BARD

FINAL REPORT

PROJECT NO. US-2451-94

Genetic and Biochemical Characterization of Fructose Accumulation: A Strategy to Improve Fruit Quality

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BARD Final Scientific Report

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Continuation of (Related to) Previous BARD Project: ☐ Yes ☒ No Number:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

tomato, carbohydrate, sugar metabolism, sweetness

Abbreviations used in the report, in alphabetical order:

Publications & Patents Summary (following page)

Summary of Cooperation (following page)

Budget: IS: $ US: $ Total: $

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	2	0	7	
Submitted, in review, in preparation			2	
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts				
Not refereed (proceedings, reports, etc.)				

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	2			
Issued (allowed)		1		
Licensed				

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	1	1		2
Sabbaticals				
Postdoctorates				

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

The cooperation between labortories was complementary with each pursuing different objectives and with somewhat different expertise. The diversity of approaches gave a much greater probability of success since each approach alone was somewhat high risk. In the end, success came from taking this broad-based and complementary approach.

ABSTRACT

The goal of the research project was to evaluate the potential to genetically modify or engineer carbohydrate metabolism in tomato fruit to enhance levels of fructose, a sugar with nearly twice the sweetness value of other sugars. The specific research objectives to achieve that goal were to:

1. Establish the inheritance of a fructose-accumulating trait identified in F_1 hybrids of an interspecific cross between L. hirsutum X L. esculentum and identify linked molecular markers to facilitate its introgression into tomato cultivars.

This objective was completed with the genetic data indicating a single major gene, termed *Fgr* (**F**ructose **g**lucose **r**atio), that controlled the partitioning of hexose in the mature fruit. Molecular markers for the gene, were developed to aid introgression of this gene into cultivated tomato. In addition, a second major gene encoding fructokinase 2 (FK2) was found to be a determinant of the fructose to glucose ratio in fruit. The relationship between FK2 and Fgr is epistatic with a combined synergistic effect of the two hirsutum-derived genes on fructose/glucose ratios.

2. Characterize the metabolic and transport properties responsible for high fructose/glucose ratios in fructose-accumulating genotypes.

The effect of both the Fgr and FK2 genes on the developmental accumulation of hexoses was studied in a wide range of genetic backgrounds. In all backgrounds the trait is a developmental one and that the increase in fructose to glucose ratio occurs at the breaker stage of fruit development. The following enzymes were assayed, none of which showed differences between genotypes, at either the breaker or ripe stage:invertase, sucrose synthase, FK1, FK2, hexokinase, PGI and PGM. The lack of effect of the FK2 gene on fructokinase activity is surprising and at present we have no explanation for the phenomenon. However, the hirsutum derived Fgr allele was associated with significantly lower levels of phosphorylated glucose, Glc-1-P and Glc-6-P and concomitantly higher levels of the phosphorylated fructose, Fru-6-P, in both the breaker and ripe stage. This suggests a significant role for the isomerase reaction.

3. Develop and implement molecular genetic strategies for the production of transgenic plants with altered levels of enzymes that potentially control fructose/glucose ratios in fruit.

This objective focused on manipulating hexokinase and fructokinase expression in transgenic plants. Two highly divergent cDNA clones (Frk1 and Frk2), encoding fructokinase (EC 2.7.1.4), were isolated from tomato (*Lycopersicon esculentum*) and a potato fructokinase cDNA clone was obtained from Dr. Howard Davies. Following expression in yeast, each fructokinase was identified to code for one of the tomato or potato fructokinase isoforms Transgenic tomato plants were generated with the fructokinase cDNA clone in both sense and antisense orientations and the effect of the gene on tomato plants is currently being studied.

ACHIEVEMENTS

Significance: Control of hexose ratios is plants is scientifically challenging because these sugars are key substrates in glycolysis, a fundamental pathway in cellular bioenergetics. It was not known to what extent the cell would tolerate engineering around these these key metabolites. In addition to the fundamental scientific significance, because fructose is twice as sweet as glucose, enhancing the fructose/glucose ratio is expected to strongly influence fruit sweetness with no change in total carbohydrate content. Because sweetness is a key flavor component it is anticipated that fruit with this trait would be recognized by consumers as being of significantly higher quality.

