This novel breeding line served as experimental material to determine the metabolic and molecular control of starch synthesis in tomato fruit.

5.2.1. Starch content and AGPase activity in the high starch line.

Immature fruit of line 904 (approximately 15 days after anthesis) was characterized by starch levels significantly higher than those of the standard industry type tomato cultivar, M-82 (Table 3). A comparative survey of enzyme activities involved in sucrose-to-starch metabolism was performed on these fruit. Immature fruit of 904 were characterized by a significantly higher activity of AGPase, compared to M-82, while the activities of the other enzymes of sucrose-to-starch metabolism were not different between the lines. Starch synthase activity was not measured in this initial comparison, but later assays showed no difference in activity of either soluble or insoluble starch synthase activities.

Table 3. Starch levels and activities of enzymes involved in the metabolism of sucrose-to-starch in young (approximately 15 days after anthesis) tomato fruit. M-82, standard industry type tomato cultivar; 904, high starch tomato line. **At least four fruits from individual plants were assayed. * Indicates a statistical significance (*P*<0.05) between the two lines.**

	Line	
	M-82	**904**
Starch (mg/gfw)	13.1	34.9*
Enzymes (nmol/gfw/min)		
Invertase	15,480	17,870
Sucrose synthase	29,570	27,570
Fructokinase	91	137
Phosphoglucomutase	5,760	7,490
Phosphoglucosisomerase	1,950	2,060
UGPase	15,080	14,760
AGPase	40	268*

5.2.2. Starch content and AGPase activity in the high starch line and F_1 genetic populations

To further test the assumption that increased AGPase activity is the causal factor in the increase in starch levels in the high starch breeding line, immature fruits from a variety of crosses with the high starch line were analyzed. These crosses included parallel hybridizations of both the high starch line 904, as well as the standard cv. M-82, with nine different lines of the *L. pennellii* ILs, each containing a small chromosome segment of the wild *L. pennellii* in the background of the cultivated *L. esculentum* cv. M-82 (described in Schaffer *et al*., 2000). The underlying assumption of this strategy was that crossing of two, 904 and M-82, with a wide range

of genotypes would supply a broad spectrum of combined genetic backgrounds in which the genetic effect of 904 could be detected in the F_1 generation.

Starch levels of the immature fruit, as well as the soluble solids levels of the mature fruit, from the nine hybrids with line 904 were significantly higher than those from the parallel hybrids with M-82 (Table 4). Immature fruit of the high starch hybrids derived from the cross with 904 and the low starch hybrids derived from the cross with M-82 were subjected to a detailed enzymatic analysis of the enzymes involved in sucrose-to-starch metabolism. Table 5 shows that of the enzymes assayed; only AGPase activity was significantly higher in the hybrids derived from the cross with line 904, compared to the hybrids derived from the cross with M-82. In addition to strengthening the correlation between AGPase activity and the high starch level, these results also indicate that the inheritance of the trait is at least partially dominant, as indicated by the expression of the trait in the F_1 hybrids.

Table 4. Starch levels of immature fruit and °Brix values of mature fruit of M-82, 904, the hybrid between them, a mix of nine hybrids between 904 and nine ILS (described in the text), and a mix of the nine parallel hybrids between M-82 and the same nine ILs.
Five plants from each cross or lines were grown. At least two fruits from each of the individual hybrids were assayed for starch and five fruits from each of the hybrids were measured for °Brix. At least three fruits from each of M-82, 904 and the hybrid between them were assayed. Different letters indicate a statistical significance (*P*<0.05) within columns.

Genotype	Starch (mg/gfw)	°Brix
M-82	23 B	4.1 B
904	58 A	8.1 A
F_1 (M-82×904)	46 A	7.1 A
ILs×M-82	25 B	5.3 B
IL_S×904	44 A	7.5 A

Table 5. Enzyme activities of immature fruit pericarp of nine hybrids between 904 and nine ILs (described in the text), and the parallel nine hybrids between M-82 and the same ILs.
From one to three fruit of each of the 18 hybrids were measured. For each of the nine ILs, the hybrid with 904 gave a minimum of a 2-fold increase in AGPase activity, compared to the cross of the same IL with M-82, and the data are, therefore, presented as an average of the results of the nine hybrids. * Indicate a statistical significance (*P*<0.05).

	Activity (nmol/gfw/min)		
Enzyme	**904×ILs**	**M-82×ILs**	**Ratio**
Invertase	520	620	0.83
Sucrose synthase	710	560	1.27
Fructokinase	225	219	1.03
Hexokinase	23	25	0.95
Phosphoglucomutase	6,900	5,900	1.31
Phosphoglucosisomerase	3,160	2,630	1.21
UGPase	8,490	7,130	1.19
AGPase	190	56	3.67 *
Soluble starch synthase	48	38	1.26
Insoluble starch synthase	5	5	0.93

Specific DNA primers for the four AGPase genes, which could distinguish between the *L. hirsutum* derived allele and the *L. esculentum* derived allele, were designed in collaboration with Dr. Ilan Levin. The analysis of the different genotypes shows that line 904 carries the *L. hirsutum* allele for the *AgpL1*, whereas the other two large subunits, *AgpL2* and *AgpL3*, and the small subunit *AgpS1* of 904 are derived from *L. esculentum* (Schaffer *et al*., 2000). Accordingly, the 904 line carries a chromosomal introgression from the wild species which harbors the *AgpL1* gene encoding for the AGPase large subunit.

5.2.3. Starch content and AGPase activity of the high starch line and F_2 genetic populations

In order to examine whether the *L. hirsutum* derived *AgpL1* is correlated with increased AGPase activity and increased starch level in the immature fruit, an F_2 population of 64 plants of the cross between the high starch line 904 and M-82 was analyzed. The 64 F_2 plants segregated for the single locus *AgpL1* in a ratio of 16:31:17, as expected for a 1:2:1 ratio. Immature fruit from five plants of each of the three F_2 genotypes were assayed for AGPase activity and starch levels. Mature fruit of all the plants were measured for TSS content. The results presented in Table 6 show that in the segregating population the *L. hirsutum* allele for AGPase *AgpL1* was associated with increased AGPase activity and increased starch levels in the immature fruit. Furthermore, the TSS values of the mature fruit were similarly influenced by the genotype of the *AgpL1* gene.

Table 6. Activity of AGPase in F_2 plants from the cross between line 904 and M-82.
AGPase activity and starch levels were measured in immature fruits (8–13 g) and presented as averages from five individual F_2 plants of each genotype. TSS (°Brix) values are the average of five fruits per plant of the entire F_2 population. Different letters indicate significant differences ($P<0.05$) within columns.

Genotype	AGPase (nmol/gfw/min)	Starch (mg/gfw)	°Brix
EE	104 C	16.4 B	5.3 B
EH	306 B	25.2 AB	5.9 AB
HH	450 A	37.3 A	6.3 A

The existence of the *AgpL1* allele from *L. hirsutum* (*AgpL1*^{H}) in segregating populations from the cross between the high starch line and M-82 was correlated with increased in AGPase activity and starch level in the immature fruit and soluble solids content in the mature fruit.

5.3 Temporally extended gene expression of the AGPase large subunit (*AgpL1*) leads to increased enzyme activity in developing tomato fruit

5.3.1 Determination of the size of the wild species introgression

Initially, the *AgpL1* locus was mapped to the distal portion of chromosome 1, and the other AGPase genes were mapped to other chromosomal locations (Schaffer *et al.*, 2000). In order to further define and minimize the introgression size and to reduce the possibility that the increase in enzyme activity is due to an unrelated but linked gene, the present research was carried out on advanced near isogenic lines differing in a smaller *AgpL1* introgression from the wild species. Polymorphisms between the two species for 9 markers spanning the distal portion of chromosome 1 (Monforte and Tanksley, 2000 and http://www.sgn.cornell.edu) were identified (from TG161 to TG90, the distal marker on the chromosome). The $AgpL1^H$ introgression in the segregating lines used in this study spanned from TG158 to TG389 (Fig.12). Distances in cM are taken from Monforte and Tanksley (2000) based on the *L.esculentum* X *L. penellii* population. The *L. esculentum* X *L. hirsutum* based map is significantly compressed (Monforte and Tanksley, 2000), so that the TG158-TG389 distance is approximated 1 cM. The most distal markers TG159, TG90 were found to be outside of the introgression responsible for the increased enzyme activity. The principle of mapping based on polymorphism between the two genotypes is illustrated on the example of the most distal marker TG159 (Table 7). By analyzing the sequenced product of the PCR reaction obtained with primers harboring the entire TG159 segment, polymorphism was found between the parental plants (Fig. 13 a, c). Based on this polymorphism, plants which segregate for TG159 marker were determined (Fig. 14) and were confirmed by analysis of sequenced amplicon (Fig. 13 b, c).

Most significantly, the results show that the *AgpL1* locus segregates independently in the lines studied here from a previously described QTL for Brix content (TG161; Monforte and Tanksley, 2000) and that the genotypes compared in the present work do not harbor this QTL for Brix.

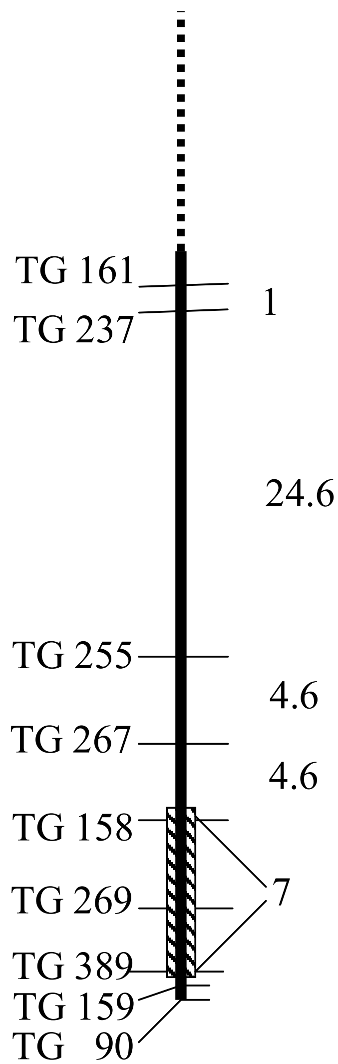

Fig. 12. Delineation of the introgressed segment harboring the *AgpL1* allele, indicated by the hatched lines covering from TG158 to TG389. Distances in cM are taken from Monforte and Tanksley (2000) based on the *L. esculentum* X *L. penellii* population.

Table 7. AGPase activity in immature fruit of tomato plants harboring different size introgressions of the *L. hirsutum* distal portion of chromosome 1. The results show that the effect on AGPase activity is unrelated to the most distal portion of the chromosome (TG159). Data are the averages of 4 fruits from 2 plants of each genotype. Different letters indicate significant differences at 0.05 level, using the Tukey-Kramer HSD.

Genotype		AGPase activity	
LS1	TG159	(nmol/gfw/min)	
hh	ee	193	A
ee	ee	40	B
ee	hh	37	B

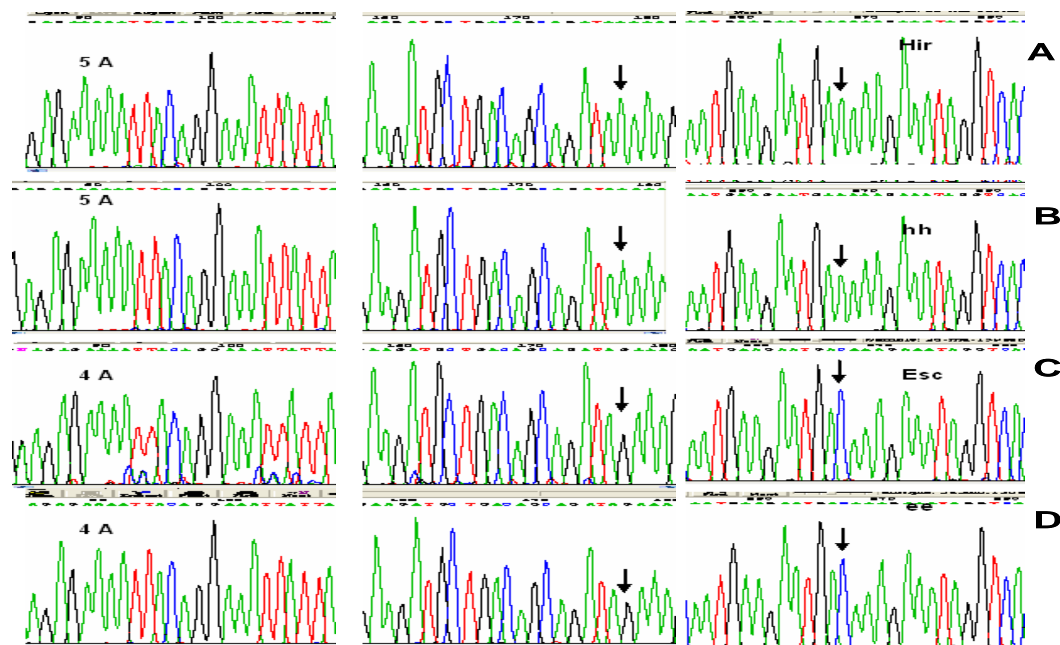

Fig.13. Chromatographically confirmed polymorphism for TG159 genomic marker found between tomato fruits of parental plants, *L.hirsutum* (A) and *L.esculentum* (C) and of segregating population, $TG159^H$ (B) and $TG159^E$ (D). The arrows mark the place of a single nucleotide polymorphism.

TG 159 (159eF, 159hF, 159R)

Fig.14. Polymorphism for TG159 genomic marker in segregating tomato population. Separation of PCR products was obtained with the specific primers (159hF, 5'GAAGATGCTGACAGCAGATAA3'; 159eF, 5'GCTTGTTAGCAATGAAGAATGAC3'; 159R, 5'CCAATGCAGTTTCATCTTAGGA3') on 2% agarose gel, as described in Materials and methods (4.4).

5.3.2 AGPase activity in developing tomato fruit

Comparison of enzyme activity in developing $AgpL1^H$ and $AgpL1^E$ fruit from the segregating lines showed that high AGPase activity was maintained for a longer period of development in the $AgpL1^H$ genotypes (Fig. 15a). The transient pattern of AGPase activity in the standard $AgpL1^H$ genotype showed a peak activity during the second week of fruit development, followed by a continuous decline in activity. In the $AgpL1^H$ fruit, the decline in activity was delayed, so that AGPase activity in the $AgpL1^H$ fruit was approximately twice of that in the $AgpL1^E$ fruit during the period between 20 and 40 days after anthesis. Similar results were observed in a series of comparative analyses spanning six growing seasons, and in many cases the differences in enzyme activity were even more pronounced. The experimental results presented here are from a single season encompassing all the variables. The transient starch accumulation pattern was observed in both genotypes; however, starch levels increased in the $AgpL1^H$ genotype. Whereas peak starch levels occurred approximately a week

following the peak of AGPase activity in the *AgpL1*^{E} genotype, the starch levels of the *AgpL1*^{H} genotype remained relatively high until after 40 days following anthesis (Fig. 15b). Since these more mature fruits, which were characterized by higher enzyme activity and higher starch per fresh weight are also larger, there was an even a larger difference in the starch reservoir of the whole fruit, reaching in the data presented to nearly 300 mg, which in a 50 gm final fruit size translates to 0.6% starch (Fig. 15c). Significantly, the increases in enzyme activity and starch levels were not accompanied by any decrease in fruit size. Rather, there was generally an increase in fruit size, as well as in soluble sugar content in fruit harboring the *AgpL1*^{H} allele (Table 13). In a range of genetic backgrounds of different mature fruit size (from 25-100 gram final fruit weight) segregating for the *AgpL1* allele, there was no negative effect of *AgpL1*^{H} on fruit size, starch levels, or TSS levels. In general, fruit size increased from 5% to 15% fresh weight.

Table 13. Impact of *AgpL1*^{H} allele on tomato fruit quality.

Data are expressed in %, as an average of analysis of at least 3 fruits per different plant of seven independent segregating lines, minimum 25 plants each, growing at different seasons. *AgpL1*^{E} and *AgpL1*^{H} , isogenic lines. Standard error <5%.

	AGPase activity	**Starch**	**TSS**	**Fruit weight**
AgpL1^{E}	100	100	100	100
AgpL1^{H}	210	114	112	110

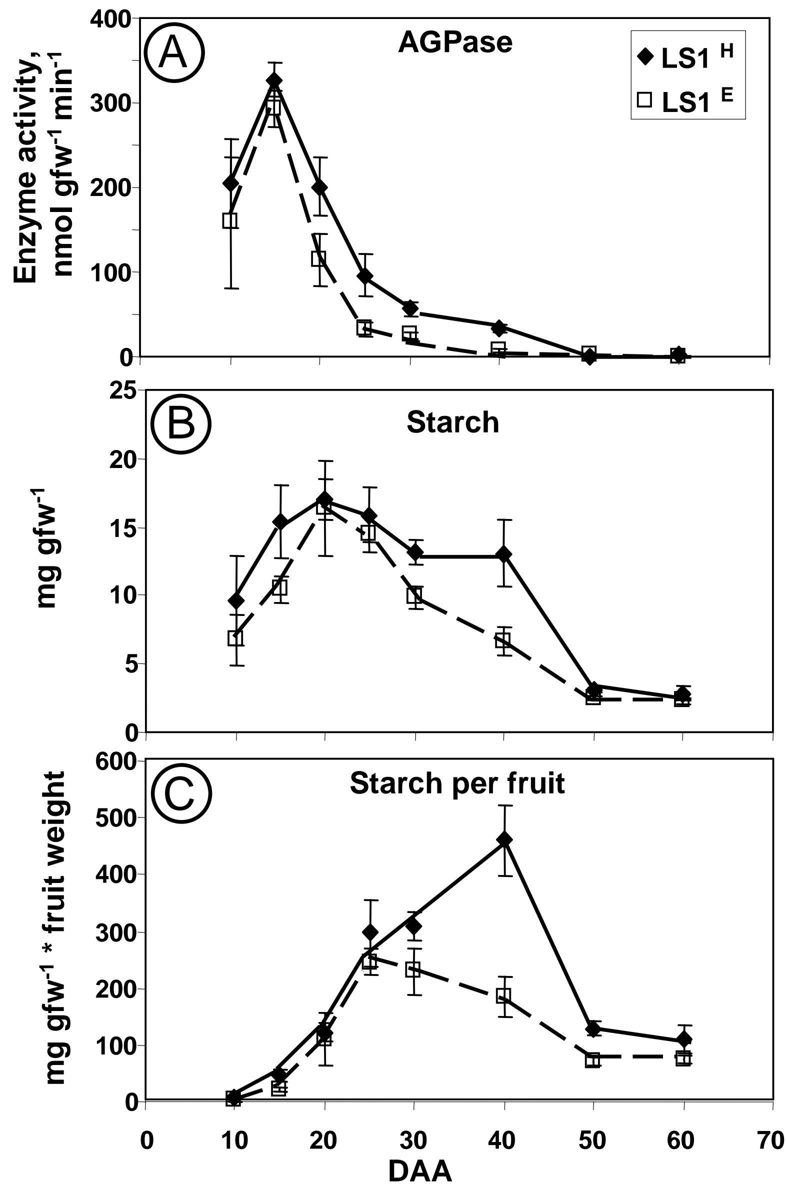

Fig. 15. AGPase activity and starch content in tomato fruit during development. A, AGPase activity measured in the pyrophosphorolytic direction in whole tomato fruit. B and C, starch content in tomato fruit expressed as mg/g fresh weight (b) or as starch content per fruit unit (mg per total fruit weight) (C). ♦ (black diamonds), tomato fruits carrying the AGPase large subunit from *L. hirsutum* (*AgpL1^H*); □ (white square), tomato fruits carrying the AGPase large subunit from *L. esculentum* (*AgpL1^E*). *AgpL1^E* and *AgpL1^H*, isogenic lines. Error bars represent standard errors of the mans from analysis of 3 to 8 fruits.

