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BARD

FINAL REPORT

PROJECT NO. IS-3441-03

**Modulating Phytonutrient Content in Tomatoes
Combining Engineered Polyamine Metabolism with
Photomorphogenic Mutants**

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**Project Title: Modulating phytonutrient content in tomatoes combining
engineered polyamine metabolism with photomorphogenic mutants**

Investigators

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Keywords: Lycopene, carotenoids, *high-pigment*, flavonoids, photomorphogenesis.

Abbreviations: *hp* – *high pigment*, yeast S-adenosylmethionine decarboxylase – ySAMdc.

Budget: IS: \$139,000

US: \$201,000

Total: \$340,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged		1	1	
Submitted, in review, in preparation			1	
Invited review papers		2	1	
Book chapters				
Books				
Master theses			1	
Ph.D. theses				
Abstracts			11	
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1		1
Longer Visits (Sabbaticals)				

Description Cooperation: The collaboration between the US and Israel was mainly underlined by the exchange of genetic material and methods between the two countries. This genetic material was used in classical breeding in Israel and in transgenic transformation in the US.

Patent Summary (numbers)

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

Abstract

Fruit constitutes a major component of our diet, providing fiber, vitamins, minerals, and many other phytonutrients that promote good health. Fleshy fruits, such as tomatoes, already contain high levels of several of these ingredients. Nevertheless, efforts have been invested in increasing and diversifying the content of phytonutrients, such as carotenoids and flavonoids, in tomato fruits. Increasing levels of phytonutrients, such as lycopene, is highly justified from the perspective of the lycopene extraction industry due to cost effectiveness reasons. Diversifying phytonutrients, in particular those that contribute to fruit color, could potentially provide an array of attractive colors to our diet.

Our major goal was to devise a novel strategy for developing tomato fruits with enhanced levels of phytochemicals known to promote good health with special emphasis on lycopene content. A further important goal was to analyze global gene expression of selected genetic lines produced throughout this study in order to dissect the molecular mechanisms regulating phytonutrients accumulation in the tomato fruit. To achieve these goals we proposed to: 1. combine, by classical breeding, engineered polyamine metabolism with photomorphogenic *high pigment* mutants in order to generate tomato plant with exceptionally high levels of phytonutrients; 2. use gene transfer technology for genetic introduction of key genes that promote phytonutrient accumulation in the tomato fruit, 3. Analyze accumulation patterns of the phytonutrients in the tomato fruit during ripening; 4. Analyze global gene expression during fruit ripening in selected genotypes identified in objectives 1 and 2, and 5. Identify and analyze regulatory mechanisms of chloroplast disassembly and chromoplast formation.

During the 3 years research period we have carried out most of the research activities laid out in the original proposal and our key conclusions are as follows: 1. the engineered polyamine metabolism strategy proposed by the US collaborators can not increase lycopene content either on its own or in combination with an *hp* mutant (*hp-2^{dg}*); 2. The *hp-2^{dg}* affects strongly the transcriptional profile of the tomato fruit showing a strong tendency for up- rather than down-regulation of genes, 3. Ontology assignment of these miss-regulated genes revealed a consistent up-regulation of genes related to chloroplast biogenesis and photosynthesis in *hp-2^{dg}* mutants throughout fruit development; 4. A tendency for up-regulation was also usually observed in structural genes involved in phytonutrient biosynthesis; however this up-regulation was not as consistent. 5. Microscopic observations revealed a significantly higher number of chloroplasts in pericarp cells of mature-green *hp-2^{dg}/hp-2^{dg}* fruits in comparison to their normal fully isogenic counterparts. 6. The relative abundance of chloroplasts could be observed from early stages of fruit development.

Cumulatively these results suggest that: 1. the overproduction of secondary metabolites, characterizing *hp-2^{dg}/hp-2^{dg}* fruits, is more due to chloroplast number rather than to transcriptional activation of structural genes of the relevant metabolic pathways, and 2. The molecular trigger increasing metabolite levels in *hp-2^{dg}* mutant fruits should be traced at early stage of fruit development.

Achievements

Objective 1. combine engineered polyamine metabolism with photomorphogenic high pigment mutants in order generate tomato plant with exceptionally high levels of phytonutrients: Plants from two transgenic lines homozygous for the yeast S-adenosylmethionine decarboxylase gene (ySAMdc) were crossed with a breeding line homozygous for the *hp-2^{dg}* mutations (n-935). F₁ plants resulting of these crosses were allowed to self-pollinate to generate F₂ plants that that segregated for both the ySAMdc transgene and the *hp-2^{dg}* mutation. We have planted these F₂ plants and further obtained F₃ plants harboring both the ySAMdc transgene and the *hp-2^{dg}* mutation in homozygous state. These F₃ plants were used in a field trail carried in Israel during the summer season of 2005.

Objective 2. To use gene transfer technology for genetic introduction of key genes that promote phytonutrient accumulation in the tomato fruit: During the first year of research we have developed, in collaboration with the laboratory of Dr. Benjamin Steinitz, Volcani, ARO transformation protocols suitable for *hp* mutants, known for their low transformation capacity. The *hp-2^{dg}* line n935 was directly transformed with ySAMdc under the fruit specific E8 promoter in the US. Several independent lines transformed with ySAMdc gene were generated and validated for the ySAMdc gene expression. Using this protocol we have also managed to introduce, under a constitutive promoter, the tomato the gene encoding UV DAMAGED DNA BINDING protein 1 (*DDB1*) that was recently identified by us as the gene that causes the *hp-1* and *hp-1^w* mutant phenotypes that are also characterized by increased fruit phytonutrient content.

