Biochemistry and molecular control of starch synthesis in tomato fruit

Thesis submitted for the degree of "Doctor of Philosophy"

by
Marina Petreikov

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This work was carried out under the supervision of

**Dr. Arthur A. Schaffer**
Institute of Plant Science
Agricultural Research Organization
The Volcani Center, Bet Dagan

**Prof. Joseph Riov**
The R.H.Smith Institute of Plant Sciences and Genetics in Agriculture
Faculty of Agricultural, Food and Environmental Quality Sciences
The Hebrew University of Jerusalem
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<th>Description</th>
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<tbody>
<tr>
<td>3-PGA</td>
<td>3-phosphoglyceric acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ADP-Glc</td>
<td>ADP-glucose</td>
</tr>
<tr>
<td>AGPase</td>
<td>ADP-Glc pyrophosphorylase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cv</td>
<td>cultivar</td>
</tr>
<tr>
<td>DAA</td>
<td>days after anthesis</td>
</tr>
<tr>
<td>dd</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DIECA</td>
<td>diethylldithiocarbamic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>1, 4-dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FK</td>
<td>fructokinase</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>fructose 6-phosphate</td>
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<tr>
<td>FW</td>
<td>fresh weight</td>
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<td>Glc-1-P</td>
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<td>Glc-6-P DH</td>
<td>glucose 6-phosphate dehydrogenase</td>
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<td>GK</td>
<td>glucokinase</td>
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<td>Hepes</td>
<td>N-(2-hydroxyethyl)piperazine-N’-2-ethane sulfonic acid</td>
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<td>HK</td>
<td>hexokinase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<tr>
<td>I</td>
<td>iodine</td>
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<td>insoluble starch synthase</td>
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<tr>
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<td>MW</td>
<td>molecular weight marker</td>
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<tr>
<td>NAD+</td>
<td>oxidised nicotinamide adenine dinucleotide</td>
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<td>sodium hydroxide</td>
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<td>(NH4)2SO4</td>
<td>ammonium sulfate</td>
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(NH₄)H₂PO₄  ammonium hydrogen phosphate
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PEP  phosphoenolpyruvate
PGI  phosphoglucoisomerase
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
SSStS  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, AgpL1, AgpL2 and AgpL3) 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 AgpL1E allele) and wild species tomato S. habrochaites S. Knapp and D.M. Spooner (formerly L. hirsutum Humb. and Bonpl., source of the AgpL1H allele). The fruit carrying the AgpL1H 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 AgpL1H allele, we compared the expression of all subunits (L1, L2, L3 and S1) in developing tomato fruit in the two near isogenic lines. The AgpL1H allele in the high starch line was expressed for a prolonged period of fruit development, compared to the AgpL1E 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 AgpL1H 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-L1H 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, AgpL1E and AgpL1H 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 AgpL1H. 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 $\alpha$-1,4 glucosidic linkage) and amylopectin (a branched $\alpha$-1,4:$\alpha$-1,6 D-glucan polymer with about 5% $\alpha$-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 al., 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 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, vacuolar invertase; SuSy, sucrose synthase; SPS, sucrose phosphate synthase; UGPase, UDP-Glc pyrophosphorylase; FKs, fructokinases;](image-url)
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).
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 \textit{in vivo} activities of SuSy and FK may be well below their \textit{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 \textit{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 \textit{et al}., 1984), potato tubers (Gardner \textit{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 \textit{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 (SSoS) and starch-granule-associated forms (ISoS), both of which utilize ADP-Glucose (ADP-Glc) as a substrate (Visser and Jacobsen, 1993). ISoS, but not SSoS, 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 ISoS does not have a significant effect on the total amount of starch produced in the waxy (or amylose-free) maize mutant; the lack of ISoS, which is associated with amylose synthesis, was compensated by SSoS (Tsai, 1974). Accordingly, ISoS 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 (Nguyen-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 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 Cys12 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 (AgpL1, AgpL2 and AgpL3) and only one for the small subunit (AgpS1) (Chen et al., 1998a; Park and Chung, 1998). Tomato isoforms have very high identities of 96-98% to the corresponding potato isoforms (Chen et 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 et 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 **Enzymes involved in starch degradation**

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 than 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 H₂O 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°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 3 min, 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 Enzyme extraction and assays

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 MgCl₂, 1 mM EDTA, 10 mM
KCl, 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 1 mM 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% (NH₄)₂SO₄ 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 MgCl₂, 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 Hapes-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\(_2\)HPO\(_4\) - 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 MgCl₂, 1 mM EDTA, 2.5 mM DTT, 3 mM DIECA, and 1 mM 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 1 ml of reaction mixture contained 50 mM Hepes-NaOH (pH 7.8), 5 mM MgCl₂, 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°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 1 ml of reaction mixture containing 50 mM Hepes-NaOH (pH 7.8), 5 mM MgCl₂, 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 1 ml of reaction mixture containing 50 mM Hepes-NaOH (pH 7.8), 5 mM MgCl₂, 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-Glucose 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 MgCl₂, 1 mM EDTA, 5 mM DTT, 3 mM DIECA, and 1 mM PMSF. The supernatant after centrifugation (13,000g, 20 min) was incubated for 4 min at 60°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\textsuperscript{H} and 7.5 μmol/mg protein/min for AGPase-L1\textsuperscript{E}).

c) Enzyme-linked assay

\textbf{Pyrophosphorolytic direction (AGP-Glc + PPI → ATP + Glc-1-P)}

\textbf{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\textsubscript{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 \textit{Leuconostoc}), 2U PGM, and up to 0.1 ml of enzyme extract in of. Following 5 min of incubation at 37\textdegree{}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.