A further study comparing the activity between an introgression line containing the portion of chromosome 7 harboring the wild species allele for the small subunit

(*AgpS1*) showed that the wild species allele for *AgpS1* had no affect on enzyme activity and starch content of the immature tomato fruit (Table 14).

Table 14. The impact of AGPase small subunit (*AgpS1*) on tomato fruit quality in a population segregating for *AgpS1*. Values are means ± SE of 5 different plants. Statistical significance is presented at the 0.05 level using the Tukey-Kramer HSD

Genotype		**Fruit weight**	**AGPase**	**Starch**
AgpS1	*AgpL1*	**(g)**	**(nmol/gfw/min)**	**(mg/gfw)**
ee	**ee**	16.87 ± 1.13	69.97 ± 17.57	11.62 ± 2.38
hh	**ee**	18.90 ± 1.37	39.18 ± 9.43	11.80 ± 1.40
significance		A/A	A/B	A/A

5.3.3 AGPase gene expression in developing tomato fruit

In order to determine the stage at which the $AgpL1^H$ allele exerts its effect, L1 gene expression was followed in developing fruit of the two genotypes. Expression of the three other AGPase subunits (L2, L3 and S1) was also compared in order to examine whether the *AgpL1* allele affects on their expression in a compensation manner. Both the $AgpL1^H$ and $AgpL1^E$ genotypes harbored only the cultivated tomato alleles of the three other subunits. Two independent experiments on different lines in different seasons gave a very similar pattern.

The data showed that the $AgpL1^H$ allele led to a prolonged and higher expression of the *AgpL1* subunit in the $AgpL1^H$ high starch lines (Fig. 16a). The effect on expression was most evident from 20 DAA. While the $AgpL1^E$ allele showed a strongly declining pattern of expression at 20 DAA, the $AgpL1^H$ allele was expressed more strongly at 20 and 30 DAA and even showed a residual expression until the ripe

fruit stage of 50 DAA. The other *AgpL* genes, *AgpL*2 and *AgpL*3 were expressed at a lower degree and there seemed to be no effect of the genotype of the *AgpL1* allele on increasing their expression, although at the earliest stages of development (10 and 15 DAA) there was an indication that the $AgpL1^{H}$ allele reduced expression of the other genes(Fig. 16b, c). *AgpL*2 was expressed at levels of approximately a third, and *AgpL*3 expression level was approximately only 1% of that of *AgpL1*. The *AgpS1* gene was the most highly expressed AGPase gene (nearly 7 fold higher than that of *AgpL1*), and its expression extended for the longest period of fruit development, so that even at the ripe stage expression of SS was as high as the peak expression of *AgpL1* (Fig. 16d). The transient developmental pattern of *AgpL1* and *AgpS1* expression followed the transient pattern of enzyme activity, suggesting the limiting role of gene expression, especially that of *AgpL1*, in AGPase activity.

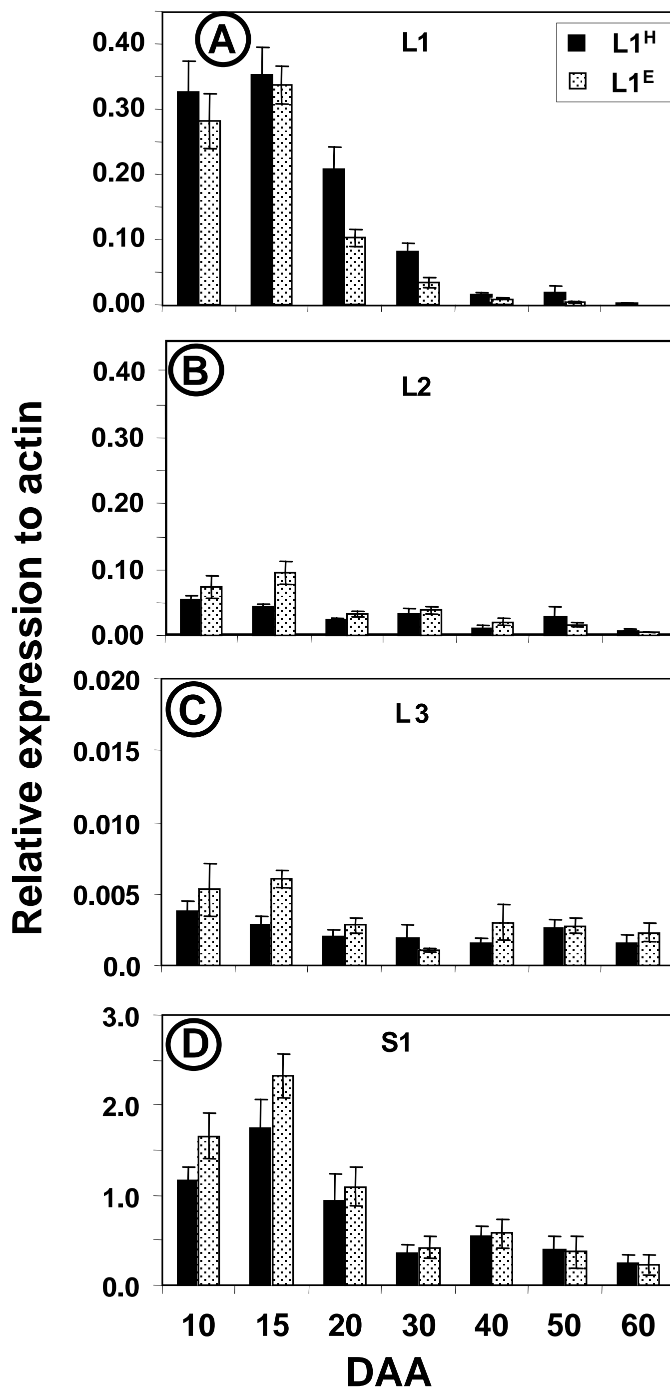

Fig. 16. Quantitative gene expression of AGPase subunits throughout tomato fruit development, determined by quantitative RT-PCR. The data were normalized to the expression of actin in each preparation. Data are means ± SE of QRT-PCR reactions of RNA extracted from three different fruits. Expression of four genes: A, L1; B, L2; C, L3; D, S1 in the two genotypes carrying the AGPase large subunit from *L. hirsutum* (*AgpL1^H*) (black bar) and from *L. esculentum* (*AgpL1^E*) (grey bars). *AgpL1^E* and *AgpL1^H* , isogenic lines.

5.3.4 AGPase protein levels in developing tomato fruit

To determine the relationship of the differences in AGPase gene expression and protein subunit levels, samples from the same fruit which were assayed for developmental enzyme activity and gene expression were analyzed for AGPase protein subunit content. Immunoblot analysis of the soluble protein in the developing tomato fruit was carried out using antibodies specific for the large and small subunits. The antibodies were prepared against potato tuber purified subunits and were a generous gift from Prof. T. W. Okita. A preliminary study confirmed the specificity of the antibodies for the LS (51kD) and SS (50kD) (Fig. 17).

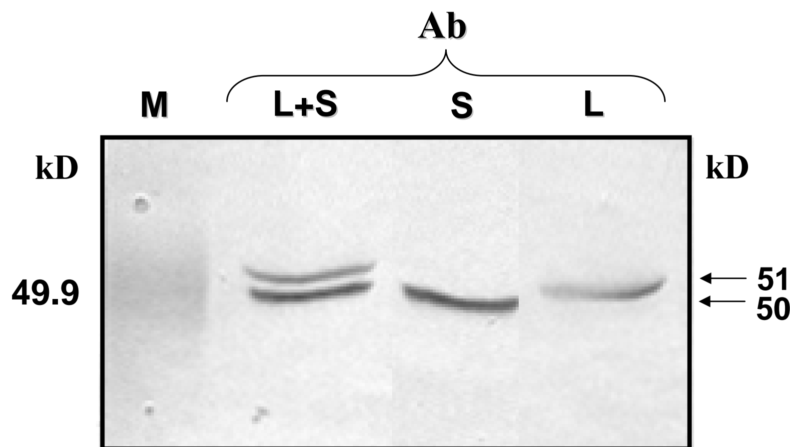

Fig. 17. Immunoblot with subunit specific antibodies. First step purified enzyme extract was separated on SDS-PAGE, (15%, 18µg protein/well) and immunoblotted with antibodies specific for the AGPase large (L) and small (S) subunits, prepared against the potato tuber subunits. Lane "L+S" is the immunblot with a mixture of both antibodies and lanes "S" and "L" are the immunoblot of the same enzyme extract with only one of the antibodies, small or large, respectively. The molecular mass of the protein marker in the blot is approx. 50 kD (M).

The results show that, in contrast to the differential pattern of gene expression of the *AgpL1* and *AgpS1* described above, both the L1 and S1 protein subunits showed coordinated levels through all developmental stages (Fig. 18). For each genetic background the transient pattern of both subunits was similar.

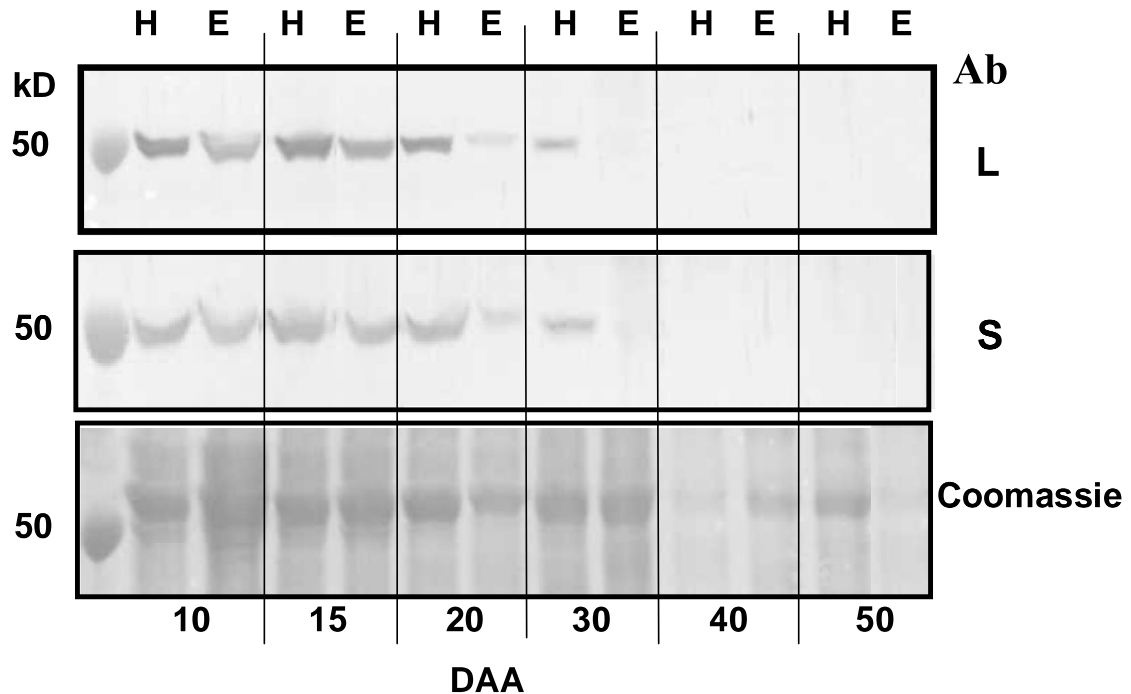

Fig. 18. Western blot analysis of developing fruit of the two genotypes of isogenic lines carrying the AGPase large subunit from *L. hirsutum* (H) and from *L. esculentum* (E). Enzyme extracts were separated on 10% SDS-PAGE, immunoblotted with subunit specific antibodies for AGPase large (L, upper panel) and small (S, middle panel) subunits, and visualized by Coomassie Brilliant Blue R-250 staining (lower panel). Protein from similar amounts of fresh tissue (65 μg fw) were loaded in each well, so that results express the AGPase subunit protein content per fresh weight of fruit, analogous to the data in Figure 15 of AGPase activity per fresh weight of fruit.

However, the most striking difference between $AgpL1^H$ and $AgpL1^E$ genotypes is the observation that the protein levels of both subunits remained at a high level for a longer period in the $AgpL1^H$ line and was still immunologically detected with our assay 30 DAA. In contrast, the transient levels of both L1 and S1 subunit protein declined earlier in the $AgpL1^E$ genotypes and were not observed at all 30 DAA.

5.3.5 Kinetic characteristics of AGPase-L1^H and -L1^E

The differences in activity of the AGPase-L1^H and AGPase-L1^E enzymes could also be due to their different sensitivity to 3-PGA/PPi regulation or to differences in their affinity for the substrates, due to the minor differences in amino acid sequence. Partially purified enzymes (approximately 200-fold) of each of the two genotypes were used to characterize their affinity constants and 3-PGA/PPi regulation. A single eluted peak with AGPase activity confirmed the existence of only one form of tomato fruit AGPase (Fig. 19).

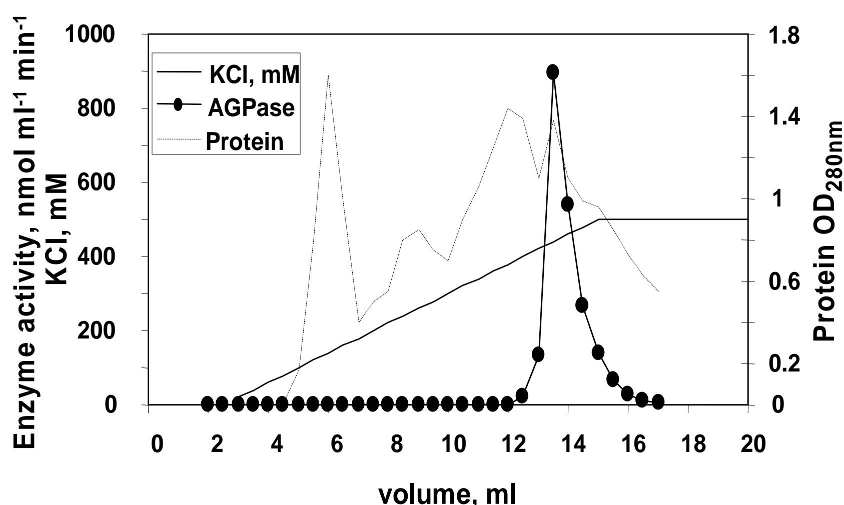

Fig. 19. HPLC-ion exchange (MonoQ) separation of AGPase activity of immature tomato fruit. Activity was measured in the pyrophosphorolytic direction. Analogous results indicating a single peak of AGPase activity was similarly observed upon separation by MonoP.

The kinetic properties of enzyme activity were characterized in both the ADP-Glc synthesis direction and the pyrophosphorolytic direction. The effect of ADP-Glc concentrations on the activity of the partially purified AGPase of both genotypes and the calculation of the kinetic characteristics are expressed in Figure 20, an example of evaluation of the enzyme kinetic constants present in this chapter. There were no differences in substrate affinity or in PGA activation between the AGPase enzymes

from the two genotypes (Fig. 21a, Table 15) indicating that the differences in activities are due to differences in amount of enzyme protein per g fresh tissue rather than to intrinsic catalytic differences in enzyme kinetics. In addition, both enzymes were identically inhibited to 50 % of maximum activity by 1.6mM Pi in the presence of 5mM 3-PGA (Fig. 21b).

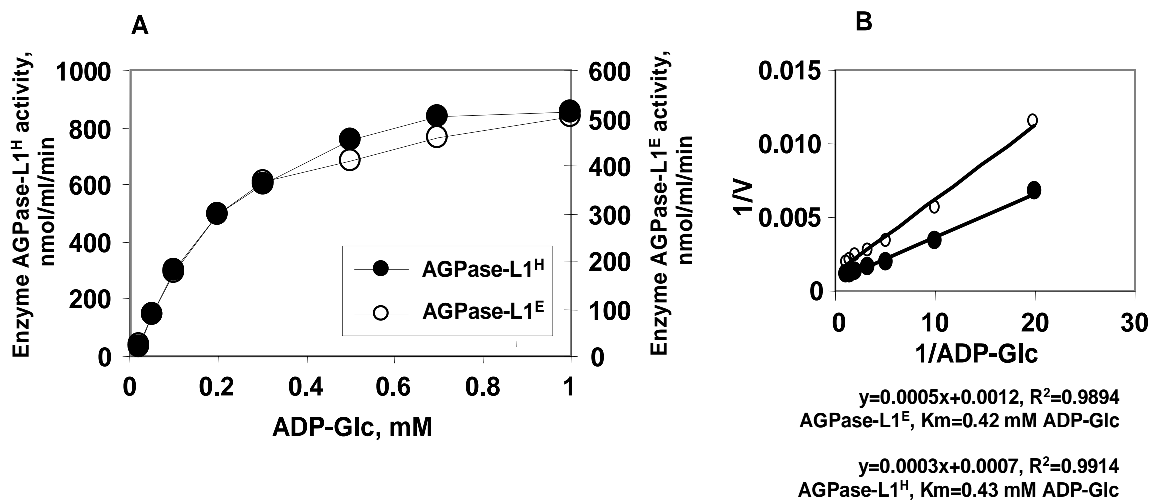

Fig. 20. The effect of ADP-Glc concentrations on the activity of partially purified AGPase in the pyrophosphorolytic direction. A, AGPase activity in response to ADP-Glc concentrations; B, Lineweaver-Burk plot of ADP-Glc saturation curves with corresponding equations and Km constants. ● (black circles), tomato fruits of isogenic lines carrying the AGPase large subunit of *L. hirsutum* (*AgpL1^H*); ○ (white circles), tomato fruits carrying the AGPase large subunit of *L. esculentum* (*AgpL1^E*).

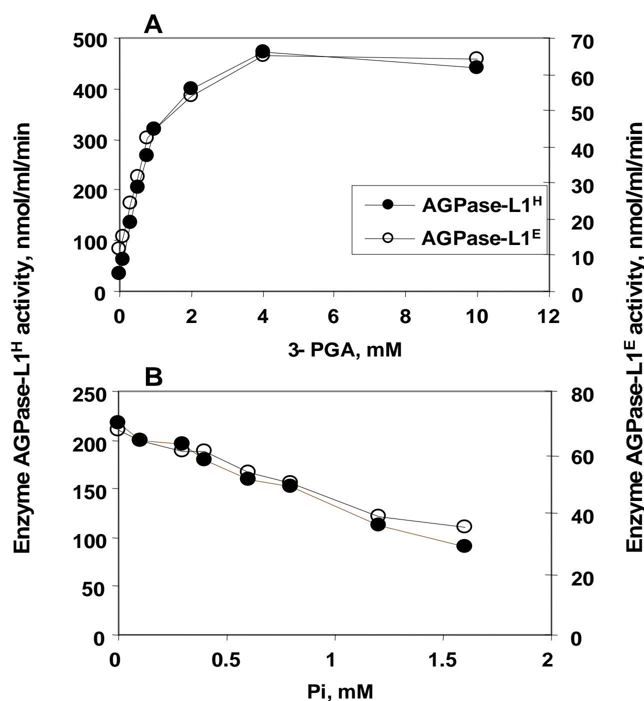

Fig. 21. Effect of activator (3-PGA, A) or inhibitor (Pi, B) on the AGPase activity in the two genotypes of isogenic lines. The activity was measured in the pyrophosphorolytic direction in first step purified enzyme preparation purified tomato fruit extracts after the first step purified carrying the AGPase large subunit of *L. hirsutum* (*AgpL1^H*), ● (black circles); and in tomato fruits carrying the AGPase large subunit of *L. esculentum* (*AgpL1^E*), ○ (white circles). The Pi inhibitory effect was measured in the presence of 5 mM 3-PGA.