Potential for application: The potential for application of this research is very high. Indeed, seed companies are now working with tomato lines developed in this project to elevate fructose/glucose ratios in commercial tomato cultivars. In the long-term, the genetic information developed by this project will provide the basis for other tomato breeders to address this trait in a wide variety of fresh and processing tomato cultivars.

Estimated economic benefits: The global fresh and processed tomato industry produces an annual crop valued in excess of $4B. Because the trait studied in this project influences quality of the fruit, it is difficult to assign a direct value. However, it is anticipated that the higher quality would contribute to increased demand for premium tomato fruit, resulting in better prices for farmers and a social benefit to consumers who have access to a higher quality product.

Estimated impact: The traits studied and developed in this project could become a dominant feature in commercial tomato cultivars, particularly for fresh tomatoes. The US fresh tomato crop is valued at approximately $1B and cultivars with high fructose/glucose ratios could easily account for 25-50% of this market within ten years. Seed companies have expressed serious interest in developing this trait in proprietary cultivars and several active breeding programs are underway to exploit the information developed in this project.

Cooperation: The cooperation between laboratories in the US and Israel developed a sufficient "critical mass" of researchers focused on this topic to ensure continuous progress. We communicated frequently and exchanged biomaterials and expertise freely to achieve the project goals. The best example of this cooperation was in the definition of the tomato FRK genes. The

cDNAs were isolated and characterized in the US but could not be rigorously identified by sequence alone. The Israel lab took the FRK clones and expressed them in yeast to provide the basis for rigorous biochemical identification (Kanayama et. al., 1997; Kanayama et al., 1998). The cooperation between the laboratories in the US and Isreal will continue long after the expiration of this project.

FINAL REPORT

Genetic and Biochemical Characterization of Fructose Accumulation: A Strategy to Improve Fruit Quality

Background and rationale for the project

Fructose has a sweetness value nearly twice that of other soluble sugars. Because in many cases sweetness, rather than total carbohydrate content, is a primary quality attribute we reasoned that increasing fructose content would enhance sweetness without increasing total sugar content. This strategy of sweetness enhancement by altering fructose/glucose ratios avoided the need to alter source/sink relationships that are implicit in other strategies of sweetness enhancement which require the elevation of total sugar levels. Preliminary research had indicated that there existed natural genetic variation for fructose/glucose ratios in tomato and this variation provided the basis for the research project. We anticipate that the general principles developed in this project should be applicable to essentially any other fruit and would provide a strategy for quality enhancement.

Research objectives

The goal of the research project was to evaluate the potential to genetically modify or engineer carbohydrate metabolism in tomato fruit to enhance levels of fructose, a sugar with nearly twice the sweetness value of other sugars. The specific research objectives to achieve that goal were to:

Objective 1. Establish the inheritance of a fructose-accumulating trait identified in F_1 hybrids of an interspecific cross between *L. hirsutum* X *L. esculentum* and identify linked molecular markers to facilitate its introgression into tomato cultivars.

Objective 2. Characterize the metabolic and transport properties responsible for high fructose/glucose ratios in fructose-accumulating genotypes.

Objective 3. Develop and implement molecular genetic strategies for the production of transgenic plants with altered levels of enzymes that potentially control fructose/glucose ratios in fruit.