\textbf{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\textdegree{}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 \textit{Leuconostoc}), 1U PGM was added. After 40 min of incubation at 37\textdegree{}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 A₀.₅ 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$_2$HPO$_4$, 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. ISStS 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 KCl, 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* (L. *esculentum* accession U81033 and *L. hirsutum* accession AF184345). The primers used were: forward-A1PF (5’GAGGGAGTTTGAACAACAATC3’), and reverse-A1PR (5’TAAGCAACCCCCAGGTTAA3’) with amplicons of 94bp and 85bp for *AgpL1* and *AgpL1E*, respectively. Alternatively, based on a 15bp insertion in *AgpL1* intron 13, primers offering better resolution were designed for genotypic analysis:

Forward - A3PF (5’GATTGCCTCCCTGTAGAG3’) and
Reverse - A3PR (5’CCTATCTTTGCCTTGGTCAA3’) with an amplicon 205bp and 190bp for *AgpL1* and *AgpL1E*, 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’CCCTTTAAATTCTTAGCCYAC3’), and
Reverse - AprR (5’GTGGAGATTAGCTGACAAT TTC3) with amplicons of 184bp and 266bp for *AgpL1* and *AgpL1E* 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):
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:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer</th>
<th>T anneal °C</th>
<th>Product, bp</th>
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<tbody>
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<td>161F</td>
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<td></td>
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<tr>
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<td></td>
<td>158R</td>
<td>5'CTCGAGAAAGAGGATAGA3'</td>
<td>60</td>
</tr>
<tr>
<td>TG269</td>
<td>269F</td>
<td>5'TCCCTCAACAAACAGCA3'</td>
<td>60</td>
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<tr>
<td>TG 90</td>
<td>90F</td>
<td>5'GTAGACACTCTTTGATCGTAACTAT3'</td>
<td>70</td>
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<tr>
<td></td>
<td>90R</td>
<td>5'GGACTACGTATACTTGGGCTGTT3'</td>
<td>72</td>
</tr>
</tbody>
</table>

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 Genomic sequencing

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 Quantitative real-time PCR

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’GATTATATGGAGTTGGTGACGACAACC 3’,
Reverse - 5’ACCAGCCCAAAATCTGATGCT 3’;

*AgpL2* (U81034) Forward - 5’GTCGGGCGAAACTAAGATACA 3’,
Reverse - 5’TCAGCTTTTCAACACCTTGCTT 3’;

*AgpL3* (U85497) Forward - 5’ AACCTTGCACTCACTGAACATCC 3’,
Reverse - 5’TTATCAATCGCTGATGGAGGTAAGT 3’;

*AgpS1* (L41126) Forward - 5’AAAATGCTTTGTGCGCATGTC3’,
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 R² =0.94-0.99.

The program used for the real-time PCR was as follows: 10 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°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 $2^{-\left(C_{t \text{ sample}}-C_{t \text{ actin}}\right)}$, 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 Laemmlli (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 Na₂HPO₄, 0.17 M NaH₂PO₄, 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°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 MgCl₂, 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 1 ml 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°C was washed with 0.125 ml dd water and the combined supernatant neutralized with 3 M KOH. After 10 min 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 13,000 g for 10 min at 4°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 MgCl₂, 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 10 min incubation at 37°C, the absorbance was monitored spectrophotometrically at 340 nm for Glc-6-P determination. 1 U 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 MgCl₂, 1 mM ATP, 20 µM NADH (in 5% NaHCO₃). 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 NH₄H₂PO₄ 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 **Protein estimation**

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 **Statistical calculations**

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.

<table>
<thead>
<tr>
<th>Maturity stage</th>
<th>Fructokinase activity (nmol/gfw/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assay 1</td>
</tr>
<tr>
<td>Immature</td>
<td>184 ± 25</td>
</tr>
<tr>
<td>Mature</td>
<td>63 ± 8</td>
</tr>
</tbody>
</table>

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.
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 fructose

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 similar 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. FKI 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. LeFRKI-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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>FKI Km, mM</th>
<th>FKII Km, mM</th>
<th>FKIII Km, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>0.33</td>
<td>0.16</td>
<td>0.27</td>
</tr>
<tr>
<td>Fructose inhibition</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mg</td>
<td>0.34</td>
<td>0.83</td>
<td>4.66</td>
</tr>
<tr>
<td>Mg inhibition</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ATP</td>
<td>0.075</td>
<td>0.14</td>
<td>0.18</td>
</tr>
<tr>
<td>CTP</td>
<td>0.50</td>
<td>0.14</td>
<td>0.50</td>
</tr>
<tr>
<td>GTP</td>
<td>0.50</td>
<td>0.38</td>
<td>2.10</td>
</tr>
<tr>
<td>UTP</td>
<td>0.50</td>
<td>3.44</td>
<td>3.00</td>
</tr>
<tr>
<td>ADP inhibition, I_{0.5}, mM</td>
<td>2.0</td>
<td>3.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

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-Glucose 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.

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

5.2.2. Starch content and AGPase activity in the high starch line and F₁ 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 F1 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 F1 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Starch (mg/gfw)</th>
<th>°Brix</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-82</td>
<td>23 B</td>
<td>4.1 B</td>
</tr>
<tr>
<td>904</td>
<td>58 A</td>
<td>8.1 A</td>
</tr>
<tr>
<td>F1 (M-82×904)</td>
<td>46 A</td>
<td>7.1 A</td>
</tr>
<tr>
<td>ILS×M-82</td>
<td>25 B</td>
<td>5.3 B</td>
</tr>
<tr>
<td>ILS×904</td>
<td>44 A</td>
<td>7.5 A</td>
</tr>
</tbody>
</table>
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 ($P<0.05$).

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

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 F₂ 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 F₂ population of 64 plants of the cross between the high starch line 904 and M-82 was analyzed. The 64 F₂ 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 F₂ 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 F₂ 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 F₂ plants of each genotype. TSS (°Brix) values are the average of five fruits per plant of the entire F₂ population. Different letters indicate significant differences (P<0.05) within columns.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AGPase (nmol/gfw/min)</th>
<th>Starch (mg/gfw)</th>
<th>°Brix</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE</td>
<td>104 C</td>
<td>16.4 B</td>
<td>5.3 B</td>
</tr>
<tr>
<td>EH</td>
<td>306 B</td>
<td>25.2 AB</td>
<td>5.9 AB</td>
</tr>
<tr>
<td>HH</td>
<td>450 A</td>
<td>37.3 A</td>
<td>6.3 A</td>
</tr>
</tbody>
</table>

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 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AGPase activity (nmol/gfw/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS1</td>
<td>TG159</td>
</tr>
<tr>
<td>hh</td>
<td>ee</td>
</tr>
<tr>
<td>ee</td>
<td>ee</td>
</tr>
<tr>
<td>ee</td>
<td>hh</td>
</tr>
</tbody>
</table>