Table 15. Kinetic characteristics (Km and $A_{0.5}$) of partially purified AGPase enzymes of immature fruit of the two L1 genotypes. Activity was assayed in both the pyrophosphorolytic and ADP-Glc synthesis directions. Values are averages of at least 5 separate extractions in the pyrophosphorolytic direction and 2 independent extractions in the synthesis direction. Km and $A_{0.5}$ values correspond to the substrate or activator concentrations, respectively, at which the reaction velocity is half-maximal.

Pyrophosphorolytic direction		**mM**	
	ADP-Glc, Km	PPi, Km	3-PGA, $A_{0.5}$
AGPase - L1^E	0.44	0.08	0.17
AGPase - L1^H	0.47	0.07	0.16
Synthesis direction		**mM**	
	ATP, Km	G-1-P, Km	3-PGA, $A_{0.5}$
AGPase - L1^E	0.51	0.18	0.4
AGPase - L1^H	0.48	0.24	0.4

In order to determine whether there might be differences in the physiological concentrations of the substrates/products and activator of AGPase activity, the concentration of these metabolites were measured in the pericarp of immature green tomato fruit (approximately 20 DPA) of both *AgpL1*^{H}and *AgpL1*^{E} genotypes. The results represented in Table 16 indicate no significant differences between the metabolite concentrations in the green fruit of the two genotypes.

Table 16. Metabolite concentrations in immature tomato fruit (*ca* 25 days DAA) of the two AGPase-L1 genotypes of isogenic lines. Recovery experiments were carried out by adding a known amount of each metabolite to the sample during extraction. Values are means ± SE of 3 separate extractions.

			nmol/gfw		
	ADP-Glc	**ATP**	**3-PGA**	**G-1-P**	**G-6-P**
AGPase - L1^{E}	9.8 ± 2.0	38.4 ± 3.0	57.8 ± 5.1	29.6 ± 5.0	109.1 ± 12.2
AGPase - L1^{H}	9.2 ± 1.4	40.3± 3.0	52.2 ± 5.2	31.9 ± 6.7	117.0 ± 16.8
Recovery,%	73	79	91	97.1	97

5.3.6. Thermostability and post-translational redox modification of tomato fruit AGPase protein

Enzymes from both genotypes showed increasing activity upon preincubation of the crude extract at temperatures above 40°C, with maximum activity after incubation at approximately 56°C (Fig. 22). This thermoactivation is presumably associated with the denaturation of other proteins which inhibit AGPase activity (Fig. 22 b). The AGPase-L1^{H} protein appears to be slightly more thermostable and maintained over 40% of maximal activity after incubation at 66°C, whereas AGPase-L1^{E} lost 90% of its maximum activity (Fig. 22 a). No significant differences were

observed in the effect of incubation time; maximum activity for both extracts was observed after 2-3 minutes of incubation at 58°C (Fig. 22 c).

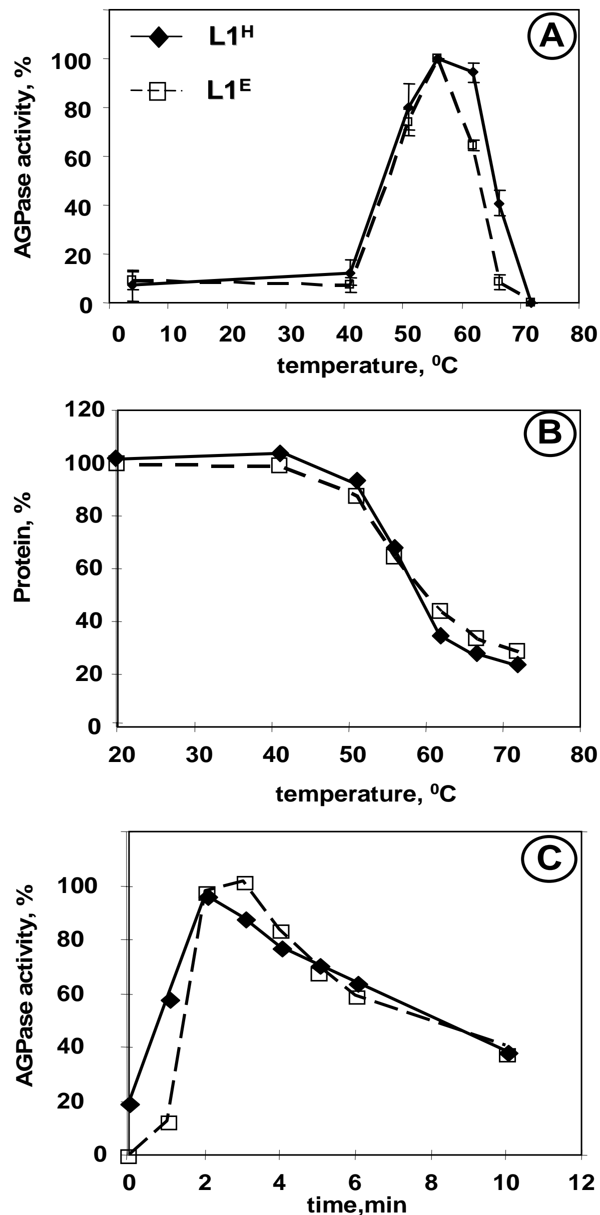

Fig. 22. Effect of temperature on AGPase activity (A) and total protein level (B) in tomato extract after 3 min incubation; (C) effect of time on AGPase activity during incubation at 58°C : ♦ (black diamonds), tomato fruits carrying the AGPase large subunit from *L. hirsutum* (*AgpL1^{H}*); □ (white square), tomato fruits carrying the AGPase large subunit from *L. esculentum* (*AgpL1^{E}*). The maximum enzyme activity was 200 nmol gfw^{-1} min^{-1} and 70 nmol gfw^{-1} min^{-1} for *AgpL1^{H}* and *AgpL1^{E}*, respectively. Standard error bars in (A) are based on four independent extractions from different fruits.

The effect of the reducing environment on dimerization of the tomato enzyme was studied in order determine whether post-translational redox modification occurs with respect to the tomato enzyme in a manner similar to that previously reported for the potato AGPase (Tiessen *et al.*, 2002). Fig. 23 shows that the two genotypes behave similarly in response to DTT in the extraction buffer and have similar active band intensity ratio. Similar to the potato tuber small subunit, the small subunit of both tomato enzymes AGPase- $L1^H$ and AGPase- $L1^E$ can dimerize (100 kD) effectively leading to the post-translational inactivation. The LS subunit does not dimerize, as indicated by only a single immunodetectable band with the LS specific antibody, irrespective of the reducing environment.

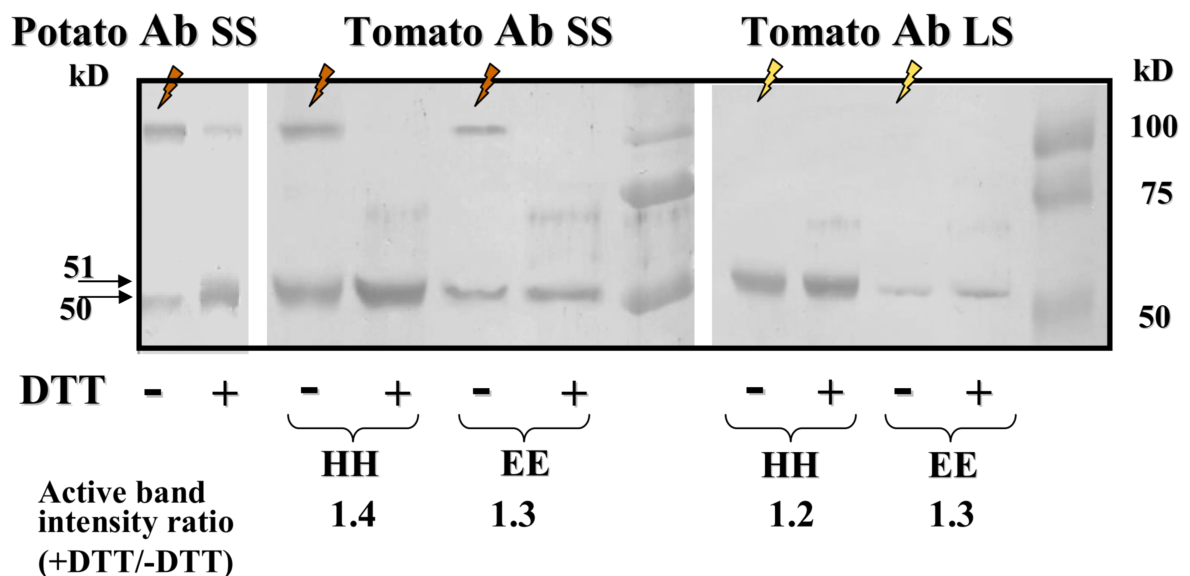

Fig. 23. Effect of reducing environment on the dimer (*ca* 100 kD) and monomer (*ca* 50 kD) form of the AGPase small subunit and monomer (*ca* 51 kD) form of the AGPase large subunit. Protein was extracted from young (*ca* 15 DPA) green tomato fruit of the two genotypes of isogenic lines as well as from immature (11g) potato tuber, under reduced (+DTT) and non-reduced (-DTT) conditions and separated on 10% SDS-PAGE (25 µg fw/well). The identical results were obtained when protein was extracted by adding sample buffer immediately to the tissue or to the first step purified enzyme preparation extract after heat treatment. Immunological detection was as described above using the specific S1 and L antibodies. The band at ca 70 kD in the +DTT lanes was unidentified and had similarly been observed by Beckles *et al.* (2001a). Quantitation of the active monomer band intensities was performed using the TotalLab v2.00 program and ratios of band intensities were calculated for each of the enzymes.

5.3.7. Determination of genomic sequences of two alleles *AgpL1^H* and *AgpL1^E*

The genomic sequences of the two *AgpL1* alleles were compared in order to attempt to detect possible polymorphisms that could serve as candidates causing the temporal differences in gene expression. The entire genomic coding sequence and approximately 2000 bp upstream to the initiation site of two *L. hirsutum* accessions (LA1777 and LA 2650), three *L. esculentum* lines (cv. M82, breeding line 361 and an unidentified cherry cultivar) as well as *AgpL1^H* and *AgpL1^E* segregants from the advanced segregating lines were sequenced and compared. Multiple sequences of each allele from different accessions were performed in order to nullify intraspecific genetic heterogeneity. The nucleotide sequences have been deposited under accession DQ322682 for the M82 gene, DQ322683 for the *AgpL1^E* NIL and DQ322684 for the LA1777 gene. Promoter sequences have been deposited as DQ322688 (M82), DQ322685 (LA1777), DQ322689 (unidentified cherry cv.), DQ322687 (*AgpL1^E* NIL), DQ322686 for (*AgpL1^H* NIL). Although the initial interspecific hybrid was based on LA1777 there were small differences between the sequences of newly obtained seeds of this accession and that of the segregating *AgpL1^H* genotype, presumably due to genetic heterogeneity in this wild species accession.

The two alleles, *AgpL1^E* and *AgpL1^H* are 4500bp and 4497bp long, respectively and both consist of 15 exons and 14 introns (Fig. 24). I confirmed the previously determined differences in *AgpL1* amino acid composition based on cDNA sequencing (I34M, EKK duplication in *AgpL1^H* at 54-56, S419T and T513I). Identity of 95% was found between the genomic sequences of segregating *AgpL1^H* and *AgpL1^E* individuals with small differences in the intronic sequences. There is a 15bp insertion in intron 12 of *AgpL1^H* (5'TCATGAACTCAAGAA3') and two insertions, 4bp (5'AAAG3') and 8bp (5'CTTTTCTTTT3'), in intron 13 of *AgpL1^E*.

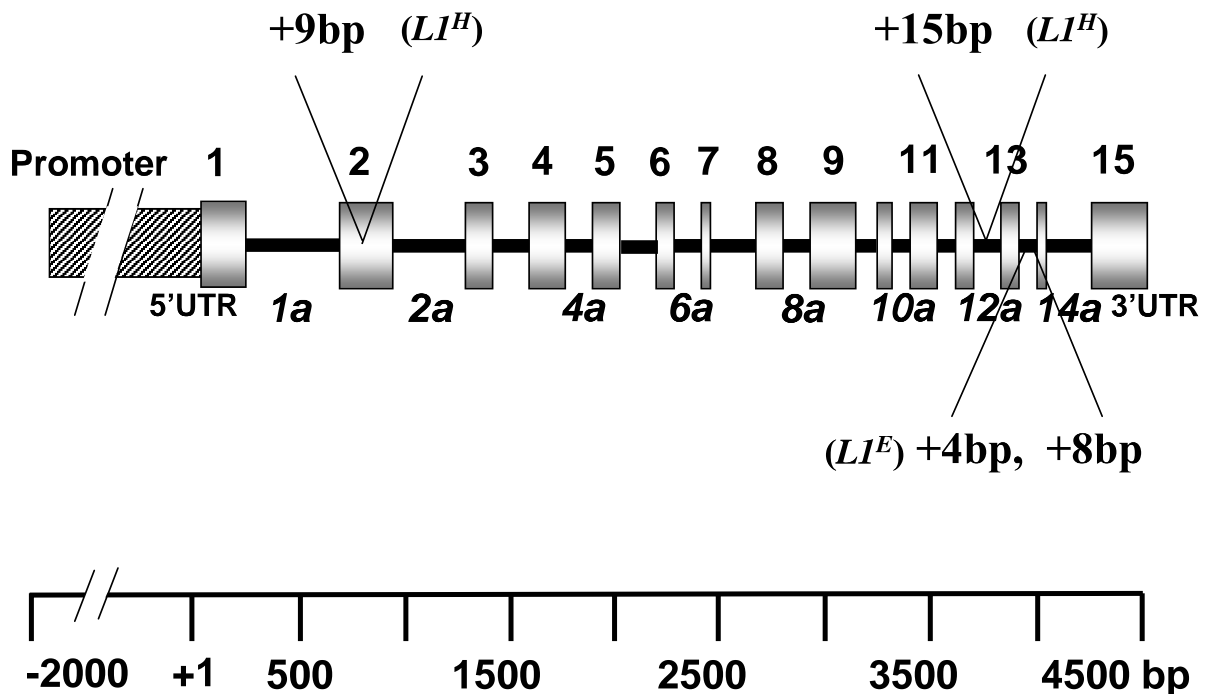

Fig. 24. A diagramatic representation of the two alleles of the AGPase large subunit gene (*AgpL1^H^* and *AgpL1^E^*) of isogenic lines. Exons are shown as blocks and numbered from 1-15. Introns are designated as thick black lines and numbered from 1a-14a. Polymorphisms distinguishing between the two alleles are indicated above (*AgpL1^H^*) or below (*AgpL1^E^*) the diagram.

The putative promoter region sequenced was 2051bp ($AgpL1^H$) and 2063bp ($AgpL1^E$) long. There were numerous polymorphisms between accessions within the individual species (Fig. 25). Considering only those differences which distinguish between the two species, there is a large 77bp deletion at -1000, and additional small insertions and deletions, as well as an 8bp Poly (A) to Poly (T) substitution at -616. Furthermore there are 70 single nucleotide polymorphisms that distinguish between the promoters of the two species. The TATA-box sequence was found 65bp upstream from the transcriptional initiation site in all the promoters (Fig. 26).