Progress towards objectives

Objective 1: *Establish the inheritance of a fructose-accumulating trait identified in F_1 hybrids of an interspecific cross between L. hirsutum X L. esculentum and identify linked molecular markers to facilitate its introgression into tomato cultivars.*

We have completely realized this objective. We have analyzed segregating F_2 populations derived from crosses between standard tomato lines and a number of genetic lines characterized by unusually high ratios of fructose to glucose. The genetic data from some of the crosses clearly showed that there was a single major gene that controlled the partitioning of hexose in the mature fruit. Molecular markers for the gene, termed *Fgr* (**F**ructose **g**lucose **r**atio) were developed, in cooperation with Dr. Ilan Levin, and these were found to be localized to the centromeric region of chromosome 4, using the *L. pennellii* introgression lines developed by D. Zamir. By comparing the marker polymorphisms in a number of independently derived lines containing the *L. hirsutum* derived *Fgr* allele, we were able to determine a very close linkage between the *Fgr* gene and the *Adh* gene, with no crossovers in any of the hundreds of plants we analyzed. The calculated distance between the two genes is less than 0.5 cM.

Further analysis of the independent segregating populations has revealed that the gene encoding fructokinase 2 (*FK2*) is a second major gene involved in determining the fructose to glucose ratio. The relationship is an epistatic one in which the *FK2-hirsutum* allele has no effect in the presence of the *Fgr-esculentum* allele. However, the combined effect of the two *hirsutum*-derived genes is synergisitic rather than additive (Table 1). The *FK2* gene was mapped to chromosome 6-3.

Table 1: Effects of the Fgr and FK2 genes on the fructose/glucose ratio in mature tomato fruit. Results presented are from a segregating F_3 population whose genotypes were determined based on molecular polymorphisms between the hirsutum (HH)- and esculentum (EE)-derived alleles.

Genotype		Glu	Fru	F/G
Fgr	FK2		(mg/gfw)	
EE	EE	14.4	15.0	1.06 c
HH	EE	11.5	18.3	1.65 b
EE	HH	14.1	15.7	1.12 c
HH	HH	9.1	19.5	2.28 a

In conclusion, the results of our research have clearly established the inheritance of the trait of fructose to glucose ratio and determined that there are two major genes, *Fgr* and *FK2*, with an epistatic complementary gene action, that control hexose partitioning in the mature fruit.

Objective 2: *Characterize the metabolic and transport properties responsible for high fructose/glucose ratios in fructose-accumulating genotypes.*

a. Developmental pattern of fructose accumulation

We have studied the effect of both the *Fgr* and *FK2* genes on the developmental accumulation of hexoses in a wide range of genetic backgrounds. In all backgrounds studied to date we have observed that the trait is a developmental one and that the increase in fructose to glucose ratio occurs at the breaker stage of fruit development. This was an important conclusion as it determined the stage of fruit development for further study of hexose metabolism in the fruit and for the comparison of metabolism between the genotypes.

b. Effects on hexose metabolism

We compared the crude enzyme activities of the key enzymes involved in hexose-P metabolism in advanced segregating populations for the trait, as determined by the molecular markers described earlier. Unfortunately, there were no significant differences in enzyme activities that could be attributed to either the *Fgr* gene or to the *FK2* gene in the presence or absence of the *Fgr-hirsutum* allele. The following enzymes were assayed, none of which showed differences between genotypes, at either the breaker or ripe stage:invertase, sucrose synthase, FK1, FK2, hexokinase, PGI and PGM. The lack of effect of the FK2 gene on fructokinase activity is surprising and at present we have no explanation for the phenomenon. However, the *hirsutum* derived *Fgr* allele was associated with significantly lower levels of phosphorylated glucose, Glc-1-P and Glc-6-P and cocomitantly higher levels of the phosphorylated fructose, Fru-6-P, in both the breaker and ripe stage. This possibly suggests a significant role for the isomerase reaction.

In an attempt to shed light on the role of the fructokinases in determining the trait we analyzed the fructokinase isozymes. We succeeded in separating three fructokinase and two hexokinase isozymes from immature and ripe tomato fruit pericarp, using HPLC ion exchange chromatography. The three fructokinase differed from one another with respect to response to fructose and Mg concentrations. FKI was characterized by both substrate (fructose) inhibition as well as Mg inhibition, FKII was inhibited by neither fructose or Mg and FKIII was inhibited by fructose but not by Mg. All three were specific to fructose with undetectable activity towards glucose. ATP was the preferred nucleotide for all three FKs but FKI showed inhibition by CTP and GTP above 1 mM. All three FKs showed competitive inhibition by ADP. The two hexokinase isozymes are more similar one to another but can be distinguished by their activities at increasing pH. The hexokinases have higher affinity to glucose, yet have activity with fructose, suggesting that they are, in fact, hexokinases. No indication of a true glucokinase was found in tomato fruits.