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.
5.3.2 AGPase activity in developing tomato fruit

Comparison of enzyme activity in developing AgpLI^{H} and AgpLI^{E} fruit from the segregating lines showed that high AGPase activity was maintained for a longer period of development in the AgpLI^{H} genotypes (Fig. 15a). The transient pattern of AGPase activity in the standard AgpLI^{H} genotype showed a peak activity during the second week of fruit development, followed by a continuous decline in activity. In the AgpLI^{H} fruit, the decline in activity was delayed, so that AGPase activity in the AgpLI^{H} fruit was approximately twice that in the AgpLI^{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 AgpLI^{H} genotype. Whereas peak starch levels occurred approximately a week
following the peak of AGPase activity in the $AgpLI^E$ genotype, the starch levels of the $AgpLI^H$ 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 $AgpLI^H$ allele (Table 13). In a range of genetic backgrounds of different mature fruit size (from 25-100 gram final fruit weight) segregating for the $AgpLI$ allele, there was no negative effect of $AgpLI^H$ on fruit size, starch levels, or TSS levels. In general, fruit size increased from 5% to 15% fresh weight.

Table 13. Impact of $AgpLI^H$ 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. $AgpLI^E$ and $AgpLI^H$, isogenic lines. Standard error <5%.

<table>
<thead>
<tr>
<th></th>
<th>AGPase activity</th>
<th>Starch</th>
<th>TSS</th>
<th>Fruit weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AgpLI^E$</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$AgpLI^H$</td>
<td>210</td>
<td>114</td>
<td>112</td>
<td>110</td>
</tr>
</tbody>
</table>
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 effect 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fruit weight (g)</th>
<th>AGPase (nmol/gfw/min)</th>
<th>Starch (mg/gfw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ee ee</td>
<td>16.87 ± 1.13</td>
<td>69.97 ± 17.57</td>
<td>11.62 ± 2.38</td>
</tr>
<tr>
<td>hh ee</td>
<td>18.90 ± 1.37</td>
<td>39.18 ± 9.43</td>
<td>11.80 ± 1.40</td>
</tr>
</tbody>
</table>

5.3.3 AGPase gene expression in developing tomato fruit

In order to determine the stage at which the AgpL1H 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 AgpL1H and AgpL1E 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 AgpL1H allele led to a prolonged and higher expression of the AgpL1 subunit in the AgpL1H high starch lines (Fig. 16a). The effect on expression was most evident from 20 DAA. While the AgpL1E allele showed a strongly declining pattern of expression at 20 DAA, the AgpL1H 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, AgpL2 and AgpL3 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 AgpL1H allele reduced expression of the other genes(Fig. 16b, c). AgpL2 was expressed at levels of approximately a third, and AgpL3 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*<sup>H</sup>) (black bar) and from *L. esculentum* (*AgpL1*<sup>E</sup>) (grey bars). *AgpL1*<sup>E</sup> and *AgpL1*<sup>H</sup>, 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).

![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 immunoblot 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).](image)

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\textsuperscript{H} and -L1\textsuperscript{E}

The differences in activity of the AGPase-L1\textsuperscript{H} and AGPase-L1\textsuperscript{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).

![Graph showing enzyme activity and protein concentration](image)

**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* (*AgpL1H*); ○ (white circles), tomato fruits carrying the AGPase large subunit of *L. esculentum* (*AgpL1E*).
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* (*AgpL1H*), ● (black circles); and in tomato fruits carrying the AGPase large subunit of *L. esculentum* (*AgpL1E*), ○ (white circles). The Pi inhibitory effect was measured in the presence of 5 mM 3-PGA.

Table 15. Kinetic characteristics (Km and A0.5) 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 A0.5 values correspond to the substrate or activator concentrations, respectively, at which the reaction velocity is half-maximal.
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 \textit{AgpL1}^{H} and \textit{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.

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

**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).

![Graphs showing effect of temperature on AGPase activity and total protein level in tomato extract](image)

**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 to 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- L1H and AGPase- L1E 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.

![Figure 23](image)

**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\textsuperscript{H} and AgpL1\textsuperscript{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 \textit{L. hirsutum} accessions (LA1777 and LA 2650), three \textit{L. esculentum} lines (cv. M82, breeding line 361 and an unidentified cherry cultivar) as well as\textit{AgpL1}\textsuperscript{H} and \textit{AgpL1}\textsuperscript{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 \textit{AgpL1}\textsuperscript{E} NIL and DQ322684 for the LA1777 gene. Promoter sequences have been deposited as DQ322688 (M82), DQ322685 (LA1777), DQ322689 (unidentified cherry cv.), DQ322687 (\textit{AgpL1}\textsuperscript{E} NIL), DQ322686 for (\textit{AgpL1}\textsuperscript{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 \textit{AgpL1}\textsuperscript{H} genotype, presumably due to genetic heterogeneity in this wild species accession.

The two alleles, \textit{AgpL1}\textsuperscript{E} and \textit{AgpL1}\textsuperscript{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 \textit{AgpL1} amino acid composition based on cDNA sequencing (I34M, EKK duplication in \textit{AgpL1}\textsuperscript{H} at 54-56, S419T and T513I). Identity of 95% was found between the genomic sequences of segregating \textit{AgpL1}\textsuperscript{H} and \textit{AgpL1}\textsuperscript{E} individuals with small differences in the intronic sequences. There is a 15bp insertion in intron 12 of \textit{AgpL1}\textsuperscript{H} (5’TCATGAACTCAAGAA3’) and two insertions, 4bp (5’AAAG3’) and 8bp (5’CTTTTCTTTT3’), in intron 13 of \textit{AgpL1}\textsuperscript{E}.  

Fig. 24. A diagramatic representation of the two alleles of the AGPase large subunit gene (AgpL1\textsuperscript{H} and AgpL1\textsuperscript{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\textsuperscript{H}) or below (AgpL1\textsuperscript{E}) the diagram.