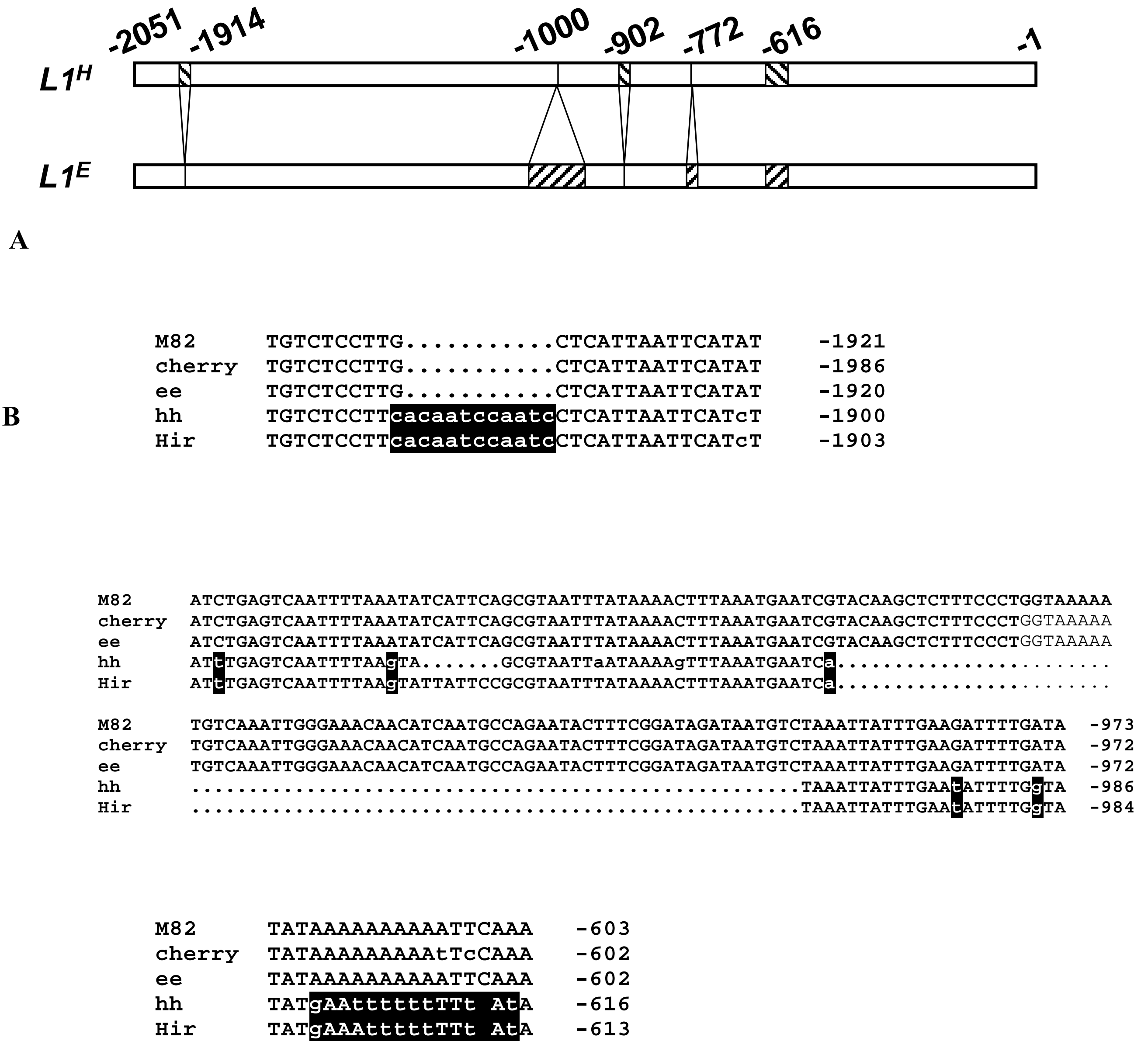

Fig. 25. Diagramatic representation (A) and sequence alignment (B) comparing the promoter sequences of the two *AgpL1* alleles. A, The major large polymorphisms (not including the single nucleotide polymorphisms, SNPs) are indicated by their position and hatched boxes, as described in the text; B, Polymorphism between accessions within the individual species.

```
                      -65bp
                                                                          +1
M82         GAGTATAAATAGAAAGA.TAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAAAGCTGATC
Cherry      GAGTATAAATAGAAAGA.TAGCAA.GTTTCTCGTGAGAGTTCACAAGCCAATAAAGCTGATC
Esc 361     GAGTATAAATAGAAAGA.TAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAAAGCTGATC
ee          GAGTATAAATAGAAAGA.TAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAAAGCTGATC
hh          GAGTATAAATAGAAAGATTAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAAAGCTGATC
Hir 1777    GAGTATAAATAGAAAGATTAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAAAGCTGATC
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Fig. 26. Promoter region of tomato fruits *AgpL1* alleles of two genotype: carrying the $AgpL1^H$ allele (parental Hir 1777 and NIL hh) and four genotypes carrying $AgpL1^E$ allele (M82, Cherry, Esc 361, and NIL ee). The putative TATA-box is designated by a frame.

Based on polymorphism found in the promoter region of the two *AgpL1* alleles, primers were designed (see Materials and methods, 4.4) to afford good resolution for screening large segregating population of tomato plants (Fig. 27), which could be used in future work.

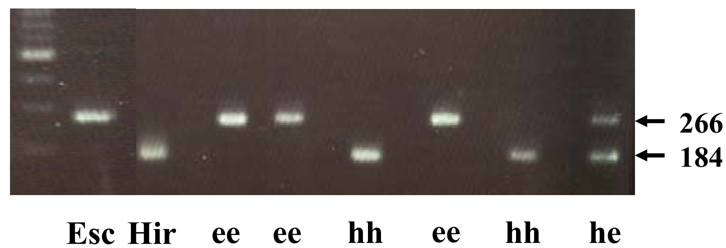

Fig. 27. *AgpL1* promoter region polymorphism. The amplicons (266bp and 184bp for $AgpL1^E$ and $AgpL1^H$ alleles of isogenic lines respectively) were separated on 2% agarose gel and after Ethidium bromide staining visualized in UV.

6. Discussion and Conclusions

The research work presented in this thesis examined in depth the two potential limiting steps in the sucrose-to-starch metabolic pathway in developing tomato fruit. The preliminary results pointed to FK and AGPase as two likely candidates for controlling the transient starch synthesis in tomato fruit and therefore the role of these two enzymes was studied. A novel genotype of tomato characterized by an increased transient starch accumulation phase served as experimental material to dissect the metabolic control on starch synthesis. The increased immature fruit starch content was found to be correlated with increased AGPase activity due to the AGPase large subunit locus (*AgpL1*) introgressed from a wild species. It was determined that the increase in activity is due to the increased expression of the regulatory large subunit and subsequent maintenance of the active heterotetrameric enzyme.

6.1 The role of FKs

Tomato fruit fructokinases (FK) was one of the foci of the present research for the following reasons: a) preliminary results based on the developmental analysis of the sucrose to starch metabolic pathway showed a decline in fructokinase activity in a synchronous manner with the loss of the transient starch content in developing fruit (Schaffer and Petreikov, 1997a); and b) preliminary results further supported a controlling role for fructokinase in sucrose to starch flux in light of the inhibitory effect of physiological fructose concentrations on FK activity and subsequent feedback control of the sucrose metabolism (Schaffer and Petreikov, 1997b). Fructose is a product of both sucrose degradation reactions (SuSy and Inv) and can be estimated as approximately 50% of the carbon provided to the tomato fruit sink. Since SuSy, the

initial enzyme of sucrose degradation during the starch synthesis phase (Ho, 1996; Schaffer and Petreikov, 1997b), is a reversible reaction, the further metabolism of fructose by phosphorylation should drive the flux in the cleavage direction and enhance the sink strength of the tissue. In view of the fact that fructokinases comprise a family of enzymes with distinct kinetic characteristics and unique spatial and temporal expression patterns (Pego and Smeekens, 2000), the different fructokinase isozymes in the developing tomato fruit were separated and characterized in order to allow the study of their individual contribution to starch synthesis. Three distinct enzymes with FK activity (FKI, FKII, FKIII), including a novel enzyme FKIII which had not been reported previously , and an additional two peaks with HK activity (HKI, HKII) were separated and characterized in both green and red tomato fruit pericarp (Petreikov *et al*, 2001). The two HK isozymes are not related to sucrose-to-starch pathway per se but they are key–regulatory enzymes catalyzing the initial step in glycolysis and are linked to the process of sugar-mediated regulation of gene expression and glucose-dependent growth and senescence (Pego and Smeekens, 2000; Xiao *et al*. 2000, Dai *et al*., 1999).

The three FK tomato fruit isozymes could be distinguished from one another with respect to substrate inhibition by fructose, Mg^{+2} and nucleoside substrate specificity (Petreikov *et al*, 2001). The trait of fructose inhibition has been proposed as a possible classifying characteristic of FK enzymes (Pego and Smeekens, 2000). The different kinetic characteristics of the three tomato fruit enzymes (inhibition by fructose and Mg^{+2}, and response to nucleotides) may be of physiological significance, possibly related to distinct localization and compartmentation of the isoforms (Petreikov *et al*, 2001).

This is the first report of a distinct functional third fructokinase in plant tissues. Multiple forms of FK have been reported in various plant tissues and the studies carried out at the onset of the research suggested the presence of only two fructokinase isoforms (Gardner *et al.* 1992; Schnarrenberger, 1990; Baysforder *et al*. 1989; Copperland and Tanner, 1988; Copperland *et al.,* 1978; Doehlert, 1989). Three FK genes were sequenced from Arabidopsis, but it is still uncertain whether the *FRK3* gene was actually transcribed (Pego and Smeekens, 2000). Previous studies of tomato fruit FKs (Delhaize and Randall 1995) Martinez-Barajas and Randall, 1996; Kanayama, 1997, 1998; Martinez-Barajas *et al*. 1997) indicated only two FK isozymes isolated from young tomato fruit, which exhibited almost identical kinetic characteristics. It is not clear whether these isoforms actually represent the products of distinct genes. The success in separating three FK isoforms in the present work was due to an improved ion-exchange chromatography technique, using an HPLC-Mono P ion-exchange column together with an extended ion gradient.

By comparing the kinetic characteristics of the three tomato FK isozymes with the products of the two previously published tomato fruit FK genes expressed in yeast (*LeFRK1* and *LeFRK2*) (Kanayama *et al*, 1997, 1998; Martinez-Barajas *et al*. 1997) with respect to fructose, Mg^{+2} and nucleotide inhibition, the FKI protein was shown to be the gene product of the *LeFRK2* gene and the FKII protein to likely be the gene product of *LeFRK1*. Accordingly, FKI functions as the predominant FK form existing in the young tomato pericarp and would be the crucial isoform controlling sucrose to starch flux. Therefore we hypothesized that the FKI activity would decline in comparison with the other FK isoforms concomitant with the decline in starch synthesis during the temporal fruit maturation. However, no significant loss of FKI activity was observed compared with the other FKs during fruit ripening. Even in the

mature fruit, in which starch synthesis is absent, FKI still remains the predominant FK form, although all FKs decline in activity during ripening.

Results from a parallel study carried out during the present research (Dai *et al*., 2002; German *et al*., 2003) on transgenic tomato plants expressing the *LeFRK* genes, confirmed that *LeFRK2*, the gene encoding FKI, the main FK isozyme in tomato fruits, is not required for starch biosynthesis in developing fruits. Transgenic tomato plants expressing antisense *LeFRK2*, without FKI activity, had normal starch content. Similarly, *LeFRK1* also did not have any impact on starch synthesis but have effected on delayed flowering at the first inflorescence (Odanaka *et al*., 2002). The *LeFRK3* gene was recently sequenced (German *et al*., 2004), and according to the biochemical characteristics of the heterologously expressed protein as well as its chromatographic behavior, FKIII is most likely the product of *LeFRK3* gene. Similarly, a recent report on transgenic potato plants with antisense inhibition of StFK1, the predominant fructokinase isoform in the tuber, indicated that starch synthesis per se is not affected by this enzyme although the plants had a reduced tuber yield (Davies *et al*., 2005). The authors suggested that FK1 is not important for the control of starch synthesis but plays an important role in maintaining a balance between sucrose synthesis and degradation, acting in concert with sucrose synthase.

In conclusion, the results of my research on the fructokinases, supplemented by the parallel studies carried out during the past two years point to the conclusion that the FKs do not play a role of limiting enzymes in transient starch accumulation in tomato fruit. This is true for the prime FK candidate in starch synthesis, FKI, encoded for by the *LeFRK2* gene. This is also true for the FKII, and the novel FKIII isoform which was discovered during the present research work. In light of this conclusion the research work focused on contribution of AGPase to starch synthesis in tomato fruit.

6.2 AGPase is limiting to the temporal synthesis of starch in tomato fruit

The results presented here indicate not only that AGPase is limiting to starch synthesis but that its temporal extension and increase of activity can further increase starch accumulation. Other report showed conclusively that reducing AGPase activity reduces starch content (Lin *et al.*1988; Müller-Röber *et al.*, 1992**;** Geigenberger *et al.*, 1999; Weber *et al.*, 2000; Obiadalla Ali, 2003). This was demonstrated in starchless mutants and or in transgenic plants expressing antisense AGPase subunits (Tsai and Nelson, 1966; Dickinson and Preiss, 1969**;** Lin *et al.*, 1988; Smith *et al.*, 1989). However, there are only two studies that have shown that an increase in AGPase activity can lead to a further increase in starch synthesis, one in transgenic potato tuber (Sweetlove *et al.*, 1996, Stark et al., 1992; Tiessen *et al*,, 2002) and the other in transgenic tomato fruit (du Jardin *et al.*, 1997). Both of these studies utilized a mutant non-regulated bacterial monomeric AGPase (Stark *et al.*, 1992). There have also been reports of correlations between increased native AGPase activity and increased seed size in graminous seeds (Giroux *et al.*, 1996; Greene and Hannah, 1996, 1998a, b; Smidansky *et al.*, 2002, 2003). Enhanced turnover of transitory starch in source leaves was also recently reported by expression of up-regulated AGPase in *A. thaliana* **(**Obana *et al***,** 2006). However, the present study is the first to show that a naturally derived increase in activity of AGPase can lead to increased starch content in the tomato fruit.

Our initial study (Schaffer and Petreikov, 1997a) showed that there is a developmentally coordinated control of the starch synthesis metabolic pathway and that at least four enzymes in the pathway (SuSy, FK, AGPase and SStSy) all decline in a synchronous manner. However, of the four enzymes AGPase is apparently the most

important enzyme in the pathway, constituting a metabolic bottleneck (Schaffer and Petreikov, 1997a). Accordingly, increased activity of this limiting enzyme alone was effective in increasing flux to starch synthesis.

An additional confirmation of the role of AGPase as limiting to starch synthesis was obtained in our comparative survey of enzyme activities involved in sucrose-to-starch metabolism of the high starch breeding line (904) and the standard cultivar of tomato (M-82). The results of this comparison showed that AGPase was definitively the only enzyme in the pathway that was significantly more active in the high starch line. AGPase activity in a F_2 segregating population of the cross of the high starch line 904 and the cultivated M-82 further support the correlation between AGPase activity and starch levels (Table 4 and 5, and published as part of Schaffer *et al*., 2000).

Upon concluding that the activity of AGPase can be further increased in tomato fruit and that this is causal to an increase in starch levels my research continued to determine the mode of action of the genetic increase in enzyme activity.

6.3 Effect of *AgpL1^H* allele

6.3.1 *The large subunit of AGPase derived from the wild tomato L. hirsutum leads to the increase in AGPase activity and elevated transient starch content in tomato fruit*

The molecular analysis of the high starch introgression line 904 showed that it carries the *AgpL1* allele derived from the wild species *L. hirsutum* (*AgpL1^H*) and that the other three AGPase loci (*AgpL2*, *AgpL3* and *AgpS1*) are all derived from the cultivated parental species (Schaffer *et al.*, 2000). Of the three large subunit loci present in the tomato genome, *AgpL1* is the gene most strongly expressed in developing fruit and encodes for the predominant large subunit of the fruit enzyme (Chen *at al.*, 1998; Park and Chung, 1998; Li *et al*., 2002 and results presented in Fig.

16). The large subunit functions as the allosteric controlling subunit of the active heterotetramer, together with the catalytic small subunit. Therefore, the contribution of the *AgpL1*^{H} to the activity of the holoenzyme is a novel example of genetic variability for an allosteric non-catalytic enzyme subunit contributing to increased in vivo activity and flux.

The results presented here indicate that both AGPase activity and fruit starch content were higher and also temporally prolonged in developing fruit harboring the *AgpL1*^{H} allele. The next portion of the research uncovered the mode of action of the *AgpL1*^{H} allele in causing the temporal increase in enzyme activity.

6.3.2 *AgpL1*^{H} *allele shows prolonged and higher expression in the high starch lines*

The study of the developmental expression of the AGPase genes during fruit development indicate that only the *AgpL1*^{H}-allele shows a differential expression pattern between the high and low starch genotypes. The *AgpL1*^{H} allele shows a prolonged and higher expression in the high starch lines. The relatively low and steady expressions levels of the two other large subunits, *AgpL2* and *AgpL3*, during fruit development indicate that they do not play a significant role in fruit transient starch accumulation and it is questionable whether the *AgpL3* is expressed to any significant level in the fruit. The *AgpS1* gene is developmentally the most highly expressed AGPase gene (nearly 7-fold of that of *AgpL1*), but it demonstrates a similar expression level in both the high and low starch genotypes.