The two FK genes recently described (Kanayama et al. 1998) were expressed in yeast and were characterized with respect to the distinguishing characteristics of fructose, Mg and nucleotide inhibition, calculated pI values of the three enzymes together with coelution on MonoQ with the tomato pericarp enzymes. Our results indicate that FKI is the gene product of *FK2,* FKII is likely the gene product of *FK1*, and FKIII is possibly the gene product of the newly described third FK gene. During the maturation of the tomato fruit FK activity decreases dramatically. The loss of activity was due to the decrease in activity of all three FKs. Nevertheless, in the ripe fruit all three FKs were still observed.

Objective 3: *Develop and implement molecular genetic strategies for the production of transgenic plants with altered levels of enzymes that potentially control Fru/Glu ratios in fruit.*

a. Hexokinase and glucokinase

To study the role of key metabolic genes on sugar metabolism in tomato fruits we cloned *A. thaliana* hexokinase cDNA by complementation of hexokinase deficient yeast cells. The cDNA clone complements growth of hexokinase deficient yeast cells on both glucose or fructose, identifying the gene as hexokinase. The biochemical features of the enzyme coded by

the cloned cDNA was analyzed in yeast extracts. Its affinity for glucose was found to be 400 times higher than its affinity to fructose (K_m glucose = 44μM compared to K_m fructose = 17mM). Transgenic tomato plants were generated with this cDNA clone and the effect of the gene on tomato plants is currently being studied.

Recently we isolated a cDNA clone of tomato hexokinase *LeHXK1* from a tomato cDNA library. The 1884 bp cDNA of *LeHXK1* contained an open reading frame encoding a 498 amino acid protein that has about 70% identity with the two *Arabidopsis* HXKs, 82% and 91% identity with potato *StHXK2* and tobacco *NtHXK* respectively, 82% identity with a recently cloned tomato hexokinase *LeHXK2* and 98% identity with potato *StHXK1*. *LeHXK1* was mapped to chromosome 3 by means of *L. pennellii* introgression lines. To confirm the identification of *LeHXK1* as hexokinase, we expressed *LeHXK1* in triple-mutant yeast cells (*hxk1 hxk2 glk1*) which lack glucose and fructose phosphorylation ability and hence are unable to grow on either glucose or fructose. Mutant yeast cells expressing *LeHXK1* grew on both glucose and fructose. The kinetic properties of *LeHXK1* expressed in yeast were determined after purification of *LeHXK1* on HPLC - ion exchange chromatography. The affinity of *LeHXK1* enzyme to glucose and mannose was over 100 times higher than that to fructose, characteristic of plant hexokinases. In addition, *LeHXK1* activity was competitively inhibited by ADP, suggesting that *LeHXK1* is regulated by ADP/ATP ratio. Expression analysis of *LeHXK1* revealed that *LeHXK1* is expressed at different levels in all tissues tested. Yet, expression of *LeHXK1* in young tomato fruits was very high suggesting that *LeHXK1* might have an important metabolic role in fruit development, while expression in leaves was very low suggesting a regulatory role rather than a metabolic role.