The putative promoter region sequenced was 2051bp (AgpL1\textsuperscript{H}) and 2063bp (AgpL1\textsuperscript{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.
Fig. 26. Promoter region of tomato fruits \textit{AgpL1} alleles of two genotype: carrying the \textit{AgpL1}^H allele (parental Hir 1777 and NIL hh) and four genotypes carrying \textit{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 \textit{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.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>M82</td>
<td>GAGTATAAATAGAAAGA.CAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAGCGTGATC</td>
</tr>
<tr>
<td>Cherry</td>
<td>GAGTATAAATAGAAAGA.CAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAGCGTGATC</td>
</tr>
<tr>
<td>Esc 361</td>
<td>GAGTATAAATAGAAAGA.CAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAGCGTGATC</td>
</tr>
<tr>
<td>ee</td>
<td>GAGTATAAATAGAAAGA.CAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAGCGTGATC</td>
</tr>
<tr>
<td>hh</td>
<td>GAGTATAAATAGAAAGA.CAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAGCGTGATC</td>
</tr>
<tr>
<td>Hir 1777</td>
<td>GAGTATAAATAGAAAGA.CAGCAAGGTTTCTCGTGAGAGTTCACAAGCCAATAGCGTGATC</td>
</tr>
</tbody>
</table>

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

\textit{Esc Hir heeehh hheeee}
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,
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, Mg²⁺ 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 et al., 1998; Park and Chung, 1998; Li et al., 2002 and results presented in Fig.
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 AgpL1H 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 AgpL1H and AgpL1E genotypes with respect to the developmental protein subunit levels. In the AgpL1H 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 L1H protein stabilizes
the small subunit protein and provides to AGPase-L1\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{H} protein appears to be slightly more heat stable then AGPase-L1\textsuperscript{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°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\textsuperscript{H} and AGPase-L1\textsuperscript{E} enzymes are heat stable, the AGPase-L1\textsuperscript{H} enzyme seemed to be slightly more heat stable, retaining up to 42% activity even at 66°C, whereas AGPase-L1\textsuperscript{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\textsuperscript{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 additional disulfide bridge, or enhanced electrostatic interactions at the dimer-dimer interface, similar to those in the tetrameric 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\textsubscript{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 AgpL1H 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 Glc-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-L1H 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* 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 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\textsuperscript{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 AgpL1E. The wild species donor itself accumulates little starch. However, we have not yet developed or characterized the heterotetrameric enzyme from the genotype AgpL1H/AgpS1H 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 Tanksley, 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 AgpL1H 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**


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9. Appendices

9.1. Promoter sequence DQ322687 (AgpL1E, NIL): 2063 bp

1      CGGAGTAAGT GACATACATA TAAAGATGGG TGGAATCTTC CAAATAGTGA AAGAAGACAA
61     GCACACTTCTT ACATTTAACC TTCAATTATC TAATTAAATC TTAATGACGT CGTCCTACAT
121    GTCTCCCTGG TCATTATATT ATATTTAAAC ATTTTAATTT ATTTTAAACT GTCTTGGCCT
181    ATGATCTCTTC AGCTCAGACT AAAAGTTTTT TTTGTTAGGT TATTGAAACA AATTTTTTCT
241    TCAATTTTTT GATGACACAT TTGAACATGA TTTTTTTTTT AAAATTTTTT TTTTAAAAAT
301    TATTTTGTGT AAGCTTTTAC TTTAAATAAG TAAAATTTAA AAAAAAATTT TTAAAAATTT
361    ATGATCCTTG AGACTTTATT AAGGTCGGGT CGAGGTCGGT CCATGGCCTT AATTTTTGTT
421    TATTTAAGTG TATTTTAAAA AAATTTTACT ACCCTTTTTC TTCCTTTTA TTTTTTTTCT
481    ATATATATAA TGAAAAATTT GTTTAAATTT CTGTAATTCT TTTTTTCTTT AAAAAATTTT
541    ATATATATATG GATTTTGCAT TTTTTTTTTT TTTTTTTTTT TTAAATTTAT TTTTAAAAAT
601    ATATATATATG GAATTTTGAT ATGAAATTGT CAGCTAATCT CCAACTAAAT AATGTCTGAC
661    GATACATACAT GATTACACTA GTTTTTTTTT TTGTTAGTGT TATTGAAACA AATTTTTTCT
721    TCATTATTAC ATAGCTTTTT CTCTTTTTTT CTCTTTTTTT CTCTTTTTTT CTCTTTTTTT
781    AACGCTTCTCC TTCTCTTTCC CTCTTTTTCC CTCTTTTTCC CTCTTTTTCC CTCTTTTTCC
841    CATCCTACC CTCTTAATTT TTTATAAAA AAAAAATTTT CTGGAATTTA CTGATTTATG
901    TTTTTTTTTT TCTTTTCTTT CTCTTTTTTT CTCTTTTTTT CTCTTTTTTT CTCTTTTTTT
961    CACGCTTAATT TTTAAAAAAT TAAATGATCT ATGTTTCTGA TGTGAATTTT TTTTTTTTTT
1021    AAATTTTGAAA AAAAAATTTT TTTTTTTTCT TTATTTTTTT TTTTTTTTCT TTATTTTTTT
1081    AGATTTTGTG ATGAAAACAA TGTGAATTTT TTATTTTTTT TTTTTTTTCT TTATTTTTTT
1141    TTTTTTACGG ACAAAAGATCT CTCTAGAAGT TTAAGATGTA TTTTTTCTTT TTTTTTCTTT
1201    TGTTGAATTG TTATAGCTAT ATTTTTAATT TTATTTTTTT TTATTTTTTT TTATTTTTTT
1261    TAATATATAC AAAAAATTTA AGATTTAAAT TCGTCGTCTCC TTTTTTTTTT TTTTTTTTTT
1321    ATCTTTGTGG ATTACCACTAC CTGTACGATC GCTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1381    GACGAGTTTT CTTGCTTTAA CCCCTTTTAA CTGTTTTTTT ATTGTTTTTT ATTGTTTTTT
1441    GACTATAAAA AAAAAATTTA CAGACTATCT AAAAAATTTT ATATTTTTTT TTATTTTTTT
1501    TCATTAAATG TTTTTTTTGT TTTTAAAAAA AGTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1561    CACCTAAAAT TTATTTTATA TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1621    GTCATATTTT GTTTCTTTTT GTTGTGTTGTT GTTTTTTTTT TTATTTTTTT TTATTTTTTT
1681    AATTTTTTTA AAAAAATTTA TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1741    GAAATCAAGT TTATAGGATT ATATATATAT ATATATATAT ATATATATAT ATATATATAT
1801    TTAAGTGCTT AAAACTTTTT ATGACGAATTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1861    ATATATAGGA TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1921    ATATATAGGA AAAATTTTTT TTATTTTTTT TTATTTTTTT TTATTTTTTT TTATTTTTTT
1981    TTTATCTAAA TAGGGGAAT CAAAAATTGTT ATATAATAGG ATGATAGGAT TTTTTTTTTT
2041    GAGTTCCAA GCAATAAAG AATGTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
9.2 Promoter sequence DQ322689 (AgpL1, unidentified cherry cv.): 2129 bp