Objective 3. Analyze accumulation patterns of the phytonutrients in the tomato fruit during ripening, with particular emphasis on carotenoids, flavonoids, vitamin C and polyamines: During the course of this research we have analyzed the accumulation patterns phytonutrients in tomato plants carrying the ySAMdc transgene. These analyses were carried out during 4 consecutive seasons on ySAMdc transgenic plants produced either by classical crosses or by transgenic methods. During these 4 seasons we have analyzed transgenic parental lines, F₁ plants heterozygous for both the *hp-2^{dg}* mutation and the ySAMdc transgene, and finally: plants homozygous for both the *hp-2^{dg}* mutation and the ySAMdc transgene. These experiments, carried out in all possible environments: in the greenhouse and open-field, during winter and summer seasons, in Israel and the US, unexpectedly failed to

reproduce the positive effect of ySAMdc transgene on our prime metabolite of interest – lycopene.

Objective 4. Analyze global gene expression during fruit ripening in selected genotypes identified in objectives 1 and 2: We have recently finalized a comprehensive analysis of global gene expression of *hp-2^{dg}* mutant plants. These results were submitted to Plant physiology. Global gene expression of ySAMdc transgenic plant is currently in progress.

Objective 5. Identify and analyze regulatory mechanisms of chloroplast disassembly and chromoplast formation: The initial plan was to achieve this objective by using electron microscopy, immunological studies and gene expression to analyze the function of polyamines in nutrient accumulation in chromoplasts during fruit ripening. We have made preliminary strides towards achieving this goal, however, we also employed an alternative strategy to address this objective in a more refined and direct manner. The alternate strategy used metabolite profiling analysis of the transgenic and non-transgenic tomato fruits to discern biochemical pathways impacted by high polyamines, spermidine (Spd) and spermine (Spm). As the sub-cellular sources of the metabolites are more or less known, we predicted, we would be able to synthesize the information derived to give information about chloroplast-chromoplast metabolism as well.

We found the same metabolites present in wild-type or azygous control tomatoes are also present in the higher polyamines-accumulating, transgenic tomato fruit. However, accumulation of Spd and Spm in transgenic fruit results in differential metabolite content as compared to the controls, data that suggest that Spd/Spm are sensed by the fruit cells as organic-N which, in turn, signals carbon metabolism. These results, for the first time, show that Spd and Spm have profound effects on cellular metabolism, likely via regulation of distinct biochemical pathways.

Significant changes were found in the levels of choline, Glu, Gln, Asp, Asn, Thr, GABA, citrate, glucose, sucrose and several unidentified compounds in the transgenic tomatoes during ripening compared to the wild-type/azygous controls. Statistical analysis validated the changes in the metabolite profiles, certain variables were active at particular stage while others were active at another phase of fruit ripening. For example: Asn, Gln and choline were active throughout ripening whereas citrate, glucose and MeOH, likely indicators of changing source of carbon, were active only during the later phase of fruit ripening.

Another linkage unraveled by the metabolite profiling analysis is the one between polyamines and the methyl donor choline. The accumulation of choline in the polyamine accumulating, transgenic tomato fruit during ripening was highly significant. Choline has been classified as a 'vital amine' for human health and has an essential role as a micronutrient required for brain development. It is the precursor of membrane phospholipids, intracellular messengers diacylglycerol and ceramide, signaling lipids platelet-activating factor and sphingosylphosphorylcholine, and neurotransmitter acetylcholine. These data show that higher Spd/Spm levels do indeed positively affect nutritional attributes of tomato fruit.

List of publications

Steinitz B, Amitay A, Gaba V, Tabib Y, Keller M, Levin I (2006) A simple plant regeneration-ability assay in a range of *Lycopersicon* species. *Plant Cell Tiss Organ Cult* 84: 269-278

Srivastava A, Handa AK (2005) Hormonal regulation of fruit development: A molecular perspective. *Journal of Plant Growth Regulation* (In Press)

Handa AK, Srivastava A, Perla V (2005) Hormonal control of fruit ripening. *In* Genetic improvement of solanaceous crops (Razdan, M.K. and Mattoo, A.K., eds). Science Publishers Inc. New Hampshire, USA. (In Press).

Mattoo AK, Sobolev A, Goyal R, Neelam A, Handa AK Segre AL (2006) NMR Spectroscopy Based Metabolite Profiling of Transgenic Tomato Fruit Engineered to Accumulate Spermidine and Spermine Reveals Enhanced Anabolic and Nitrogen-Carbon Interactions. *Plant Physiology* (in press, on line).

Levin I, de Vos CHR, Tadmor Y, Bovy A, Lieberman M, Oren-Shamir M, Segev O, Kolotilin I, Keller M, Ovadia R, Meir A, and Bino R J (2006) *High pigment* tomato mutants- more than just lycopene (a review). *Israel Journal of Plant Sciences* (in press).

Kolotilin I, Koltai H, Tadmor Y, Bar-Or C, Reuveni M, Meir A, Nahon S, Shlomo H, Chen L, Levin I (2006) Transcriptional profiling of *high pigment-2^{dg}* tomato mutant links early fruit plastid biogenesis with its overproduction of phytonutrients. *Plant Physiology* (Submitted).

Caspi N (2006) Functional analysis of the tomato DAMAGED DNA BINDING PROTEIN 1 (DDB1). M.Sc. thesis, Tel-Aviv University.

Appendix

Table of Content

Published, in press or submitted papers	Page
Steinitz et al. (2006) Plant Cell Tiss. Organ Cult. 84: 269-278.	
Levin et al. (2006) Israel Journal of Plant Sciences, in press	
Mattoo et al. (2006) Plant Physiology, in press, on line	
Kolotilin et al. (2006) Plant Physiology, submitted	