In our efforts to isolate sugar phosphorylating enzymes from tomato and from *Arabidopsis* cDNA libraries, we have not obtained any true glucokinase genes (GK). In light of the enzymatic results described in the previous section, we question whether tomato has a genuine GK.

b. Fructokinase

Fructokinase genes were studied in tomato. Two highly divergent cDNA clones (*Frk1* and *Frk2*), encoding fructokinase (EC 2.7.1.4), were isolated from tomato (*Lycopersicon esculentum*). The *Frk2* cDNA encoded a deduced protein of 328 amino acids that was more than 90% identical with potato fructokinase at both the nucleotide and amino acid levels. In contrast, the *Frk1* cDNA encoded a deduced protein of 347 amino acids with only 55% amino acid identity with potato fructokinase. To confirm their identity as genuine fructokinases, the *Frk1* and *Frk2* cDNAs were expressed in a mutant yeast (*Saccharomyces cerevisiae*) line which lacks the ability to phosphorylate Glc and Fru and is unable to grow on Glc or Fru. Yeast cells expressing *Frk1* or *Frk2* were complemented to grow on fructose, but not glucose, indicating that both Frks phosphorylate fructose but not glucose. This activity was verified biochemically in extracts of the transformed yeast. Interestingly, analysis of the Frk1 enzyme expressed in yeast indicated that this fructokinase is not substrate inhibited and thus may play a major role in fructose metabolism in tomato fruit, where high levels of fructose are prevalent. Southern analysis indicated that both *Frk1* and *Frk2* hybridized to distinct genomic restriction fragments, suggesting the presence of two divergent genes each encoding a fructokinase isoform. The mRNA corresponding to Frk2 accumulated to high levels late in fruit development. Overall, the results indicated that fructokinase in tomato is encoded by at least two divergent genes which exhibit a differential pattern of expression during fruit development and also appear to have distinct kinetic properties, especially with regard to fructose inhibition.

We also obtained from H.V. Davies a fructokinase cDNA clone isolated from potato tubers and analyzed its biochemical features. Following expression in yeast, the potato fructokinase was identified to code for one of the potato fructokinase isoforms whose $K_{m\ fructose}$ is 110 mM. The enzyme produced by this clone in yeast is substrate inhibited at fructose concentrations above 1 mM similar to the potato isoform. Furthermore, *in vivo* substrate inhibition within yeast cells was also observed following proliferation of yeast cells (see attached figure). Transgenic tomato plants were generated with the fructokinase cDNA clone in both sense and antisense orientations and the effect of the gene on tomato plants is currently being studied.

c. Fructose-6-phosphatase and glucose-6-phosphatase

We have tried to isolate phosphatases of phsophorylated sugars by a functional screen with yeast cells. Yeast cells undergo sugar induced cell death (SICD) when are exposed to sugar alone. Using the Arabidopsis HXK gene we have shown that SICD phneomena is entirely dependent on the rate of sugar phosphorylation (Granot and Dai, 1997). Assuming that dephsophoruylation of phsophorylated sugars may inhibit SICD, we have trasformed yeast cells with Arabidopsis cDNA library and searched for genes that suppress SICD. Eight clones were identified and extracts from yeast cells expressing those clones were assayed for Fru-6-Pase and Glu-6-Pase activity. None of these clones appeared to have a Fru-6-Pase or Glu-6-Pase activity.

d. Production of transgenic plants

Transgenic tomato plants were generated with the potato fructokinase cDNA clone (Frk) as this clone was available before the tomato *Frk1* and *Frk2* cDNAs were cloned. Both sense and antisense Frk transgenic plants were obtained. Following regular Northern analysis and enzymatic activity of fructokinase in the transgenic tomato plants, we analyzed the effect of increased and reduced activity of fructokinase on the transgenic plants. The growth of plants with low activity of fructokinase was dramatically inhibited. However, plants with increased activity of fructokinase grew normally. It is inconclusive yet whether mature fruits have higher level of sugars.

Transgenic tomato plants expressing a ripening-specific antisense tomato *Frk1* gene were also produced and analyzed. Among 40 transgenic lines, none were determined to have reduced fructokinase mRNA or enzyme levels. This result may indicate that the ripening-specific E8 promoter may activate too late in development to effectively suppress expression of the *Frk1* gene. Additional transgenic plants were produced using a variety of alternative strategies to suppress *Frk1* and/or *Frk2* gene expression and these are still being evaluated.