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121  AGTCTCTCTAA AAGTCTCTATT CTTATATATT TTGATACGTT AGTCACTGTT
181  AATTCTTCTG TAGCTCTCTTT CTTATCTCTT CTTATCTCTT CTTATCTCTT
241  GTTCATCTCAT TTTTATTAAA TTATTAAA TTATTAAA TTATTAAA
301  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
361  AATTCTTCTG TAGCTCTCTTT CTTATCTCTT CTTATCTCTT CTTATCTCTT
421  GTTCATCTCAT TTTTATTAAA TTATTAAA TTATTAAA TTATTAAA
481  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
541  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
601  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
661  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
721  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
781  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
841  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
901  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
961  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
1021  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
1081  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
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1921  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
1981  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
2041  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT
2101  TTTGTTACAGTC AGTCTCTCTATT CTTATCTCTT CTTATCTCTT CTTATCTCTT

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9.3 Promoter sequence DQ322688 (AgpL1, M82): 2064 bp

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181 GTTAAACGG TTTTACGCT GAATACGGCT CAAATCAATTT CATATTAGGGG ATAGGTTTTG
241 GACATAAGTT GCTACTGCT TTTAAATTTA GAATTTTTTT ATTGTGTTGT TATTGAAACA
301 ATAGTCTCGT ATGCTTTTAT AAGTGCACCT GCAATGCTAT CTTAACTGTTT AACATTTTAC
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421 TCTTCGGATA CGGCTGTAAA ATATAATAGG CATGCTTTTC TTTTTTACGCT TTCTACAGAT
481 GCATGCTGTC AATGAAATTT AAGAATGCAA ATGCTAATTT TTTTTTTTTT TTTGTTAGTG
541 ATATATAAAC TATGAAATTT CGCTGACCTA AAAAATGTTA TTTTATTTAA TTAAAAATTT
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661 ATATATAAAC TATGAAATTT CGCTGACCTA AAAAATGTTA TTTTATTTAA TTAAAAATTT
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841 GACATAAGTT GCTACTGCT TTTAAATTTA GAATTTTTTT ATTGTGTTGT TATTGAAACA
901 TTTTAAAAAC TTTTTTTTTT TTTTTTTTTT TTTGTTAGTG
961 AATATAGGTCA AATATAGGTCA AATATAGGTCA AATATAGGTCA AATATAGGTCA AATATAGGTCA
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1981 TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTGTTAGTG
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116
9.4 Promoter sequence DQ322686 (*AgpL1*, NIL): 2051 bp

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121  TCCTCTCACA ATCGAAATCTCCT TATTTTTAACCT TCTTTATTTA TTTTTCAAGG
181  TTCTTTTTTTA ATTTTTTATTT AAGTGTAACT TACAATAATT TTACTAGAAT ATAATCTAAT
241  TTGGAGATAAG TTTGGGATAT AGATTCTTAA GCTCTTTTAA AATTAGGGGT TTATAAGTCT
301  CGTCTCTTTTG CTTTACTAGA ATTTTGAGCC GTCAAAACAG TTTTAACTCA TGAATCAGCC CAATTCAATT
361  TCTTTACCTA ATTAATTCAA CAAAACACCC AACATATCAT CTCCTTTTTG CTTTACTAGA
421  TCTTTATTTA TTTTCTGACT ATTATATTTA TTTTTTAAGG CCAATGTATCT CGAAATGTAT AGACTCAAAA TTAGTTTAAT
481  TTTTTTTTTG CTAGTGTTAT TGAAACAACA ACATTAGTCA TATTTTGATT
541  TTGAAAATCTT CTCAATTTCT TGATATTATA AAATTAGCAG TTCGAAAAGA AGAAAAAAAA
601  CAGTCTGGCCCT TTCCTCCTCCG CTCTTTTCTAG AAAAAAAAAC TTAGAATTTT TGTTAGAAAA
661  CTCTTTGAGGC AGGTGTTTGG CCTGTAATGG GTCTCTATTG TCCAGATCTT GGTGGACCCT ACACACTATG
721  AGTGATTTTA TTTAATAATG ACTAAGTTTT ATCGCGAATA TGAATTTTTT TTTATTATAT
781  CATCTAAAAT TTTAGGCATA ACAGATAATA ATATCTCCTT TAACTGTAGTT TAAATTTATA
841  ATTATGCTCT TCAACTTTGA GTGTACACAA GTAGACACTT AAATTTATAT AAAATTGAAC
901  AAATAGACAC ACATGTCCTA TATGTCATCC TACATGTCAT TTTTTGTCCT ACGTGGTGTC
961  CTACATGTAT TTTGCCATGT AGGATTCATA TGTTTATTTA TTTAAAAGTT GGATAGTTAA
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2041 CAATAAAGCT G
9.5 Promoter sequence DQ322685 (AgpL1, S.habrochaites S. LA1777): 2054 bp