The developmental expression pattern of the large and small subunits is partially but not completely coordinated. The *AgpL1* is silenced earlier than *AgpS1* in development and this suggests that the allosteric subunit may be limiting to the heterotetrameric holoenzyme and therefore limiting to enzyme activity in tomato fruit

(Li *et al*, 2002; present research). Analogous results pointing to the limiting factor of expression of the large subunit can also be seen in AGPase activity during the development of the wheat grain (see Ainsworth *et al*, 1995). In this starch synthesizing tissue of the wheat grain, the small subunit expression begins earlier than that of the large subunit but activity correlates with the onset of the large subunit expression, which allows for the formation of the active holoenzyme complex.

6.3.3 L1 *and* S1 *protein subunits show prolonged and higher expression in the* *AgpL1^H* *high starch lines*

The differential developmental patterns of AGPase subunit gene expression was compared to the developmental pattern of subunit protein levels in the developing fruit of the two genotypes. By utilizing antibodies which were specific for the large subunit and small subunit, respectively, we were able to follow the levels of subunit proteins in the developing fruit. Surprisingly, the protein levels of each subunit were strictly coordinated. In each genetic background the immunologically detectable levels of the large subunit and the small subunit proteins were linked temporally. This was in sharp contrast to the differential pattern of gene expression between the *AgpL1* and *AgpS1* observed above. The presence of the S1 protein ceased to be observable at the same time as the L1 protein. However, there was a striking difference between the *AgpL1^H* and *AgpL1^E* genotypes with respect to the developmental protein subunit levels. In the *AgpL1^H* high starch line, both subunit proteins exhibited a prolonged presence.

The above phenomenon is possibly due to the effect of the subunit interaction on subunit stability. Interaction between the large and small subunit proteins creates a stable and active enzyme complex. This stable complex itself functions in preventing the small subunit protein degradation. Accordingly, the extended L1^H protein stabilizes

the small subunit protein and provides to AGPase-L1^{H} an extended enzyme activity. Upon cessation of the *AgpL1* transcription and protein synthesis the small subunit is also degraded, in spite of its continuing transcription. Therefore, the *AgpL1^{H}* genotypes with the longer *AgpL1* expression are able to protect and preserve the small subunit protein as well. This model explains the effect of the *AgpL1^{H}* introgression on the AGPase activity and starch content of the high starch genotypes. This explanation is also in agreement with the data of Greene and Hannah (1998 a, b). They reported that a mutation in the large subunit *SH2* protein which increases maize AGPase stability also elevates levels of the small subunit *BT2* protein. Accordingly, *SH2* subunit alters the level or stability of the *BT2* subunit, pointing to altered interactions between the two subunits. The reciprocal relationship is also evident. Wang *et al*. (1998) reported that the presence of a functional small subunit is required for large subunit stability in *A. thaliana*.

6.3.4 *AGPase-L1^{H} protein appears to be slightly more heat stable then AGPase-L1^{E} protein*

The dicot tomato and potato AGPase enzymes are relatively heat stable (Sowokinos and Preiss, 1982; Okita *et al*., 1990; Chen and Janes, 1997), in contrast to the monocot AGPases that are more heat labile (Hannah *et al*., 1980). In order to determine whether the different *AgpL1* subunits impart different heat stability characteristics to the active AGPase enzymes of the two genotypes, the enzyme activity and protein levels were compared following various heat treatments. The enzymes of both tomato genotypes were found to be relatively heat stable. In fact, heat treatment was necessary to activate the enzyme, which is likely due to the effect on heat lability of specific inhibitory substances. A 90% increase in activity after pre-

incubation at 58^{O}C for 5 min was observed. This thermo-activation is presumably associated with the denaturation of 65% of other proteins, some of which may inhibit AGPase activity (Fig 22b).

Although both the AGPase-L1^{H} and AGPase-L1^{E} enzymes are heat stable, the AGPase-L1^{H} enzyme seemed to be slightly more heat stable, retaining up to 42% activity even at 66^{O}C, whereas AGPase-L1^{E} lost 90% of maximum activity under the same conditions. The reason for this difference may be the result of 3 amino acid EKK duplication at the N-terminus of $AgpL1^{H}$ (Schaffer *et al*., 2000). N-terminus area has been shown to be involved in heat stability of the AGPase large subunit of potato and critical for enzyme activity (Kavakli *et al*., 2002). Previous studies showed the importance of amino acids from the N-terminus of both the S and L subunits for the active conformation and stability of potato tuber AGPase (Iglesias *et al*., 1993; Ballicora *et al*., 1995; Laughlin *et al*, 1998). Similarly, by modifications of the N-terminal region of S subunit it is possible to turn the heat labile maize protein (Hannah *et al*., 1980) into a heat stable one (Boehlein *et al*. 2005; Linebarger *et al*., 2005). Enhanced heat stability involves alternations in the aggregation status of the enzyme. An additonal disulfide bridge, or enhanced electrostatic interactions at the dimer-dimer interface, simular to those in the tetrametic malate dehydrogenase (Bjork, Dalhus et al. 2004), can explain enhanced stability. Future research studying heterologously expressed chimerical enzymes can shed light on the role of the EKK N-terminus duplication in protein stability.

6.3.5 *Post-translational redox activation of AGPase is similar in the high and standard starch lines*

The higher activity and stability of AGPase-L1^H protein was not a result of differences in the post-translational redox modification. The formation of S1 dimers under non-reducing conditions of the tomato enzyme of both *Agp-L1* genotypes was similar to that reported for potato S1 (Tiessen *et al*., 2002; Hendrics *et al*, 2003; Jin *et al.*, 2005), due to existence of Cys in the position 12 of the mature protein. Due to the absence of Cys in N-termini of the L1, the large subunits do not form dimers in non-reducing conditions. This is the first time that the tomato enzyme has been characterized in this regard. Post-translational redox modification is proposed to be the preferred strategy of plastidial enzymes to regulate various metabolic processes such as carbon fixation, starch metabolism, lipid synthesis, and amino acid synthesis in response to physiological and environmental inputs like light and sugars. Post-translational redox activation of AGPase in response to sugars is part of a signaling mechanism linking the rate of starch synthesis to the availability of carbon in diverse plant tissues (Geigenberger *et al*., 2005) but it is not involved in the genetic differences in transient starch synthesis of tomato fruit.

6.3.6. *Effect of the AgpL1 allele on the kinetic properties of the enzyme*

The sensitivity to allosteric effectors is different for cytosolic and plastidic forms of AGPase: the wheat and barley endosperm enzyme, in which the cytosolic isoform accounts for 65-95% of the total AGPase, is less sensitive to 3-PGA activation and Pi-inhibition than plastidic isoforms (Tetlow *et al*., 2004). Tomato fruit plastidic AGPase is almost inactive in the absence of 3-PGA, with less than 10% of maximum activity (Fig. 21a). This is in agreement with the results of Chen and Janes (1997)

which also showed a high sensitivity of the tomato fruit AGPase to 3-PGA/PPi regulation, similar to the plastidial enzyme from potato tuber and other higher plants (Preiss, 1982; Kavakli *et al*., 2001; Burger *et al*., 2003; Ballicora *et al*., 2004).

We did not find any differences in the substrate affinity or sensitivity to 3-PGA/PPi regulation of the AGPase enzymes from the two genotypes (Fig. 21b, Table 15). This, despite of the fact, that the *AgpL1*^{H} allele encodes for a protein with a substitution of one amino acid (Ile to Thr) in the PGA activation site at the C-terminus that may impact on PGA activation/Pi inhibition (Schaffer *et al*., 2000).

The concentrations of metabolites (substrates or products), including Glc-6-P, which can affect AGPase activity *in vivo* were also found to be unchanged in the high starch genotype. The concentration of Glc-6-P was assayed since it is the principal hexose-phosphate which crosses the amyloplast membrane in tomato (N'tchobo, 1998). According to Nguyen-Quoc and Foyer (2001) there are two potential factors which are involved in control of starch synthesis: AGPase activity in the plastid and transfer of Gcl-6-P between compartments (cytosol to amyloplast). Since the measured Glc-6-P concentration was similar in the two genotypes having normal and elevated enzyme activity, AGPase remains the main factor in for tomato fruit starch regulation. The cause of increased activity in the system studied here is due, perhaps solely, to the effect of temporal *AgpL1* gene expression and not to protein sequence differences that could lead to modified kinetic properties of the AGPase-L1^{H} enzyme or to metabolic fine control of enzyme activity.

6.3.7 *Sequence comparison of the AGPase gene and promoter region of two genotypes*

The genomic sequences of the two *AgpL1* alleles were compared in attempt to detect possible sequence differences that could account for the temporal differences in gene expression. The importance of sequencing full length alleles including introns lies in the possibility that lesions within the intron lead to aberrant splicing, producing proteins with different qualities (Lal *et al*, 1999). Comparative analysis of the two alleles of the *AgpL1* gene indicated 97% homology between the full length sequences and 86% homology between the two promoter regions. The genomic sequence comparison raised some candidates for the observed differences in temporal expression; however, further promoter analysis focusing on these candidate regions is necessary to elucidate the causal factors determining the expression differences.

Differences in amino acid composition are located at the N-terminus (three amino acids duplication and one amino acids substitution) and at the C-terminus (two amino acid substitutions in addition to the polymorphism in two introns) and not in the central part of the $AgpL1^H$ allele. These differences can affect the stability of the protein heterotetramer, in which two small and two large subunits stick together by their N- and C-terminal regions, according to the recently proposed 3-D crystal structure of potato tuber AGPase (Jin *et al*., 2005). The mature protein of the potato large subunit begins at residue 7, which corresponds to the residue 64 of tomato *AgpL1*. The functional N-terminus position of the tomato mature L1 protein is not yet determined. Nevertheless, the stability of a mature $L1^H$ protein could be enhanced by two factors: the substitution of an aliphatic amino acid for a one containing sulfur (M34) and the duplication of a very polar EKK sequence (E, glutamic acid is acidic and K, lysine is a basic amino acid) in close proximity of 4 residues upstream to the

possible starting point of the mature protein, as indicated in the study of the large subunits of potato (Iglesias *et al.*, 1993), spinach (Morell *et al.*, 1987) and our own recent MS/MS analysis of the tomato LS protein (unpublished). The observed higher stability and heat stability of the AGPase-$L1^{H}$ protein is in agreement with the differences in the N- and C-terminal regions of the two genotypes.

Irrespectively, these results emphasize the role of L1 not only in the allosteric control and modulation of AGPase activity but also in the stability of the L1-S1 heterotetramer.

6.4 Concluding remarks

The understanding of the metabolic pathway from the point of sucrose unloading in the immature fruit sink to starch synthesis is of a great importance, since accumulated starch serves as a reservoir for soluble solids synthesis in the mature fruit. The contribution of starch accumulation in the immature fruit to soluble sugar levels in the mature fruit will depend on the size of the transient reservoir of storage carbohydrates. This, in turn, will be a function of the starch concentration and the size of the reservoir, which is a function of the duration of the starch accumulation period, the growth rate of the fruit, and the distribution of starch within the fruit. A simple calculation (ignoring the role played by daily starch turnover) shows that a mid-size tomato cultivar (100 g) that accumulates a transient maximum of 2% starch on the basis of the fresh weight of the bulk fruit tissue when the fruit is small (i.e. 10g) will contain a potential reservoir of only 200 mg of starch, an insignificant contribution to the carbon economy of the mature fruit. However, a genotype that continues active starch accumulation for an extended period and reaches the same 2% starch when the fruit weight is 50 g will have a reservoir of 1000 mg and this could have an impact

upon the final fruit sugar content. Thus, multiple strategies are available for increasing the starch reservoir of the developing fruit: increasing the rate of starch synthesis, expanding the relative contribution of starch- synthesizing tissues to the fruit bulk, and extending the period of starch accumulation.

Three different FK isozymes, including a novel one, which was discovered during the present research, do not play a role of limiting enzymes in transient starch accumulation in tomato fruit.

AGPase is the most prominent limiting enzyme in starch synthesis. The increased starch content in the immature fruit, as well as higher soluble sugars in the mature fruit following the breakdown of the transient starch, are correlated with increased AGPase activity. The results of this research indicate that the increased activity of AGPase in tomato fruits harboring the wild tomato *L. hirsutum AgpL1* introgression is due to the increased expression and protein levels of the regulatory large subunit, which subsequently leads to an extended stability and activity of the functional holoenzyme.