Transgenic tomato plants expressing an *Arabidopsis thaliana* hexokinase (HK) gene under the 35S promoter were also produced. Independent, single-copy transgenic tomato plants

expressing the heterologous hexokinase were used to develop isogenic populations with 0, 1 or 2 copies of the *A. thaliana* hexokinase gene. Plants homozygous for the *A. thaliana* hexokinase gene were characterized by decreased chlorophyll content in the leaves, reduced rate of photosynthesis, extreme inhibition of growth, formation of necrotic lesions, and early senescence. All these phenotypes were directly correlated with increased expression and activity of the *A. thaliana* hexokinase. Reciprocal grafting experiments suggested that the inhibitory effects occurred when the hexokinase was expressed in photosynthetic tissues. In addition, seeds expressing *A. thaliana* hexokinase germinated slowly. We concluded that hexokinase activity and sugar phosphorylation regulate seed germination, plant growth, photosynthesis and senescence.

Publications

a. Refereed papers

1. Dai N., Schaffer A., Petreikov M. and Granot D. (1995). Cloning of *Arabidopsis thaliana* hexokinase cDNA by complementation of yeast cells. *Plant Physiology* 108: 879-880

2. Granot D. and Dai N. (1996). The 5' untranslated region of *Arabidopsis thaliana* calmodulin cDNA is an independent cDNA containing an open reading frame. *Planta* 198: 162-163

3. Dai N., Schaffer A., Petreikov M. and Granot D. (1996). Potato (*Solanum tuberosum* L.) fructokinase expressed in yeast exhibits inhibition by fructose of both in vitro enzyme activity and rate of cell proliferation *Plant Science* 128:191-197

4. Granot D. and Dai N. (1997). Sugar induced cell death in yeast is dependent on the rate of sugar phosphorylation as determined by *Arabidopsis thaliana* hexokinase. *Cell Death and Differentiation* 4: 555-559

5. Kanayama Y, Dai N, Granot D, Petreikov M, Schaffer A, Bennett AB (1997) Divergent fructokinase genes are differnetially expressed in tomato (*Lycopersicon esculentum* Mill.). Plant Physiol 113:1379-1384

6. Kanayama Y, Granot D, Dai N, Petreikov M, Schaffer A, Powell, A, Bennett AB (1998) Tomato fructokinases exhibit differential expression and substrate regulation. Plant Physiol 117:85-90

7. Schaffer AA, Petreikov M (1997a) Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. Plant Physiol 113:739-746

8. Schaffer AA, Petreikov M (1997b) Inhibition of fructokinase and sucrose synthase by physiological levels of fructose in young tomato fruit undergoing transient starch accumulation. Physiol Plant 110:800-806

9. Levin I, Gilboa N, Yeselson E, Shen S, Schaffer AA (2000) Fgr, a major locus that modulates the fructose to glucose ratio in mature tomato fruits. Theor Appl Genet 1000:256-262

b. Papers in preparation

1. Levin I. Cincarevsky F, Gilboa N, Yeselson E, Shen S, Schaffer AA. (2000) The fructose to glucose ratio in developing tomato fruits is determined by an epistatic relationship between the Fgr and FK2 genes.

2. Petreikov M, Granot D, Dai N, Schaffer AA (2000) The characterization of fructokinases and hexokinases in developing tomato fruit.

c. Patents and patent applications:

1. Fructokinase genes and their use in metabolic engineering of fruit sweetness. (Inventors: Bennett A.B., Kanayama Y.) U.S. Patent #6,031,154.

2. A molecular marker for the gene determining the fructose to glucose ratio in mature tomato fruit. (Inventors:I. Levin, A. Schaffer, D. Granot, N. Gilboa). State of Israel patent application C:28107..

3. A molecular marker based on the fructokinase gene determining the fructose to glucose ratio in tomato fruit. (Inventors: I. Levin, A. Schaffer, F. Cincarevsky). State of Israel patent application C:34466.