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181  TCTTTAGCTA ATTAATTCAA CAAAACACCC AACATATCAT CTCCTTTTTG CTTTACTAGA
241  ATTTGAGCCG TCAAAACAGT TTTAGCTCAT GAATCAGTCC AATTCAATTA AATTCATTAT
301  TGAGATAGGT TTGGGATATA AATTCTTAAG CTCTTTTAAA ACTAGGGGTT TATAAGTTTG
361  ACATCATGTAA GCTCATGATC CTTGAGACTT GATCAAGGCC GTGTCGAGGT CGGCCCATGA
421  CAAATACTTC TCAATTTCCT TTAATTTATA AATTACGCTG TCGAAAGAAA AAAAAAGAGG
481  ATTTAAATAC ACTATATGTT GTAATATGAT GAATCTTACC AGACTTTGGT AGTCCAAATA
541  CATGTATCTC GAAATGTATA GACTCAAAAT TAGTTTAATT TGTTTTAGAC ACACTCTACC
601  CCCTAATTAT TGTTTATTTA AGTGTAACTT ATAATAATTT TACTAGAATA TAATCTAATC
661  ATTTAAATAC ACTATATGTT GTAATATGAT GAATCTTACC AGACTTTGGT AGTCCAAATA
721  CATGTATCTC GAAATGTATA GACTCAAAAT TAGTTTAATT TGTTTTAGAC ACACTCTACC
781  CCCTAATTAT TGTTTATTTA AGTGTAACTT ATAATAATTT TACTAGAATA TAATCTAATC
841  ATTTAAATAC ACTATATGTT GTAATATGAT GAATCTTACC AGACTTTGGT AGTCCAAATA
901  CATGTATCTC GAAATGTATA GACTCAAAAT TAGTTTAATT TGTTTTAGAC ACACTCTACC
961  CCCTAATTAT TGTTTATTTA AGTGTAACTT ATAATAATTT TACTAGAATA TAATCTAATC
1021 GTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
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1141 TCTAAGACTA TAGTAAACTA ATGATAAATA ATGATATCTA TCAAGTACGT AGATTAGTAT
1201 AGCTAATCTC CAACTAAAATA ATGTCGACCC CCAACAAATT TTAAAGGCAG CAAAAGATCTC
1261 AGCTAATCTC CAACTAAAATA ATGTCGACCC CCAACAAATT TTAAAGGCAG CAAAAGATCTC
1321 ACTATGACTGCT GTGCAATTTT ATGTATTTTG CTATGTAGGA ATCATATGTT TATTTATTTA
1381 ATTATTTGTT ATTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
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1681 AGCTAATCTC CAACTAAAATA ATGTCGACCC CCAACAAATT TTAAAGGCAG CAAAAGATCTC
1741 ATTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1801 ACTATGACTGCT GTGCAATTTT ATGTATTTTG CTATGTAGGA ATCATATGTT TATTTATTTA
1861 ATTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1921 GTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
1981 TAGGGATATG GAAATGACTC TAAATGACAA GATTAGCAAG GATTGGCATG AGACTTTACA
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9.6 Full genome sequence DQ322683 (AgpL1<sup>E</sup>, NIL) : 4500 bp

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121  ATCTTGCACT TTTTTCACCA 
181  AAAATACAAG
241  AGGAGATTTT TTTTTTCTGC 
301  AAATTTCTGC 
361  GTTCTCTCAG
421  TACAGTCTG
481  GTTCTCTCAG
541  TACAGTCTG
601  GTTCTCTCAG
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961  TACAGTCTG
1021  TACAGTCTG
1081  TACAGTCTG
1141  TACAGTCTG
1201  TACAGTCTG
1261  TACAGTCTG
1321  TACAGTCTG
1381  TACAGTCTG
1441  TACAGTCTG
1501  TACAGTCTG
1561  TACAGTCTG
1621  TACAGTCTG
1681  TACAGTCTG
1741  TACAGTCTG
1801  TACAGTCTG
1861  TACAGTCTG
1921  TACAGTCTG
1981  TACAGTCTG
2041  TACAGTCTG
2101  TACAGTCTG
2161  TACAGTCTG
2221  TACAGTCTG
2281  TACAGTCTG
2341  TACAGTCTG
2401  TACAGTCTG
2461  TACAGTCTG
2521  TACAGTCTG
2581  TACAGTCTG
2641  TACAGTCTG
2701  TACAGTCTG
2761  TACAGTCTG
2821  TACAGTCTG
2881  TACAGTCTG
2941  TACAGTCTG
3001  TACAGTCTG
3061  TACAGTCTG
3121  TACAGTCTG
3181  TACAGTCTG
3241  TACAGTCTG
3301  TACAGTCTG
3361  TACAGTCTG
3421  TACAGTCTG
3481  TACAGTCTG
3541  TACAGTCTG
3601  TACAGTCTG
3661  TACAGTCTG

1 AAATTTACTC CTTCTGTCAC CTTGATACCT CAAATTATTA ATACACACTT
61  CTTCCTAAAC CTTTTTCTGC AAAAAATTG CTTACACACT
121  ATCTTGCACT TTTTTCACCA 
181  AAAATACAAG
241  AGGAGATTTT TTTTTTCTGC 
301  AAATTTCTGC 
361  GTTCTCTCAG
421  TACAGTCTG
481  GTTCTCTCAG
541  TACAGTCTG
601  GTTCTCTCAG
661  TACAGTCTG
721  TACAGTCTG
781  TACAGTCTG
841  TACAGTCTG
901  TACAGTCTG
961  TACAGTCTG
1021  TACAGTCTG
1081  TACAGTCTG
1141  TACAGTCTG
1201  TACAGTCTG
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1321  TACAGTCTG
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1441  TACAGTCTG
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1621  TACAGTCTG
1681  TACAGTCTG
1741  TACAGTCTG
1801  TACAGTCTG
1861  TACAGTCTG
1921  TACAGTCTG
1981  TACAGTCTG
2041  TACAGTCTG
2101  TACAGTCTG
2161  TACAGTCTG
2221  TACAGTCTG
2281  TACAGTCTG
2341  TACAGTCTG
2401  TACAGTCTG
2461  TACAGTCTG
2521  TACAGTCTG
2581  TACAGTCTG
2641  TACAGTCTG
2701  TACAGTCTG
2761  TACAGTCTG
2821  TACAGTCTG
2881  TACAGTCTG
2941  TACAGTCTG
3001  TACAGTCTG
3061  TACAGTCTG
3121  TACAGTCTG
3181  TACAGTCTG
3241  TACAGTCTG
3301  TACAGTCTG
3361  TACAGTCTG
3421  TACAGTCTG
3481  TACAGTCTG
3541  TACAGTCTG
3601  TACAGTCTG
3661  TACAGTCTG
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
Intron 7    2371 – 2573
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
9.7 Full genome sequence DQ322682 (AgpL1, M82): 4482 bp