This may be considered as an example of intra-molecular heterosis, in which each of the individual subunits contributes advantages to the hybrid heterotetramer enzyme function and activity. The hybrid heterotetramer consisting of the *L. hirsutum AgpL1* and *L. esculentum AgpS1* accumulates higher levels of starch than the corresponding heterotetramer with $AgpL1^E$. The wild species donor itself accumulates little starch. However, we have not yet developed or characterized the heterotetrameric enzyme from the genotype $AgpL1^H/AgpS1^H$ in the *L. esculentum* background which would be necessary to conclude that the hybrid enzyme does in fact display heterotic characteristics. Nevertheless, based on starch levels alone, these facts make the wild species *L. hirsutum*, which itself accumulates low starch levels, an unexpected genetic

source for increasing starch content in the cultivated tomato. The low starch level of the wild species is at least partially due to a large proportional makeup of gelatinous portion in the fruit, which has low starch content even in the cultivated tomato (Schaffer and Petreikov, 1997a). Exotic germplasm is a potential resource for improving the existing elite cultivars of tomato fruits (Fridman *et al*., 2002). The genetic potential of the wild species cannot be predicted by its own phenotype since unexpected recombinations can occur when using the wild species as a source of genetic material (Bernacchi *et al*., 1998). It is possible that the high starch line is the result of the genetic recombination of traits for increased starch synthesis (the *L. hirsutum*-derived $AgpL1^H$) together with *L. esculentum* traits of fruit morphology.

The transfer of horticultural traits from the wild species is often accompanied by the transfer of undesirable linked genes (Monforte and Tanskley**,** 2000). Limiting the size of the introgression containing the $AgpL1^H$ allele to approximately 1 cM reduces the possibility that the increase in enzyme activity is due to an unrelated linked enzyme transferred simultaneously from the wild genome. Bernacchi *et al.* (1998) reported on the transfer and identification of unexpected quantitative trait loci (QTL) for horticultural traits from *L. hirsutum* (*S. habrochaites S.*) including an *L. hirsutum*-derived QTL for soluble solids content on chromosomal segment 1–4. However, the present research shows that the previously described QTL for Brix segregates independently from the *AgpL1* locus and that the increase in Brix in the $AgpL1^H$ genotypes is not due to this more proximal locus linked to the TG161 marker (Monforte and Tanksley, 2000). Nevertheless, to prove causality of the $AgpL1^H$ allele for high starch accumulation, the generation and analysis of transgenic tomato plants with either silencing or overexpression of the large subunit gene would need to be carried out.

To the best of our knowledge, this is the first report of a non-transgenically caused increase in AGPase activity associated with increased starch content, showing that elevating activity above normal levels may increase flux towards starch, and indicating that AGPase activity is naturally limiting to starch accumulation in tomato fruit. The transient starch accumulation may be an effective strategy for increasing "sink strength" of the developing fruit. By temporally extending AGPase activity, starch accumulation continues in larger fruit, since the period of 20-30 days following anthesis is a period of major fruit expansion. Therefore, the increase in enzyme activity and starch accumulation on a fresh weight basis is further compounded when expressed on a whole fruit basis since the more developed fruit contains more fresh weight, further increasing the starch reservoir of the fruit unit. The increase in starch and mature fruit sugar levels of the $AgpL1^H$ genotypes were not accompanied by decreases in fruit size or yield (frequently fruit size was increased) so that the final sink size even further increased in response to the increased metabolic flux to starch.

7. List of publications containing results presented in this thesis

1. **Schaffer AA, Levin I,Ogus I, Petreikov M, Cincarevsky F, Yeselson E, Shen S, Gilboa N, Bar M** (2000) ADP-glucose pyrophosphorylase activity and starch accumulation in immature tomato fruit: the effect of a Lycopersicon hirsutum-derived introgression encoding for the large subunit. Plant Science **152**:135-144

2. **Petreikov M, Dai N, Granot D, Schaffer AA** (2001) Characterization of native and yeast-expressed tomato fruit fructokinase enzymes. Phytochemistry **58**: 841-847

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9. Appendices

9.1. Promoter sequence DQ322687 (*AgpL1^{E}*, NIL): 2063 bp

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1       CGGAGTAAGT GACATACATA TAAAGATGGG TGGAATCTTC CAAATAGTGA AAGAAGACAA
61      GCACATCTTT ACATTAAACC TTCAATTATC TAACTAATCT CTAACATGCT CTGTCTCATT
121     GTCTCCTTGC TCATTAATTC ATATTTTAAC ATCTTTATTT ATTTTTCAAG GTCTTGAGCT
181     AATTAATTCA ACAAAACACC CAACATATCA TCTCCTTTTT GCTTTACTAG AATTTGAGCG
241     GTTAAAACGG TTTTAGCTCA TGAATCAGAT CAATTCAATT CATTATGGGG ATAGGTTTGG
301     GACATAAGTT CTTAAGCTCT TTTAAAATTA GGACTTTATA AGTCTGATTC ATATAAGTGT
361     ATGATCCTTG AGACTTTATT AAGGTCGGGT CGAGGTCGGT CCATGGCCTT AATTTTTGTT
421     TATTTAAGTG TATCTTATAA TAATTTTACT AGTATATAAT TTAATCATTT AAATACACTA
481     TATGTTGTAA AAATGATGAA TCTTACCAGA CTTTGTTAGT CCAAATACAT GTATCTCGAA
541     ATATATAAAC TGAAAATTAG TTTAATTTGT TTTAGACACA CTCTATCCAA ATCTGGCACT
601     CTTCCTACCC TTTCATCTTG CTTGTCACTG TCTTATATTG CTCACGTATC TAGTATGCGA
661     GATACATACT GATTACACTA GTTTTTTTTT TTGTTAGTGT TATTGAAACA ACAATGTTAG
721     TCATGTTTTG ATTTGAAAAT CTTCTCAGTT TCTTGAATTA GCAGTTTGAA AAGAAAAAAT
781     AAGCTAGCCC CTCCCTTTCC CTTTATGATT GGGTTGGATT GAATATTTTC AAGATCAACC
841     CATCCTAACC CTTTAAATTT CTTAGCCTAC AAAGCTTGGA CCAGATAAAG TTGAACCAAT
901     TTTTTTAATC GCTTCAACTA GAATAAAATA ATAGTATCTG AGTCAATTTT AAATATCATT
961     CAGCGTAATT TATAAAACTT TAAATGAATC GTACAAGCTC TTTCCCTGGT AAAAATGTCA
1021    AATTGGGAAA CAACATCAAT GCCAGAATAC TTTCGGATAG ATAATGTCTA AATTATTTGA
1081    AGATTTTGAT ATGAAATTGT CAGCTAATCT CCAACTAAAT AATGTCTGAC CCCACCAAAT
1141    TTTTTAACGG ACAAAGATCT CTCTAAGAAG TATAGTAGTA TATAAAATGA CATTCATGAG
1201    TGTGGAAATT GTATATAGTT TTATTTAACT ATTTTCTTGC AAATCATATG GTTCATAGAA
1261    TAATATTAAC AATAAAAAGA CAGGTGTTTG GCCTGTAATG GGTCGGTCTC TATTGTCCAG
1321    ATCTTGGTGG ACCCTACACA CTATGACGTC TGTCAATTAA TCTTGGAAAA ATAACTGTTA
1381    GCACGAGTTT TCCAGTCTAA TTTTCAGTGA TTTTATTTAA TAATGATTAA GTTTTATTGC
1441    GAATATAAAA AAAAAATTCA AATATTCATC TAAAATTTTA GGGATAATAT ATAAATATAC
1501    TCTTTAACTT GATTTTCAAA TCACAATTAT GCCTTTCAAC TTTGGGTGTG CACAAGTAGA
1561    CACTTAAATT TATATAAAAT TGAACAAATA GACACACATG TCCTATATGT CATCCTACAT
1621    GTCATATTTT GTCCTACGTG GCGTTCTACG TGTATTTTGT CATGTAGGAC TCGTATGTTT
1681    ATTTATTTAA AAGTTGGTCT GTTTGTTCAT TATGAAAGTT GAAACTCAAA GTTAAAATTT
1741    GAAATCAAGT TTAGGATTTA ATATATGTAT TATGTCAAAA ATTTATATTA TTTAATTATT
1801    TTAAAGTGAT AAAAAAATCT AATGAAGATT TTATCAAAGT AAAAGTGAAT AAAAGGAATA
1861    AATATGAAGA TTTTCAAATG TTTTATAATT TGAGGATCTA AATAAATTTA AAGAGTTGTT
1921    AATATACTTG GGGAAAATAA AATATTATTT AAGTTATGGG GTGTATAAGT TAAAAAACTT
1981    TTTATGTAAA TAGGGAATAT GAAAATGAGT ATAAATAGAA AGATAGCAAG TTTCTCGTGA
2041    GAGTTCACAA GCCAATAAAG CTG
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9.2 Promoter sequence DQ322689 (*AgpL1*, unidentified cherry cv.): 2129 bp

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1       CGGAGTAAGT GACATACATA TAAAGATGGG TGGAATCTTC CAAATAGTGA AAGAAGACAA
61      GCACATCTTT ACATTAAACC TTCAATTATC TAACTAATCT CTAACATGCT CTGTCTCATT
121     GTCTCCTTGC TCATTAATTC ATATTTTAAC ATCTTTATTT ATTTTTCAAG GTCTTGAGCT
181     AATTAATTCA ACAAAACACC CAACATATCA TCTCCTTTTT GCTTTACTAG AATTTGAGCG
241     GTTAAAACGG TTTTAGCTCA TGAATCAGAT CAATTCAATT CAATTCATTA TTAAGATAGG
301     TTTGGGATAT AAGTTCTTAA GCTTTTTTAA AACTAGAGGT TTATAAGTCT GATCCATATA
361     AGTGTATGAT CCTTGAGACT TGATCAAGGC CGGGTCGAGG TCGGATCAAG GCCGGGGTCG
421     AGGTCGGCCC ATGGCCCTAA TTTTTGTTTA TTTAAGTGTA TCTTATAATA ATTTTACTAG
481     TATATAATCT AATCATTTAA ATATACTATA TGTTGTAATA TGATGAATCT TACTAGACTT
541     TGGTAGTCCA AATACATGTA TCTCAAAATA TATAGACTTA AAATTAGTTT AATTTGTTTT
601     AGACACACTC TATCCAAGTA GATTCTCATG AATCTGTGAT ACACAGACAA ATCTGGCACT
661     CTTCCTACCC TTTCATCTGC TTGTCACTGT CTTATATTGC TCACGTATCT AGTATGCGAG
721     ATACATGCTG ATTACACTAG TTTTTTTTTG TTAGTGTTAT TGAAACAACA ATGTTAGTCA
781     TGTTTTGATT TGAAAATCTT CTCAGTTTCT TGATATTATA GAATTAGCAG TTTGAAAAGA
841     AAAAATAAGC TAGCCCCTCC CTTTCCCTTT ATGATTGGAT TGGATTGAAT ATTTTCAAGA
901     TCAACCCATC CTAACCCTTT AAATTTCTTA GCCTACAAAG CTTGGACCAG ATAAAGTTGA
961     ACCAATTTTT TTAATCGCTT CAACTAGAAT AAAATAATAG TATCTGAGTC AATTTTAAAT
1021    ATCATTCAGC GTAATTTATA AAACTTTAAA TGAATCGTAC AAGCTCTTTC CCTGGTAAAA
1081    ATGTCAAATT GGGAAACAAC ATCAATGCCA GAATACTTTC GGATAGATAA TGTCTAAATT
1141    ATTTGAAGAT TTTGATATGA AATTGTCAGC TAATCTCCAA CTAAATAATG TCTGACCCCA
1201    CCAAATTTTT TAACGGACAA AGATCTCTCT AAGAAGTATA GTAGTATATA AAATGACATT
1261    CATGAGTGTG GAAATTGTAT ATAGTTTTAT TTAACTATTT TCTTGCAAAT CATATGGTTC
1321    ATAGAATAAT ATTAACAATA AAAAGACAGG TGTTTGGCCT GTAATGGGTC GGTCTCTATT
1381    GTCCAGATCT TGGTGGACCC TACACACTAT GACGTCTGTC AATTAATCTT GGAAAAAATAA
1441    CTGTTAGCAC GAGTTTTCCA GTCTAATTTT CAGTGATTTT ATTTAATAAT GATTAAGTTT
1501    TATTGCGAAT ATAAAAAAAA ATTCCAAATA TTCATCTAAA ATTTTAGGGA TAATATATAA
1561    ATATACTCTT TAACTTGATT TTCAAATCAC AATTATGCCT TTCAACTTTG GGTGTGCACA
1621    AGTAGACACT TAAATTTATA TAAAATTGAA CAAATAGACA CACATGTCCT ATATGTCATC
1681    CTACATGTCA TATTTTGTCC TACGTGGCGT TCTACGTGTA TTTTGTCATG TAGGACTCGT
1741    ATGTTTATTT ATTTAAAAGT TGGTCTGTTT GTTCATTATG AAAGTTGAAA CTCAAAGTTA
1801    AAATTTGAAA TCAAGTTTAG GATTTAATAT ATGTATTATG TCAAAAATTT ATATTATTTA
1861    ATTATTTTAA AGTGATAAAA AAATCTAATG AAGATTTTAT CAAAGTAAAA GTGAATAAAA
1921    GGAATAAATA TGAAGATTTT CAAATGTTTT ATAATTTGAG GATCTAAATA AATTTAAAGA
1981    GTTGTTAATA TACTTGGGGA AAATAAAATA TTATTTAAGT TATGGGGTGT ATAAGTTAAA
2041    AAACTTTTTA TGTAAATAGG GAATATGAAA ATGAGTATAA ATAGAAAGAT AGCAAGTTTC
2101    TCGTGAGAGT TCACAAGCCA ATAAAGCTG
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9.3 Promoter sequence DQ322688 (*AgpL1*, M82): 2064 bp

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1       CGGAGTAAGT GACATACATA TAAAGATGGG TGGAATCTTC CAAATAGTGA AAGAAGACAA
61      GCACATCTTT ACATTAAACC TTCAATTATC TAACTAATCT CTAACATGCA CTGTCTCATT
121     GTCTCCTTGC TCATTAATTC ATATTTTAAC ATCTTTATTT ATTTTTCAAG GTCTTGAGCT
181     AATTAATTCA ACAAAACACC CAACATATCA TCTCCTTTTT GCTTTACTAG AATTTGAGCC
241     GTTAAAACGG TTTTAGCTCA TGAATCAGCC CAATTCAATT CATTATGGGG ATAGGTTTGG
301     GACATAAGTT CTTAAGCTCT TTTAAAATTA GGACTTTATA AGTCTGATTC ATATAAGTGT
361     ATGATCCTTG AGACTTTATT AAGGTCGGGT CGAGGTCGGT CCATGGCCTT AATTTTTGTT
421     TATTTAAGTG TATCTTATAA TAATTTTACT AGTATATAAT TTAATCATTT AAATACACTA
481     TATGTTGTAA AAATGATGAA TCTTACCAGA CTTTGTTAGT CCAAATACAT GTATCTCGAA
541     ATATATAAAC TGAAAATTAG TTTAATTTGT TTTAGACACA CTCTATCCAA ATCTGGCACT
601     CTTCCTACCC TTTCATCTTG CTTGTCACTG TCTTATATTG CTCACGTATC TAGTATGCGA
661     GATACATACT GATTACACTA GTTTTTTTTT TTGTTAGTGT TATTGAAACA ACAATGTTAG
721     TCATGTTTTG ATTTGAAAAT CTTCTCAGTT TCTTGAATTA GCAGTTTGAA AAGAAAAAAT
781     AAGCTAGCCC CTCCCTTTCC CTTTATGATT GGGTTGGATT GAATATTTTC AAGATCAACC
841     CATCCTAACC CTTTAAATTT CTTAGCCTAC AAAGCTTGGA CCAGATAAAG TTGAACCAAT
901     TTTTTTAATC GCTTCAACTA GAATAAAATA ATAGTATCTG AGTCAATTTT AAATATCATT
961     CAGCGTAATT TATAAAACTT TAAATGAATC GTACAAGCTC TTTCCCTGGT AAAAATGTCA
1021    AATTGGGAAA CAACATCAAT GCCAGAATAC TTTCGGATAG ATAATGTCTA AATTATTTGA
1081    AGATTTTGAT ATGAAATTGT CAGCTAATCT CCAACTAAAT AATGTCTGAC CCCACCAAAT
1141    TTTTTAACGG ACAAAGATCT CTCTAAGAAG TATAGTAGTA TATAAAATGA CATTCATGAG
1201    TGTGGAAATT GTATATAGTT TTATTTAACT ATTTTCTTGC AAATCATATG GTTCATAGAA
1261    TAATATTAAC AATAAAAAGA CAGGTGTTTG GCCTGTAATG GGTCGGTCTC TATTGTCCAG
1321    ATCTTGGTGG ACCCTACACA CTATGACGTC TGTCAATTAA TCTTGGAAAA ATAACTGTTA
1381    GCACGACTTT TCCAGTCTAA TTTTCAGTGA TTTTATTTAA TAATGACTAA GTTTTATCGC
1441    GAATATAAAA AAAAAATTCA AATATTCATC TAAAATTTTA GGCATAATAT ATAAATATAT
1501    TCTTTAAATT GACTTTCAAA TCACAATTAT GACTTTCAAC TTTGGGTGTG CACAAGTAGA
1561    CACTTAAACT TGTAAAATAG ACACACATAT CCTATATGTC ATCCTACATA TCATTTTTTG
1621    TCCTACGTGG TGTCCTACAT GTATTTTGTC ATGTAGGACT CGTATGTTTA TTTATTTAAA
1681    AGTTGAATAA TTAAAGTGTC TGTTTGTTCA TTATGAAAGT TGAAACTCAA AGTTAAAATT
1741    TGAAATCAAA TTTAGGTTCA ATATATGCAT TATGTCAAAA TTGTATATTA TTTAATTATT
1801    TTAAATGATA AAAATAATCT AATGAAGATT TTATCAAAGT AAAAGTGATT AAAAGGAATA
1861    AATGTGAAGA TTTTCAAATG TTTTATATTT TGAGGGATCT AAATAAATTT AAAGAGTTGT
1921    TAATATACTT GGGGAAACAT AAAATATTAT TTAAGTTATG GGGTGTATAA GTTAAAAACT
1981    TTTTATGTAA ATAGGGATAT GGAAATGAGT ATAAATAGAA AGATAGCAAG GTTTCTCGTG
2041    AGAGTTCACA AGCCAATAAA GCTG
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9.4 Promoter sequence DQ322686 (*AgpL1*^{H}, NIL): 2051 bp

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1      CGGAGTAAGT GACATACATA TAAAGATGGG TGGAATCTTC CAAATAGTGA GAAGACAAGC
61     ACATTTTTAC ATTAAACCTT CAATTATCTA ACTAATCTCT AACATGCACT GTCTCATTGT
121    CTCCTTCACA ATCCAATCCT CATTAATTCA TCTTTTAACA TCTTTATTTA TTTTTCAAGG
181    TCTTTACCTA ATTAATTCAA CAAAACACCC AACATATCAT CTCCTTTTTG CTTTACTAGA
241    ATTTTGAGCC GTCAAAACAG TTTTAACTCA TGAATCAGCC CAATTCAATT CAATTCATTA
301    TTGAGATAAG TTTGGGATAT AGATTCTTAA GCTCTTTTAA AATTAGGGGT TTATAAGTCT
361    GATTTATATA AGCTTATGAT CCTTGACACT TGATCAAGGC CGTGTCGAGG TCGGCCCATG
421    ACCCTAATTA TTGTTTATTT AAGTGTAACT TACAATAATT TTACTAGAAT ATAATCTAAT
481    CATTTAAATA CACTATATGT TGTAATATGA TGAATCTTAC CAAACTTTGG TAGTCCAAAT
541    ACATGTATCT CGAAATGTAT AGACTCAAAA TTAGTTTAAT TTGTTTTAGA CACACTCTAT
601    CCAAGTAGAT TCTCATGAAT CTATGATACA CAGACAAATC TCGTGCTCTC CCTGCCCTCT
661    CATCTTGCTT GTCACTATCT TATATTGCTC ACATATCTAG TATGCAAGAT ACATGTTGAT
721    TACACTAGTT TTTTTTTTTG CTAGTGTTAT TGAAACAACA ACATTAGTCA TATTTTGATT
781    TGAAAATCTT CTCAATTTCT TGATATTATA AAATTAGCAG TTCGAAAAGA AGAAAAAAAA
841    GCTAGCCCCT TCCTCTCCCT CTATGATTGG GTTGGATTCA ACATTTTCAA GATCAACCCA
901    TTCTAGCCCT TTAAATTTCT TAGCCCACAA AGCTTGGATC AGATGAAGCT AAATCAATCT
961    TTTTAACCGC TTTAACTAGA ATAAAATAAT AGTATTTGAG TCAATTTTAA GTAGCGTAAT
1021   TAATAAAAGT TTAAATGAAT CATAAATTAT TTGAATATTT TGGTATGAAA TTGTCAGCTA
1081   ATCTCCAACT AAATAATGTC TGACCCCACC AAATTTTTTA ACGGACAAAG ATCTCTCTAA
1141   GACTATAGTT ATAGTAGTAT ATAAAATGAC ATTCATGAGT GTGGAAATTG TATATAGTTT
1201   TATTTAACTA TTTTCTTGCA AATCATATGG TTCATAGAAT AATATTAACA ATAAAAAGAC
1261   AGGTGTTTGG CCTGTAATGG GTCTCTATTG TCCAGATCTT GGTGGACCCT ACACACTATG
1321   ACGTCTGTCA ATTAATCTTG GAAAAATAAC TGTTAGCACG ACTTTTCCAG TCTAATTTTC
1381   AGTGATTTTA TTTAATAATG ACTAAGTTTT ATCGCGAATA TGAATTTTTT TTTATATATT
1441   CATCTAAAAT TTTAGGCATA ACAGATAAAT ATATTCTTTT TAACTTGACT TTAAATTATA
1501   ATTATGCTCT TCAACTTTGA GTGTACACAA GTAGACACTT AAATTTATAT AAAATTGAAC
1561   AAATAGACAC ACATGTCCTA TATGTCATCC TACATGTCAT TTTTTGTCCT ACGTGGTGTC
1621   CTACATGTAT TTTGCCATGT AGGATTCATA TGTTTATTTA TTTAAAAGTT GGATAGTTAA
1681   AGTGTCTGTT TGTTCATTAT GAAAGTTGAA GGTCAAAGTT AAAATTTAAA GTCAAGTTTA
1741   GAGTTCAATA TATATATTAT GTCAAAATTT TATATTATTT AATTATTTTA AACGATAAAA
1801   AAAATCTATT GAAAATTTTA TCAAAGTAAA AGTGAATAAA AGGAATAAAT GCGAAGATTT
1861   TCAAATGTTT TATAATTTGA GGATCTAAAT AAATTTAAAG AGTTGTTAAT ATATTTGGGG
1921   ATAATAAAAT ATTATTTAAG TTATGGGGTG TATAAGTTAA AAAACTTTTT ATGTAAATAG
1981   GGATATGGAA ATGAGTATAA ATAGAAAGAT TAGCAAGGTT TCTCGTGAGA GTTCACAAGC
2041   CAATAAAGCT G
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9.5 Promoter sequence DQ322685 (*AgpL1*, *S.habrochaites S*. LA1777): 2054 bp

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1       CGGAGTAAGT GACATACATA TAAAGATGGG TGGAATCTTC CAAATAGTGA GAAGACAAGC
61      ACATTTTTAC ATTAAACCTT CAATTATCTA ACTAATCTCT AACATGCACT GTCTCATTGT
121     CTCCTTCACA ATCCAATCCT CATTAATTCA TCTTTTAACA TCTTTATTTA TTTTTCAAGG
181     TCTTTAGCTA ATTAATTCAA CAAAACACCC AACATATCAT CTCCTTTTTG CTTTACTAGA
241     ATTTGAGCCG TCAAAACAGT TTTAGCTCAT GAATCAGTCC AATTCAATTA AATTCATTAT
301     TGAGATAGGT TTGGGATATA AATTCTTAAG CTCTTTTAAA ACTAGGGGTT TATAAGTTTG
361     ATCTATGTAA GCTCATGATC CTTGAGACTT GATCAAGGCC GTGTCGAGGT CGGCCCATGA
421     CCCTAATTAT TGTTTATTTA AGTGTAACTT ATAATAATTT TACTAGAATA TAATCTAATC
481     ATTTAAATAC ACTATATGTT GTAATATGAT GAATCTTACC AGACTTTGGT AGTCCAAATA
541     CATGTATCTC GAAATGTATA GACTCAAAAT TAGTTTAATT TGTTTTAGAC ACACTCTATC
601     CAAGTAGATT CTCATGAATC TATGATACAC AGACAAATCT CGTGCTCTCC CTGCCCTCTC
661     ATCTTGCTTG TCACTATCTT ATATTGCTCA CATATCTAGT ATGCAAGATA CATGTTGATT
721     ACACTAGGTT TTTTTTTTGC TAGTGTTATT GAAACAACAA CATTAGTCAT ATTTTGATTT
781     GAAAATCTTC TCAATTTCTT GATATTATAA AATTAGCAGT TCGAAAAGAA AAAAAAAAGC
841     TAGCCCCTTC CTCTCCCTCT ATGATTGGGT TGGATTCAAC ATTTTCAAGA TCAACCCATT
901     CTAGCCCTTT AAATTTCTTA GCCCACAAAG CTTGGATCAG ATGAAGCTAA ATCAATCTTT
961     TTAACCGCTT TAACTAGAAT AAAATAATAG TATTTGAGTC AATTTTAAGT ATTATTCCGC
1021    GTAATTTATA AAACTTTAAA TGAATCATAA ATTATTTGAA TATTTTGGTA TGAAATTGTC
1081    AGCTAATCTC CAACTAAATA ATGTCTGACC CCACCAAATT TTTTAACGGA CAAAGATCTC
1141    TCTAAGACTA TAGTTATAGT AGTATATAAA ATGACATTCA TGAGTGTGGA AATTGTATAT
1201    AGTTTTATTT AACTATTTTC TTGCAAATCA TATGGTTCAT AGAATAATAT TAACAATAAA
1261    AAGACAGGTG TTTGGCCTGT AATGGGTCTC TATTGTCCAG GATCTTGGTG GACCCTACAC
1321    ACTATGACGT CTGTCAATTA ATCTTGGAAA AATAACTGTT AGCACGACTT TTCCAGTCTA
1381    ATTTTCAGTG ATTTTATTTA ATAATGACTA AGTTTTATCG CGAATATGAA ATTTTTTTTA
1441    TATATTCATC TAAAATTTTA GGCATAACAG ATAAATATAT TTTCTTTAAC TTGGATTCAA
1501    ATTATAATTA TGCCCTTCAA CTTTGAGTGT GCACAAGTAG ACACTTAAAT TTATATAAAA
1561    TTGAACAAAT AGACACACAT GTCCTATATG TCATCCTACA TGTCATTTTT TGTCCTACGT
1621    GGTGTCCTAC ATGTATTTTG CCATGTAGGA ATCATATGTT TATTTATTTA AAAGTTGAAT
1681    AGTTAAAGTG TCTGTTTGTT CATTATGAAA GTTGAAGGTC AAAGTTAAAA TTTAAAGTCA
1741    AGTTTAGAGT TCAATATATG TATTATGTCA AAATTTTATA TTATTTAATT ATTTTAAACA
1801    ATCAAAAAAA TCTAATGAAG ATTTTATCAA AGTAAAAGTG AATAAAAGGA ATAAATATGA
1861    AGATTTTCAA ATGTTTTATA ATTTGAGGAT CTTAAATTTA AAGAGTTGTT AATATATTTG
1921    GGGATAATAA AATATTATTT AAGTTATGGG GTGTATAAGT TAAAAAACTT TTTATGTAAA
1981    TAGGGATATG GAAATGAGTA TAAATAGAAA GATTAGCAAG GTTTCTCGTG AGAGTTCACA
2041    AGCCAATAAA GCTG
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9.6 Full genome sequence DQ322683 (*AgpL1^{E}*, NIL) : 4500 bp

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1       ATCACACTCC CCTTTGTCCA CTTGATACCT CAATAACACA ACTTCTTGTG ATTCACTTAC
61      AATTCTAGAT CTCCTTAAAA ACTTTTTCTT GATTCTACAC CACTAGCAAT TTACATTTTC
121     TCTTTCACTG ATTTTGGTTA CTTATTTGAC ATTCTTGTTT TCAAGATCCA AAAATCATCA
181     CTTTCCAGGT TCAAAATCTT GTTTTTTTTT TTAATTTTGT CTTTTAATTG CTGTATATTG
241     GGGAACTCCA CAGGTGAATT TTTATGATAT GAGTTCACCC TTTAGCTTTC TTTGTAATAT
301     GAGTGAATTT TTATACATAT ATTTTCTTTG TATCAATTTT AAAAAATATA TGTATAAAAT
361     ACCTAATTTT ATGTAAAGAT TCATGTTTTT GGGGTAATGC TATGAGGTAT TAGTACTGAG
421     CATATAGCTA GCTTGGGTTT TGGGTTTACC GACCTTTTTT TTTTTTTTTA ATTAGTGATA
481     TTTTCTTTTA TGTATTTTAT ACTTTTCTTG GTTGCTTAGA AATTACACAT ACTTTATTGA
541     GATTTGAATA AATCTATTTG ATTTGGATCC GTTGATATGG GATTGCTGAT TTGTTGATTG
601     GCTGCAGAAA TGGATACTTG TTGTGCGGCT ATGAAATCGA CGGTTCATTT GGGGAGAGTG
661     AGCACTGGTG GCTTTAACAA TGGAGAGAAG GAGATTTTTG GGGAGAAGAT CAGAGGGAGT
721     TTGAACAACA ATCTCAGGAT TAATCAGTTG TCGAAAAGTT TGAAACTTGA GAAGAAGATT
781     AAACCTGGGG TTGCTTACTC TGTGATCACT ACTGAAAATG ACACAGAGAC TGTGGTAATA
841     ATCAATTCTT TTCTCTTTAT TAGGACATGT CTGCTTCAAA ATAATGCCAT AATAGTGTTG
901     TAGATATGGT GGAAACAATC TCATATGAAC TATATTGTGA ATGGAAGTGA ATCCAATAAT
961     TAAGATTGTT AGCATTGAAC TCACCACACT TAAAAGATTA TGGGTTGTGA GTTTAATTTT
1021    GTTGAAGTTC TATTACTAGT CTTCTGCTAT ATATTTGTTT CGTGTTGAGA AGATATTGTG
1081    TTCGGTTAAG CCTACAATAT TTATCTGCAT CAGTCTGTGA TTGTGATTGT TTGTTGGACT
1141    TATGTAGTTC GTAGATATGC CACGTCTTGA GAGACGCCGG GCAAATCCCA AGGATGTGGC
1201    TGCAGTCATA TTAGGAGGAG GCGAAGGGAC CAAGTTATTC CCACTTACAA GTAGAACTGC
1261    AACCCCTGCT GTAAGAGATT TATCTTTGCT CATGAAAGTT TTATCAATTT TTTGTTTTAT
1321    ATGCTTAATG TCCAGTTAAA ACTCTCTTCC AAGTTAATTT TGTTTATCTC GTGTTAAATT
1381    TCAGATTTTT TTTTTTAATG ATATGTTATT TTTTGATTTC TACATCGATT AAATATAGAT
1441    AGAATAGGTG TACAAGATTG CAAGTTGATG TCTACTCCGT GGTCAATTTC TTAAGATTCT
1501    GATGTTTTGT GCAACATGGC ACCAGGTTCC GGTTGGAGGA TGCTACAGGC TGATAGACAT
1561    CCCAATGAGC AACTGTATCA ACAGTGCTAT TAACAAGATT TTTGTGCTGA CACAGTACAA
1621    TTCTGCTGCC CTGAATCGTC ACATTGCTCG AACGTATTTT GGCAATGGTG TGAGCTTTGG
1681    AGATGGATTT GTCGAGGTTC GAGACTATAC TTCTGGATTA GTCTTGCTAG ATATGACAGC
1741    TGAGCTAATT AATATGAGTA AATTTGCTGG TCCTCCAAGT GATTTGTTCA CAAAAAATCT
1801    GTAACAATTT CTGATGATTA TTTTCTTTTT TTTTCTGCTT TCTTGCTTCG TTAACTGAAA
1861    GGAAATCCGT CATTAAACAG GTACTAGCTG CAACTCAGAC ACCTGGGGAA GCAGGAAAAA
1921    AATGGTTTCA AGGAACAGCA GATGCTGTCA GAAAATTTAT ATGGGTTTTT GAGGTTTGTC
1981    TCTACAAATC TTTAGGACAC TAATCCACCA CTGCTCTCTT CTGTCGACCG GATATGATTT
2041    CTGAAAAATG TCTCATTATT TTTTCTTGAA TCAGTTGTTA TGTACAGACA TATACTACTT
2101    TTGTGTTTAC AAGGCAACCA ATTCTTGCAG GACGCTAAGA ACAAGAATAT TGAAAATATC
2161    CTTGTATTAT CTGGGGATCA TCTTTATAGG ATGGATTATA TGGAGTTGGT GCAGGTATGT
2221    CAACTTTAAC TACCTACACA CTCCTCATTC TTTGTTTGCC TTTTTTTTTT CTTTTAAAAA
2281    ACAAAGTTTC TGACTGCCTC CGATCTTCAA ACAGAACCAT ATTGACAGAA ATGCTGATAT
2341    TACTCTTTCA TGTGCACCAG CTGAGGACAG GTTTGTTTAA ATTCTTATAA GGAACAACTA
2401    GTATAGGTTA AGTGAATTGA GTGAGATCAG AAACAAGATG GAGAAAGAAT GCTTCTCCTC
2461    TAATACTCAT ATGCATACGC TCGCTATACA TTACTTCGAA GTAAAAACAT CAACACATTT
2521    TTTAATACCT TATTGTGATT ATGTTAAGCT GAGATTTAAG CTTTACTTAT CAGCCGAGCA
2581    TCAGATTTTG GGCTGGTCAA GATTGACAGC AGAGGCAGAG TTGTCCAGTT TGCTGAAAAA
2641    CCAAAAGGTT TTGAGCTTAA AGCAATGGTA GGTTTAGTTG AAATCTTTCT TTTAGTATAT
2701    GTGCTGATTT CTTTCTAAAC AAGTTTTTGT GGTTTATATT CAGACTAGTG TTCCTTTAGA
2761    ATTTAACTCA TTTATTTCTT GTGCTGCAGC AAGTAGATAC TACTCTTGTT GGATTATCTC
2821    CACAAGATGC GAAGAAATCC CCTTATATTG CTTCAATGGG AGTTTATGTA TTCAAGACAG
2881    ATGTATTGTT GAAGCTCTTG AAATGGAGCT ATCCCACTTC TAATGATTTT GGCTCTGAAA
2941    TTATACCAGC AGCTATTGAT GATTACAATG TTCAAGTAAA GGATCTTGGA TCTTTCGATT
3001    GAAATTATCT TTATGAATTG AGTACATTAG TTGCTCAAAC GAAAAATTAG TTGTAGTTTT
3061    AACGAATTTA TGCTATCTTC TCAGGCATAC ATTTTCAAAG ACTACTGGGA GGACATTGGA
3121    ACAATTAAAT CTTTCTATAA TGCTAGCTTG GCGCTCACAC AAGAGGCATG TTGCAAATTC
3181    ATCTTTATAC TTCTAACGAC TAGCTCAGAA ATTATTTTAC ATCAGTGCAT AATCCAACTG
3241    ATTAATTTCC ATTGCAGTTT CCAGAGTTCC AATTTTACGA TCCAAAAACA CCTTTTTACA
3301    CATCTCCTAG GTTCCTTCCA CCAACCAAGA TAGACAATTG CAAGGTAAGT GGGTAAATAC
3361    TGCAAGTGTT TTGCATTAAT TGGTCTTTTG TGGGTTCTAA TAAGTTTTGT TTTCTGTGCT
3421    TTTTCAGATT AAGGATGCCA TAATTTCTCA TGGATGTTTT TTGCGAGATT GCACTGTGGA
3481    ACACTCCATA GTGGGTGAAA GATCGCGCTT AGACTGTGGT GTTGAACTGA AGGTTTGCGT
3541    GTAGCCCTAA TTTTTATCTT CCTTTAAAAC TATTCCTGAA CAAACACATT TATTTTCTTT
3601    AGTTTGTGAA AAATGCAGCA GCTTATTGTT TCTAGTACAT TTTGTTGTAA AGTGTAGATC
3661    TCTAACACAT TTTACAACCA CAGGATACTT TCATGATGGG AGCAGACTAC TACCAAACAG
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3721    AATCTGAGAT TGCCTCCCTG TTAGCAGAGG GGAAAGTACC GATTGGAATT GGGGAAAATA
3781    CAAAAATAAG GTAAATGAAC ATTGAATATT ATCTTTTTTT GACAAATGTA TTTCATTTTC
3841    ATACTTGAGA ATTCTTTAAT TTGATTATGG TTTCACTCAT ACAGGAAATG TATCATTGAC
3901    AAGAACGCAA AGATAGGAAA GAATGTTTCA ATCATTAACA AAGATGTAAG TTTTTTTTTT
3961    GCATCCTCCG ACTTTGTTTA GTGTGTCTTA TTTTCATAGT ACTTATAGGG CGGAGGTTAG
4021    TTTCCCAACA ACTATTGTTC ACACAGTCAA GTTCAAAACA CAAATTGCTG GTGTGCTTCT
4081    TGCTCGCGAG AAAGAAAGAA AGCAGAAGAG TGTTCAATTC AGATTTGATT GTTTGTAGCA
4141    TGGGAGCACT TATATTTTTG AGACATAATT GATTGAATGA GACTCCCTGA TGTTCTTTTC
4201    TTTGAAATAT ATTTAGGGTG TTCAAGAGGC AGACCGACCA GAGGAAGGAT TCTACATACG
4261    ATCAGGGATA ATCATTATAT CAGAGAAAGC CACAATTAGA GATGGAACAG TTATATGAAC
4321    TAGAAAAGCA TCTCTTGTTG AACTAGTGGA AATCCAAATC TCAACTTGAA GAAGGTCAAG
4381    GGTGATGACC TATTTTCCCA CGAAAACCTA GCACATTCAG CTCCGGAAGA AGCTTTCTAT
4441    GCTCATGTGA AAAGAGCAAG TATAATCGAG ACACGTGTCT AAAATAAACT AAAGTTGCTG
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Exon 1          1 – 188
Intron 1      189 – 607
Exon 2        608 – 834  start 610
Intron 2      835 – 1147
Exon 3       1148 – 1270
Intron 3     1271 – 1525
Exon 4       1526 – 1696
Intron 4     1697 – 1880
Exon 5       1881 – 1973
Intron 5     1974 – 2130
Exon 6       2131 – 2214
Intron 6     2215 – 2314
Exon 7       2315 – 2370
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Exon 8       2574 – 2667
Intron 8     2668 – 2789
Exon 9       2790 – 2975
Intron 9     2976 – 3084
Exon 10       3085 - 3165
Intron 10     3166- -3257
Exon 11      3258- -3344
Intron 11    3345 - 3427
Exon 12      3428 - 3532
Intron 12    3533 - 3683
Exon 13      3684 - 3790
Intron 13    3791 - 3884
Exon 14      3885 - 3945
Intron 14    3946 – 4216
Exon 15      4217 – 4500  stop 4316
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9.7 Full genome sequence DQ322682 (*AgpL1*, M82): 4482 bp

1 ATCACACTCC CCTTTGTCCA CTTGATACCT CAATAACACA ACTTCTTGTG ATTCACTTAC
61 AATTCTAGAT CTCCTTAAAA ACTTTTTCTT GATTCTACAC CACTAGCAAT TTACATTTTC
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181 CTTTCCAGGT TCAAAATCTT GTTTTTTTTT TTAATTTTGT CTTTTAATTG CTGTATATTG
241 GGGAACTCCA CAGGTGAATT TTTATGATAT GAGTTCACCC TTTAGCTTTC TTTGTAATAT
301 GAGTGAATTT TTATACATAT ATTTTCTTTG TATCAATTTT AAAAAATATA TGTATAAAAT
361 ACCTAATTTT ATGTAAAGAT TCATGTTTTT GGGGTAATGC TATGAGGTAT TAGTACTGAG
421 CATATAGCTA GCTTGGGTTT TGGGTTTACC GACCTTTTTT TTTTTTTTTA ATTAGTGATA
481 TTTTCTTTTA TGTATTTTAT ACTTTTCTTG GTTGCTTAGA AATTACACAT ACTTTATTGA
541 GATTTGAATA AATCTATTTG ATTTGGATCC GTTGATATGG GATTGCTGAT TTGTTGATTG
601 GCTGCAGAAA TGGATACTTG TTGTGCGGCT ATGAAATCGA CGGTTCATTT GGGGAGAGTG
661 AGCACTGGTG GCTTTAACAA TGGAGAGAAG GAGATTTTTG GGGAGAAGAT CAGAGGGAGT
721 TTGAACAACA ATCTCAGGAT TAATCAGTTG TCGAAAAGTT TGAAACTTGA GAAGAAGATT
781 AAACCTGGGG TTGCTTACTC TGTGATCACT ACTGAAAATG ACACAGAGAC TGTGGTAATA
841 ATCAATTCTT TTCTCTTTAT TAGGACATGT CTGCTTCAAA ATAATGCCAT AATAGTGTTG
901 TAGATATGGT GGAAACAATC TCATATGAAC TATATTGTGA ATGGAAGTGA ATCCAATAAT
961 TAAGATTGTT AGCATTGAAC TCACCACACT TAAAAGATTA TGGGTTGTGA GTTTAATTTT
1021 GTTGAAGTTC TATTACTAGT CTTCTGCTAT ATATTTGTTT CGTGTTGAGA AGATATTGTG
1081 TTCGGTTAAG CCTACAATAT TTATCTGCAT CAGTCTGTGA TTGTGATTGT TTGTTGGACT
1141 TATGTAGTTC GTAGATATGC CACGTCTTGA GAGACGCCGG GCAAATCCCA AGGATGTGGC
1201 TGCAGTCATA TTAGGAGGAG GCGAAGGGAC CAAGTTATTC CCACTTACAA GTAGAACTGC
1261 AACCCCTGCT GTAAGAGATT TATCTTTGCT CATGAAAGTT TTATCAATTT TTTGTTTTAT
1321 ATGCTTAATG TCCAGTTAAA ACTCTCTTCC AAGTTAATTT TGTTTATCTC GTGTTAAATT
1381 TCAGATTTTT TTTTTTAATG ATATGTTATT TTTTGATTTC TACATCGATT AAATATAGAT
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1621 TCACATTGCT CGAACGTATT TTGGCAATGG TGTGAGCTTT GGAGATGGAT TTGTCGAGGT
1681 TCGAGACTAT ACTTCTGGAT TAGTCTTGCT AGATATGACA GCTGAGCTAA TTAATATGAG
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1861 AGGTACTAGC TGCAACTCAG ACACCTGGGG AAGCAGGAAA AAAATGGTTT CAAGGAACAG
1921 CAGATGCTGT CAGAAAATTT ATATGGGTTT TTGAGGTTTG TCTCTACAAA TCTTTAGGAC
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2041 TTTTTTCTTG AATCAGTTGT TATGTACAGA CATATACTAC TTTTGTGTTT ACAAGGCAAC
2101 CAATTCTTGC AGGACGCTAA GAACAAGAAT ATTGAAAATA TCCTTGTATT ATCTGGGGAT
2161 CATCTTTATA GGATGGATTA TATGGAGTTG GTGCAGGTAT GTCAACTTTA ACTACCTACA
2221 CACTCCTCAT TCTTTGTTTG CCTTTTTTTT TTCTTTTAAA AAACAAAGTT TCTGACTGCC
2281 TCCGATCTTC AAACAGAACC ATATTGACAG AAATGCTGAT ATTACTCTTT CATGTGCACC
2341 AGCTGAGGAC AGGTTTGTTT AAATTCTTAT AAGGAACAAC TAGTATAGGT TAAGTGAATT
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2581 AAGATTGACA GCAGAGGCAG AGTTGTCCAG TTTGCTGAAA AACCAAAAGG TTTTGAGCTT
2641 AAAGCAATGG TAGGTTTAGT TGAAATCTTT CTTTTAGTAT ATGTGCTGAT TTCTTTCTAA
2701 ACAAGTTTTT GTGGTTTATA TTCAGACTAG TGTTCCTTTA GAATTTAACT CATTTATTTC
2761 TTGTGCTGCA GCAAGTAGAT ACTACTCTTG TTGGATTATC TCCACAAGAT GCGAAGAAAT
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2941 ATGATTACAA TGTTCAAGTA AAGGATCTTG GATCTTTCGA TTGAAATTAT CTTTATGAAT
3001 TGAGTACATT AGTTGCTCAA ACGAAAAATT AGTTGTAGTT TTAACGAATT TATGCTATCT
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3121 AATGCTAGCT TGGCGCTCAC ACAAGAGGCA TGTTGCAAAT TCATCTTTAT ACTTCTAACG
3181 ACTAGCTCAG AAATTATTTT ACATCAGTGC ATAATCCAAC TGATTAATTT CCATTGCAGT
3241 TTCCAGAGTT CCAATTTTAC GATCCAAAAA CACCTTTTTA CACATCTCCT AGGTTCCTTC
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3481 AAGATCGCGC TTAGACTGTG GTGTTGAACT GAAGGTTTGC GTGTAGCCCT AATTTTTATC
3541 TTCCTTTAAA ACTATTCCTG AACAAACACA TTTATTTTCT TTAGTTTGTG AAAAATGCAG
3601 CAGCTTATTG TTTCTAGTAC ATTTTGTTGT AAAGTGTAGA TCTCTAACAC ATTTTACAAC
3661 CACAGGATAC TTTCATGATG GGAGCAGACT ACTACCAAAC AGAATCTGAG ATTGCCTCCC

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3841   ATTTGATTAT GGTTTCACTC ATACAGGAAA TGTATCATTG ACAAGAACGC AAAGATAGGA
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3961   TAGTGTGTCT TATTTTCATA GTACTTATAG GGCGGAGGTT AGTTTCCCAA CAACTATTGT
4021   TCACACAGTC AAGTTCAAAA CACAAATTGC TGGTGTGCTT CTTGCTCGCG AGAAAGAAAG
4081   AAAGCAGAAG AGTGTTCAAT TCAGATTTGA TTGTTTGTAG CATGGGAGCA CTTATATTTT
4141   TGAGACATAA TTGATTGAAT GAGACTCCCT GATGTTCTTT TCTTTGAAAT ATATTTAGGG
4201   TGTTCAAGAG GCAGACCGAC CAGAGGAAGG ATTCTACATA CGATCAGGGA TAATCATTAT
4261   ATCAGAGAAA GCCACAATTA GAGATGGAAC AGTTATATGA ACTAGAAAAG CATCTCTTGT
4321   TGAACTAGTG GAAATCCAAA TCTCAACTTG AAGAAGGTCA AGGGTGATGA CCTATTTTCC
4381   CACGAAAACC TAGCACATTC AGCTCCGGAA GAAGCTTTCT ATGCTCATGT GAAAAGAGCA
4441   AGTATAATCG AGACACGTGT CTAAAATAAA CTAAAGTTGC TG
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Exon 1         1 - 188
Intron 1     189 - 607
Exon 2       608 - 834   Start 610
Intron 2     835 - 1147
Exon 3       1148 - 1270
Intron 3     1271 - 1507
Exon 4       1508 - 1678
Intron 4     1679 - 1862
Exon 5       1863 - 1955
Intron 5     1956 - 2112
Exon 6       2113 - 2196
Intron 6     2197 - 2296
Exon 7       2297 - 2352
Intron 7     2353 - 2555
Exon 8       2556 - 2649
Intron 8     2650 - 2771
Exon 9       2772 - 2957
Intron 9     2958 - 3066
Exon 10      3067 - 3147
Intron 10    3148 - 3239
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Intron 11    3327 - 3409
Exon 12      3410 - 3514
Intron 12    3515 - 3665
Exon 13      3666 - 3772
Intron 13    3773 - 3866
Exon 14      3867 - 3927
Intron 14    3928 - 4198
Exon 15      4199 - 4482  Stop 4298
```