1 ATCACACTCC CTTTGTCCA CTTGATACCT CAATAACACA ACTTCTTGTG ATTCACTTAC
61 AATCTTGAAT CTTCTTTAAA ACTTTTCTCT GTATCTCAC ACTAAGCAAATT TATCACTTTC
121 TTCATTTGCA TCTTTGCTG TTTTGTGAT TTATCGTCTTTG ATCTGTTGAT TTATCTCTTC
181 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
241 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
301 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
361 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
421 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
481 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
541 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
601 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
661 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
721 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
781 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
841 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
901 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
961 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
1021 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
1081 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
1141 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
1201 CTTTTCAGGT CTATTCTTTTT TTATTTTGTG ATCTGTTGAT TTATCTCTTC
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
Exon 11  3240 – 3326
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

1  ATCACACTCC CTTTGTCACA CTGGTAAAC CTGCTATATG CTGGCTTGTG AGTGCAATAC
61  AGTGGAAATCT CATTGCTTCC ACTTTGCTTT TTAATATGTT TATTTATGTG AGTCAAATAC
121  CTTTGTCACA CTTTGTCACA CTGGTAAAC CTGCTATATG CTGGCTTGTG AGTGCAATAC
181  TCTCTTGGT ACACTGCTGT CTTTGTCACA CACTCTTGGT ACACTGCTGT CTTTGTCACA
241  GTGGAGCTGC GCATCGCTGC GTGGAGCTGC GCATCGCTGC GTGGAGCTGC GCATCGCTGC
301  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
361  ATCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
421  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
481  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
541  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
601  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
661  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
721  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
781  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
841  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
901  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
961  TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1021 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1081 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1141 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1201 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1261 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1321 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1381 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1441 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1501 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1561 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1621 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1681 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1741 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1801 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1861 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1921 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
1981 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2041 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2101 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2161 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2221 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
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2341 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2401 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2461 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2521 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2581 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2641 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2701 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2761 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2821 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2881 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
2941 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3001 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3061 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3121 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3181 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3241 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3301 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3361 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3421 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3481 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3541 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3601 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
3661 TCTTGGT CTGGAACTAC GCTGGAACTAC TCTTGGT CTGGAACTAC TCTTGGT CTGGAACTAC
123
Exon 1 1 – 187
Intron 1 188 – 599
Exon 2 600 – 835  Start 599
Intron 2 836 – 1147
Exon 3 1148 – 1522
Intron 3 1523 – 1693
Exon 4 1694 – 1882
Intron 4 1883 – 1975
Exon 5 1976 – 2132
Intron 5 2133 – 2216
Exon 6 2217 – 2315
Intron 6 2316 – 2371
Exon 7 2372 – 2570
Intron 7 2571 – 2664
Exon 8 2665 – 2786
Intron 8 2787 – 2972
Exon 9 2973 – 3082
Intron 9 3083 – 3163
Exon 10 3164 – 3255
Intron 10 3256 – 3342
Exon 11 3343 – 3425
Intron 11 3426 – 3530
Exon 12 3531 – 3681
Intron 12 3682 – 3788
Exon 13 3789 – 3897
Intron 13 3898 – 3958
Exon 14 3959 – 4214
Intron 14 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:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer</th>
<th>N nucl</th>
<th>T ann  °C</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG161</td>
<td>161F: 5’CTCGCATTACTGGAGATGAT3’</td>
<td>20</td>
<td>58</td>
<td>1300</td>
</tr>
<tr>
<td></td>
<td>161R: 5’AAAGGCTTACATTAAGATGAT3’</td>
<td>23</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>TG237</td>
<td>237F: 5’ATTCGCTTATATGGAATGAT3’</td>
<td>22</td>
<td>57</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>237R: 5’GTCGCCCTGAAACCTGAGAG3’</td>
<td>19</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>TG255</td>
<td>255F: 5’GCTGCGATACAAACAGG3’</td>
<td>20</td>
<td>60</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>255R: 5’GAAGAGTCTAAAACATAGATGA3’</td>
<td>22</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>TG158</td>
<td>158F: 5’CCCTAAGACTCCAGAT3’</td>
<td>20</td>
<td>60</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>158R: 5’CTCGGAGAAAGGATGAG3’</td>
<td>20</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>TG269</td>
<td>269F: 5’TCTCCCCAAACATTAAACAGCA3’</td>
<td>20</td>
<td>60</td>
<td>329</td>
</tr>
<tr>
<td></td>
<td>269R: 5’AAGGCAAACCATGATAC3’</td>
<td>20</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>TG 90</td>
<td>90F: 5’GTAGACCATCTTTGATGACTAAT3’</td>
<td>26</td>
<td>70</td>
<td>esc-400</td>
</tr>
<tr>
<td></td>
<td>90R: 5’GGACTACGTATACCTTGGGCTTT3’</td>
<td>24</td>
<td>72</td>
<td>hir-200</td>
</tr>
</tbody>
</table>

Three more markers were obtained by transformation of relevant plasmids pGEM4Z (RLFP 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, SP6 primers:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer</th>
<th>N nucl</th>
<th>T ann  °C</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG267</td>
<td>267F: 5’CGCCCTCTTATCAAGAGG3’</td>
<td>19</td>
<td>62</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>267R: 5’GAGAGTTAAGTTGATGATCA3’</td>
<td>22</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>TG389</td>
<td>389F: 5’GCATGCTAACATTGATGACT3’</td>
<td>21</td>
<td>58</td>
<td>480</td>
</tr>
<tr>
<td></td>
<td>389R: 5’GCAGACTACGATGACTCAATG3’</td>
<td>20</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>TG159</td>
<td>159F: 5’GCCATAACACAGATGTCAG3’</td>
<td>21</td>
<td>62</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>159R: 5’GGACACAGATGTCATCAG3’</td>
<td>21</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>
ביוכימיה ובק妧ת מולקולרית של סינתזה עכילה

בפרירי העבינה

הنتشر לשון קבילה תואר ודוקטור לפילוסופיה

מאת

מרינה יקוב

האוניברסיטה העברית

לשדרוג את תואר דוקטור לפילוסופיה

הוגש בירחון, ירושלים

אוגוסט 2006
דר' שפר א. נועשת работе מטעם פרופ' יוסי הלל, מנכ''ל המכון לחקלאות והחקלאים במכון וינגייט, וסגן Hancock, בראשית דן, פרופ' אריה איינשטיין, ראש המכון לחקלאות והחקלאים במכון וינגייט. בלגיון ובעוד מספר מערכות נדירים באורוぱ, מקרית ובירושלים, האוניברסיטת העברית, מחקר, החינוך והחברה.
תודה

לבזרוג ילבבי: מק"ד אורי שפר על האמונת, ב"ר על הדרכה, התמקדות והנורה והמגשセット על כל אופר ודור, ע"ע הזמנ

הברח שיתוף פעולה בתקופה והאפסופיות עלibaba סוף טוב של הפרוגי

וזהו הודות

להם.