9.8 Full genome sequence DQ322684 (*AgpL1*, *S.habrochaites S*. LA1777): 4497 bp

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1       ATCACACTCC CCTTTGTCCA CTTGATACCT CAATAACACT ACTTCTTGTG ATTCACTAAC
61      AATTCTAGAT CTCCTTAAAA AAATTTCTTG ATTCTACACC ACTAGCAATT TACATTTTTT
121     CTTTCACTGA TTTTGGTTAC TTATTAAACA TTCTTGTTTT CAAGATCCAA ACATCATCAC
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241     GGAACTCCAC AGGTGAATTT TTATGATATG GGTTCACCCT TTAGCTTTCT TTGTAATATG
301     AGTGAATTTT TATACATATA TTTTCTTTGT ATCAATTTTA GAAAATATAT GTATAAAATA
361     CCTAATTTTA TGTAGAGATT CATGTTTTTG TGGTAATGCT ATGAGGTATA AGTACTGAGC
421     ATATAGCTAG CTTGGGTTTT GGGTTTACCG ACCATTTTTT TTTAATTAGT GATATTTTCT
481     TTATGTATTT TATATTTTCT TGGTTGCTTA GAAATTACAC ATACTTTATT GAGATTTGAA
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601     AATGGATACT TGTTGTGCGG CTATGAAATC GACGGTTCAT TTGGGGAGAG TGAGCACTGG
661     TAGCTTTAAC AATGGAGAGA AAGAGATTTT TGGGGAGAAG ATGAGAGGGA GTTTGAACAA
721     CAATCTCAGG ATTAATCAGT TGTCGAAAAG TTTGAAACTT GAGAAGAAGG AGAAGAAGAT
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841     AATCAATTCT TTTCTCTTTA TTAGGACATG TATGCTTCAA ATAATGCCAT AATAGTGTTG
901     TAGATATGGT GGAAACAATC TCATATGAAC TATATTGTGA ATGGAAGCAA ATCCAATATT
961     TAAGATTGTT AGCATTGAAC TCACCATACT TATAAGATTA TGGGTTGTGA GTTTAATTTT
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1141    TATGTAGTTC GTAGATATGC CACGTCTTGA GAGACGCCGG GCAAATCCCA AGGATGTGGC
1201    TGCAGTCATA TTAGGAGGAG GCGAAGGGAC CAAGTTATTC CCACTTACAA GTAGAACTGC
1261    AACCCCTGCT GTAAGAGATT TATCTTTGCT CATGAAAGTT TTGTCAATTT TTGTTTTTAG
1321    ATGCTTAATG TCCAGTTAAA ACTATCTTCC AAGTTACTTT TGTTTATCTC GTGTTAAATT
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1501    GTTTTGTGCA ACATGGCACC AGGTTCCGGT TGGAGGATGC TACAGGCTCA TAGACATCCC
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1621    TGCTGCCCTG AATCGTCACA TTGCTCGAAC GTATTTTGGC AATGGTGTGA GCTTTGGAGA
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1741    GCTAATTAAT ATTAGTAAAT TTGCTGGTCG TCCAAGTGAT TTGTTCACAA AAAATCTATA
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1861    AAGGAATTCC GTCATTAAAC AGGTACTAGC TGCAACTCAG ACACCTGGGG AAGCAGGAAA
1921    AAAATGGTTT CAAGGAACAG CAGATGCTGT CAGAAAATTT ATATGGGTTT TTGAGGTTTG
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2101    TTTTGTGTTT ACAACGCAAC CAATTCTTGC AGGACGCTAA GAACAAGAAT ATTGAAAATA
2161    TCCTTGTATT ATCTGGGGAT CATCTTTATA GGATGGATTA TATGGAGTTG GTGCAGGTAT
2221    GTCAACTTTA ATTACCTACA TACTCCTCAT TGTTTGTTTG CCTTTTTTTT TCTTTTAAAA
2281    AACAAAGTTC CTGACTGCCT CCGATCTTCA AACAGAACCA TATTGACAGA AATGCTGATA
2341    TTACTCTTTC ATGTGCACCA GCTGAGGACA GGTTTGTTTA AATTCCAATA AGAAACAACT
2401    AGTATTGGTT AAGTGAATTG AGTGAGATCA GAAACAAGAT GGAGAAAGAA TGCTTCTCCT
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2521    AATATCTTAT TGTGATTATG TTAAGCTGAG ATTTAAGCTT TACTTATCAG CCGAGCATCA
2581    GATTTTGGGC TGGTCAAGAT TGACAACAGA GGCAGAGTTG TCCAGTTTGC TGAAAAACCA
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2701    CTGATTTCTT TCTAATCAAA TTTTTGTGGT TTATATTCAG ACTAGTGTTC CTTTAGAATT
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3121    AATTAAATCT TTCTATAATG CTAGCTTGGC GCTCACACAA GAGGCATGTT GCAAATTCAT
3181    CTTTATACTT CTAACGACTA GCTCAGAAAT TATTTTACAT CAGTGCATAA TCCAACTGAT
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3481    ACTCCATAGT GGGTGAAAGA TCACGCTTAG ACTGTGGTGT TGAACTGAAG GTTTGCATGT
3541    AGCCCTAATT TTTATCTTCC TTTGAATCTA TTCCTGAACA AACACATTTA TTTTCTTTAG
3601    TTTGTGAAAA ATGCAGCAGC TTATTGTTTC TAGTACATTT TGTTGTGAAG TGTAGATCTC
3661    TAACACGTTT TACAACCACA GGATACTTTC ATGATGGGAG CAGACTACTA CCAAACAGAA
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3901   ATGTATCATT GACAAGAACG CAAAGATAGG AAAAAATGTT TCAATCATTA ATAAAGATGT
3961   AAGCTTTTTT TGCATCCTCC GACTTTCTTT AGTGTGTGTT ATTTTTATAG TACTTACAGG
4021   GCGGAGGTTA GCTTCCCAAC AACTATTGTT CACACAGTCA AGTTCAAAAC ACAAATTGCT
4081   GGTGTGCTTC TTGCTCGCGA GAAAGAAAGC AGAAGAGTGT TCAATTCAGA TTTGATTGTT
4141   TGTAGCTAGG GAGCACTTAT ATTTTTGAGA CATAATTGAT TGAATGAGAC TCCCTGATGT
4201   TGAAATATAT TTAGGGTGTT CAAGAGGCAG ACCGACCAGA GGAAGGATTC TACATACGAT
4261   CAGGGATAAC CATTATATCA GAGAAAGCCA CAATTAGAGA TGGAACAGTT ATATGAAGTA
4321   GGGAAGCATC TCTTGTTGAA CTAGTGGAAA TCCAAATCTC AACTTGAAGA AGGTCAAGGG
4381   TGATGAGCTA TTTCCCACAA AAACCTAGCA CATTCAGCCC CGGAAGAAGC TTTCTATGCT
4441   CATGTGAAAA GAGCAAGTAT AATCGAGACA CGTGTCTAAA TAAAACTAAA GTTGCTG
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Exon 1          1 - 187
Intron 1      188 - 599
Exon 2        600 - 835   Start 599
Intron 2      836 - 1147
Exon 3       1148 - 1270
Intron 3     1271 - 1522
Exon 4       1523 - 1693
Intron 4     1694 - 1882
Exon 5       1883 - 1975
Intron 5     1976 - 2132
Exon 6       2133 - 2216
Intron 6     2217 - 2315
Exon 7       2316 - 2371
Intron 7     2372 - 2570
Exon 8       2571 - 2664
Intron 8     2665 - 2786
Exon 9       2787 - 2972
Intron 9     2973 - 3082
Exon 10      3083 - 3163
Intron 10    3164- -3255
Exon 11      3256- -3342
Intron 11    3343 - 3425
Exon 12      3426 - 3530
Intron 12    3531 - 3681
Exon 13      3682 - 3788
Intron 13    3789 - 3897
Exon 14      3898 - 3958
Intron 14    3959 - 4214
Exon 15      4215 - 4497   Stop 4314
```

9.9 Primers used for determination of the size of the wild species introgression

The primers for the most chromosome mapping markers were designed based on published in GenBank database sequences:

Marker		Primer	N nucl	T ann ^{O}C	Product,bp
TG161	**161F**	5'CTCGCATTACTGGAGATGAT3'	20	58	1300
	161R	5'AAAGGCTTACATCATTAAGATTC3'	23	60	
TG237	**237F**	5'ATTGGCTTGTTATGAAGTTATA3'	22	57	1500
	237R	5'GTCGCCTGAAACCTGAGAG3'	19	61	
TG255	**255F**	5'GCTGCGATATACACAACAGG3'	20	60	238
	255R	5'GAAGAGTCCTAACAACATAGAT3'	22	60	
TG158	**158F**	5'CCCCTAAGACTCTCCAGATT3'	20	60	188
	158R	5'CTCCGAGGAAAGAGGATAGA3'	20	60	
TG269	**269F**	5'TCTCCCCAACATAAACAGCA3'	20	60	329
	269R	5'AAGGCAAAACCATGTGATAC3'	20	59	
TG 90	**90F**	5'GTAGACCATCTTTGATCGTAACTAAT3'	26	70	esc-400
	90R	5'GGACTACGTATACTTGGGCCTGTT3'	24	72	hir-200

Three more markers were obtained by transformation of relevant plasmids pGEM4Z (RLFP Stock of tomato at the Plant Genome Center, Weizmann Institute, Israel) in to competent cells (JM109, Promega, USA) and sequencing of the purified product (AccuPrep Plasmid Extraction Kit, BioNeer, Korea, Hy-Labs) with T7,SP6 primers.

Marker		Primer	N nucl	T ann ^{O}C	Product,bp
TG267	**267F**	5'CGCCCCTCTTAGCAAAGGT3'	19	62	1000
	267R	5'GAGAGTTAACTGTTCGTATCCA3'	22	62	
TG389	**389F**	5'GCATGCTAACATTTATGCATC3'	21	58	480
	389R	5'GCAGACTTACAGATCCAATG3'	20	58	
TG159	**159F**	5'GCCATAACACAGATTCCACAG3'	21	62	420
	159R	5'GGAACAGAATGTCATCCAATG3'	21	60	

ביוכימיה ובקרה מולקולרית של סינתזת עמילן
בפרי העגבניה

חיבור לשם קבלת תואר דוקטור לפילוסופיה

מאת

פטרייקוב מרינה

הוגש לסינט האוניברסיטה העברית, בירושלים

אוגוסט 2006

עבודה זו נעשתה בהדרכתם של:

דר' ארתור א. שפר
המכון למדעי הצמח
מנהל המחקר החקלאי, בית דגן

פרופ' יוסי ריוב
המכון למדעי הצמח וגנטיקה בחקלאות
הפקולטה למדעי החקלאות, המזון ואיכות הסביבה
האוניברסיטה העברית בירושלים, רחובות

תודות

ברצוני להביע את תודתי לכל מי שסייע לי:

למדריכי ד"ר ארי שפר על האמון בי, על הדרכה, התמיכה והעזרה הממושכת לכל אורך הדרך, על הזמן הרב שהשקיע להגהת הכתיבה והאופטימיות לגבי סוף טוב של הפרויקט הזה.
למדריכי פרופ' יוסי ריוב על היחס הטוב, העצות הנבונות והזמן שהשקיע להגהה.

הנני מודה מכל הלב לחברי במחלקה לחקר ירקות על האווירה החמה והידידותית. במיוחד הנני רוצה להודות לחברי במעבדה: ד"ר ילנה יסלסון, שמואל שן, מרים פוגלמן, רן חובב, מיכל מוי, שחר כהן, נועם צ'כנובסקי ואריק שמאי, אשר עזרו לי והיו לצידי כל אחד בזמנו במשך תקופה ארוכה של עבודה זו.

לד"ר אילן לוין, ד"ר דוד גרנות, ד"ר ניר דאי וד"ר ניר כרמי תודות רבות על עזרה מיידית, עניינית והדרכה בכל השלבים, ולד"ר לאה קרני על הגהת הכתיבה.

במיוחד תודות חמות לד"ר רבקה הדס על חברותה תנאמנה, התמיכה מלאה, והגהת הכתיבה.

תודות אין סופיות למשפחתי היקרה, ובעיקר לבעלי מיכאל, ילדי יולי ורוני, והורי אשר תמכו בי ועזרו להתגבר על כל הקשיים בדרך ולהוציא עבודה זו לאור.

תוכן העניינים

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תקציר

העמילן המצטבר זמנית בפרי העגבניה הירוק יכול לתרום כ- 20% למשקל היבש של הפרי. התפרקות העמילן עם הבשלת פרי העגבניה מביאה להצטברות סוכרים מסיסים בפרי הבשל, והם הגורמים העיקריים המשפיעים על טעמו ואיכותו של פרי העגבניה. מטרת המחקר היתה להעמיק את ההבנה של התהליכים המטבוליים והבקרה המולקולארית של סינתזת עמילן מסוכרוז בשלבי התפתחות הפרי, כדי להבין את אסטרטגיות עליית רמת העמילן בפרי.

במחקר ראשוני השוואתי של אחד עשר אנזימים המעורבים במסלול הביוכימי, מסוכרוז לסינתזת עמילן, נמצא כי ארבעה אנזימים הראו תבנית דומה בפעילות הבאה לידי ביטוי בירידה משמעותית בפעילות תוך כדי התפתחות הפרי, בהתאמה עם ירידת רמת העמילן והפסקת הסינתזה שלו. שניים מהם, פרוקטוקינז (FK) ו-ADP-Glc pyrophosphorylase (AGPase) יכולים להיחשב כגורם מגביל פוטנציאלי לסינתזת העמילן בזמן צבירתו בפרי העגבנייה. לכן המחקר התמקד בשני אנזימים אלו.

שלושה איזוזימים של פרוקטוקינז (FKI, FKII, FKIII), ובתוכם אנזים חדש FKIII שנמצא בעבודה זו לראשונה, ושני איזוזימים של הקסוקינז (HKI,HKII) מפרי העגבניה בשלבי התפתחות הופרדו באמצעות כרומטוגרפיה, נוקו חלקית ואופיינו קינטית. שלושה איזוזימי ה-FK נבדלים זה מזה באפיניות ובפעילות עם פרוקטוז, Mg^{2+} ונוקלאוטידים. האנזים FKI מעוכב על ידי פרוקטוז ו-Mg^{+2}, FKII לא מעוכב על ידם ו- FKIII מעוכב על ידי פרוקטוז וכמעט שאינו מעוכב על ידי Mg^{+2}. מבין שני האיזוזימים של HK, ל-HKI ישנה אפיניות גבוהה יחסית לפרוקטוז. כל האיזוזימים עוכבו על ידי התוצר ADP ברמות שונות. FKI הוא האנזים היחיד שהראה עיכוב ספציפי על ידי הנוקלאוטידים CTP ו-GTP. כמו כן, נמצא שבמהלך התפתחות הפרי, הפעילות של כל האיזוזימים של שני האנזימים הנ"ל יורדת. אולם התוצאות מצמחי עגבניה טרנסגניים עם פעילות של FK שעבר שינוי מלמדות שהריאקציה הזו היא לא הגורם המגביל הישיר של סינתזת עמילן בפרי העגבניה.

AGPase מקטלז את ההמרה של G-1-P ל-ADP-Glc, שהינו סובסטרט לסינתזת עמילן. תפקידו היחיד הוא השתתפות ביצירת עמילן והוא נחשב כאנזים המגביל ביצירת עמילן. AGPase מפרי

העגבניה הוא אנזים הטרוטטרמרי, המורכב משתי תת-יחידות גדולות (המקודדות על ידי שלושה גנים *AgpL : AgpL1*, *AgpL2*, *AgpL3*) ושתי תת-יחידות קטנות (המקודדות על ידי גן יחיד *AgpS1*).

במעבדתנו פותחו מספר קווים כמעט איזוגניים של עגבנייה הנבדלים רק במקור של אלל *AgpL1* , שהתקבלו מהכלאה בין מינית של עגבניה תרבותית (*S. lycopersicum* L., שקודם נקראה

L. esculentum Mill., המקור לאלל $AgpL1^E$) ועגבנית בר

S. habrochaites S. Knapp and D.M. Spooner) , שקודם נקראה

L. hirsutum Humb. and Bonpl., המקור לאלל $AgpL1^H$). פרי שמכיל את האלל ממין הבר $AgpL1^H$ מאופיין בפעילות גבוהה יותר של AGPase ורמת עמילן מוגברת בפרי הירוק. בנוסף לכך מאופיין ברמת כלל חומרים מסיסים (TSS) גבוה יותר בפרי הבשל, כתוצאה מהתפרקות עמילן הנצבר בפרי הירוק ומתפרק עם הבשלת הפרי. לא נמצאו הבדלים בפעילותם של שאר האנזימים המעורבים במטבוליזם של יצירת עמילן. האינטרוגרסיה המכילה את האלל *AgpL1* הממופה לחלק המרוחק של כרומוזום מס' 1 הוערכה בעבודה זו לגודל של כ- 1 cM ואינה כוללת אינטרוגרסיה אחרת בכרומוזום 1 שגם בה דווח על QTL לרמת סוכר (TSS).

כדי לגלות את דרך ההשפעה של האלל $AgpL1^H$ על פעילות האנזים, נעשתה השוואת ביטוי של כל תת-היחידות (L1 ,L2, L3 ו-S1) במהלך התפתחות הפרי בשני קווים כמעט איזוגניים. נבדקו רמות החלבון של תת-היחידות ומאפיינים קינטיים של שני האנזימים המנוקים. האלל $AgpL1^H$ בקו עם רמת עמילן גבוהה הראה ביטוי גבוה וממושך יותר במהלך התפתחות הפרי בהשוואה לביטוי של האלל של $AgpL1^E$ בקו עם הרמת עמילן נורמאלית. הביטוי של שאר תת-היחידות בשני הגנוטיפים היה דומה במהלך התפתחות הפרי. התוצאות שהתקבלו היו בהתאמה לעלייה הממושכת בפעילות האנזים AGPase, יחד עם העלייה ממושכת ברמת החלבון של תת-היחידה והתבנית של צבירת עמילן במהלך התפתחות הפרי בקווים עם רמת עמילן גבוהה. אף על פי שלא נמצא הבדל בביטוי של הגן *AgpS1* בשני הגנוטיפים, רמת החלבון S1 הושפעה מהביטוי של L1. משמעות תוצאה זו היא שנוכחות של L1 תורמת לשמירה גם על S1 וכתוצאה מכך על הרכב הטרוטטרמר.

בהשוואה של שני אנזימי AGPase מנוקים של שני הגנוטיפים לא נמצאו הבדלים במאפיינים ביוכימיים. שניהם עוברים זירוז ע"י חום ב- 58^0C ופעילותם עולה ב- 90%, בעוד שהחלבון AGPase-$L1^H$ יציב יותר בחום. לא נמצא הבדל ב- post-translational redox modification של חלבוני תת-יחידות L1 ו-S1 של שני הגנוטיפים. בדיקות קינטיות של האנזימים לא הראו הבדלים משמעותיים באפיניות לכל הסובסטרטים / התוצרים, או בתגובה למעכב Pi ומזרז 3-PGA בשני כווני הריאקציה (סנתוז ופירוק). בנוסף לא נמצאו הבדלים ברמת המטבוליטים (הסובסטרטים ותוצרי הפירוק של האנזים בפרי הירוק) בשני הגנוטיפים.

ריצוף של שני האללים לא הראה שינויים משמעתיים: גודלם נמדדו ל-4500 bp ($AgpL1^E$) ו-4497 bp ($AgpL1^H$) ושניהם מכילים 15 אקסונים, בעלי דמיון של 97% באזור המקודד ו-14 אינטרונים. נמצאו ביניהם מספר הבדלים ברצף: indels ו-SNP המאפיינים את $AgpL1^H$, במיוחד בשניים מ-15 האינטרונים. ריצוף של 2000 bp באזור של הפרומוטר הראה דמיון של 80%.

תוצאות המחקר מצביעות על AGPase כאנזים העיקרי המבקר את ההצטברות הזמנית של העמילן בפרי ירוק של עגבניה. הפעילות הגבוהה יותר של AGPase בקווי עגבניה המכילים $AgpL1^H$ ממין הבר קשורה להתבטאות יתר של תת-היחידה הגדולה הרגולטורית ויציבות ופעילות של ההטרוטטרמר AGPase השלם. תופעה זו יכולה לשמש כדוגמא של הטרוזיס בין מולקולות, כשאללים של כל תת-היחידה משלימים זה את זה.