למדיריך פרפור ימי רווח על זיים בזוב, הנהו הבנ诘ות והזמנת שיתוף פעולה

הנגנה פנימית ובכלל זה בלוב מתחילה להקרير ירקות על האופירד והמגש והידני

תודה עלúbובד: ד"ר ילנה יסלו, שמואל, שן, מרים פוגלומ, רימ וגב, מיכה פום, שחר כון, גות

ע"ף נבוסים ואריך שמאם, אפי עזרו לי והו ליצידיל כל אופר בנס אופים קשר אחרון של עובד

עד"ל ד"ר אילן צ"ד רוז גרצות, ד"ר יירו ומ"ד"ר יירו בכרמי הדרחת ברוח על צורת מידה, עניין

הדרכה בכל השלבונים, ו"ד"ה לאו הקרין על הגנה התחבוזה.

במיוחד תודה להמה לד"ר רבקה הדס על הזרויה התנאנה, התמקדות מלאה, הגרות ושיחרהו.

תודה אחת שפויית הלמשחת היצורה, עבצקה על başka מיכאל, ילי וייל ודניאל, והורר אישר תמצית בזום

הנכר על כל הקשיות בזבז ולזריזה עבזוודlund לאור.
## תקציר

1. **מבוא**
   - העגבניות
     - ב פרי
     - בצימית
   - עמילן סינתזת
     - במזון
   - סינתזת עמילן
     - במזון
   - סינתזת סוכרוז
     - במזון
   - פרוקטוקינזות
     - במזון
   - ADP-Glc pyrophosphorylase
     - במזון
   - עמילן במורב
     - במזון
   - זהירות

## מטרות

## המחקר

### שיטות

- מתן צפיפות
- בדיקות סוכרים
- בדיקות אנזימים
- בדיקת ביצוע
- בדיקות אנזימים
- בדיקת ביצוע
- בדיקות אנזימים
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AGPase

5.3.7

قربת רכז גנום של שן האללים

.6

Di ולמסקנות

6.1

שיבורות של FKs

6.2

AgpL1H

6.3

העגבניה במפרי L. hirsutum

6.3.1

הצורות האלה

6.3.2

AgpL1H

6.3.3

מקביל האנזים הוא ב

6.3.4

העגבניה במפרי עמילן

6.3.5

בצפיפות האנזיםقالב

6.3.6

AgpL1H

6.3.7

מקבל המברה

6.4

סיכום

7

בעבודה המוצגות התוצאות הם מאמרים ורשימת 102

8

בבלוגופית

9

נספחים

9.1

פרומיטר של רצף DQ322687 (AgpL1E, NIL)

9.2

פרומיטר של רצף DQ322689 (AgpL1, unidentified cherry cv.)

9.3

פרומיטר של רצף DQ322688 (AgpL1, M82)

9.4

פרומיטר של רצף DQ322686 (AgpL1H, NIL)

9.5

פרומיטר של רצף DQ322685 (AgpL1, L. hirsutum LA1777)

9.6

פרומיטר של רצף DQ322683 (AgpL1E, NIL)

9.7

פרומיטר של רצף DQ322682 (AgpL1, M82)

9.8

פרומיטר של רצף DQ322684 (AgpL1, L. hirsutum LA1777)

9.9

הגן של סלם רצף DQ322683 (AgpL1E, NIL)

9.10

הגן של סלם רצף DQ322682 (AgpL1, M82)

9.11

הגן של סלם רצף DQ322684 (AgpL1, L. hirsutum LA1777)

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העונים של בטומי

11
The summary text is in Hebrew, discussing the role of fructokinase (FK) and ADP-glucose pyrophosphorylase (AGPase) in the conversion of fructose to fructan. The text explains that the decrease in the activity of these enzymes is associated with the decrease in fructan synthesis in mature fruit.

Fructokinase (FK) and ADP-glucose pyrophosphorylase (AGPase) are key enzymes in the conversion of fructose to fructan. The decrease in the activity of these enzymes is associated with the decrease in fructan synthesis in mature fruit.

Fructokinase (FK) is the enzyme responsible for the conversion of fructose to fructan, and ADP-glucose pyrophosphorylase (AGPase) catalyzes the formation of fructose-1,6-bisphosphate from fructose-1-phosphate and ADP-glucose. The decreased activity of these enzymes results in decreased fructan synthesis in mature fruit, affecting the quality and taste of the fruit.
The unprocessed unwritten form of the text is as follows:

"The two-terminal tetramer (the monomer on the other side) (AgpS1, AgpL2, AgpL1: AgpL), which forms a complex of large units (three proteins of the macromolecule, S. lycopersicum, L.) (AgpL1E, AgpL1H: AgpL)."
In the comparison between the two AGPase enzymes, significant differences were found for the genotypes between L1 and L1H alleles.

The enzymes were post-translationally modified. This modification affected the activity of the enzymes, with Pi and 3-PGA substrates. The effect was observed in both genotypes, with the AGPase-L1H enzyme being more stable than the AGPase-L1 enzyme.

The reaction was monitored in the presence of Pi and 3-PGA substrates, with the enzyme activity being measured in both genotypes.

The differences were also observed in the kinetic properties of the enzymes in the two genotypes.

The enzymes were further characterized by studying the effect of temperature on their activity. The AGPase-L1H enzyme was found to be more stable at higher temperatures than the AGPase-L1 enzyme.

The study also investigated the effect of temperature on the activity of the enzymes, with the AGPase-L1H enzyme being more stable than the AGPase-L1 enzyme.

The enzymes were further characterized by studying the effect of temperature on their activity. The AGPase-L1H enzyme was found to be more stable at higher temperatures than the AGPase-L1 enzyme.