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BARD

FINAL REPORT

PROJECT NO. IS-2709-96

**Biotechnology of Tomato Volatiles for Flavor
Improvement**

E. Lewinsohn, E. Pichersky, S. Gepstein

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Cover Page**



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Project Title: "Biotechnology of Tomato Volatiles for Flavor improvement"

Investigators

Institutions

Principal Investigator (PI):

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Collaborating Investigators:

Shimon Gepstein

The Technion

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

Abbreviations **commonly** used in the report, in alphabetical order:

Budget: IS: \$

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US: \$

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Total: \$

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Signature
Principal Investigator

Signature
Authorizing Official. Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	3			3
Submitted, in review, in preparation	1			1
Invited review papers				
Book chapters				
Books				
Master theses			1	1
Ph.D. theses				
Abstracts	3			3
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.

Orit Amar	I.D. 22897227
David Gang	S.S. 528-25-2326
Kyoung Hee Nam	S.S. 375-21-9124

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	2	1		3
Longer Visits (Sabbaticals)				

Description of Cooperation:

During these visits, relevant information was exchanged to jointly address research problems and also planned for the future. Transgenic tomato samples were jointly analyzed. Thanks in part to BARD funding, we have established a lasting long-term relationship accompanied by many fruitful collaborative projects.

Patent Summary (numbers)

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

BARD Project No. IS- 2709-96.--- Final Report
"Biotechnology of Tomato Volatiles for Flavor improvement"

Efraim Lewinsohn, Eran Pichersky and Shimon Gepstein

Abstract

The main objectives of the research project were:

1. The manipulation, by genetic engineering techniques, of the terpenoid pathway in tomato fruit. Specifically, to test the hypothesis whether overexpression of linalool synthase in tomato fruits will result in the diversion of intermediates of the carotene biosynthetic pathway to linalool, demonstrating that linalool synthase is a key regulatory enzyme, and possibly improving tomato flavor.
2. The elucidation of the biochemical pathway leading to eugenol and methyl eugenol, and the manipulation of this pathway to determine key enzymes and to improve flavor in tomato.

Background, conclusions and implications

The different proportions of volatile components present in foods often determine their flavor properties. Two of the ten most important flavor compounds in tomatoes, linalool and eugenol, are emitted by the flowers of *Clarkia breweri*, (Onagraceae), a plant native to California, and are also present in sweet basil (*Ocimum basilicum*, Lamiaceae). We have studied the key enzymes and genes involved in the production of these flavorants. Linalool synthase, the key enzyme in linalool biosynthesis and its corresponding gene were isolated and characterized from *Clarkia breweri*. The gene was coupled to a fruit-specific tomato promotor (E8) and was used to transform tomatoes. The transgenic tomatoes produced S-linalool and 1-hydroxylinalool, compounds absent from the fruits of controls. The transgenesis did not adversely affect the overall appearance of the plants nor the levels of other terpenoids present such as carotenoids and vitamin E. Our work has proven that the terpenoid pathway in tomatoes can be modified by the introduction and expression of foreign genes coding for the enzymes controlling the production of monoterpenoid flavor compounds. We have also isolated novel enzymes and genes that are involved in the formation of eugenol and methyl eugenol from *Clarkia breweri* and basil. An EST library of basil glandular trichomes (the site of eugenol and methyl eugenol biosynthesis) was prepared. More than 1,200 genes have been preliminary characterized and a few of them have been confirmed by functional expression, to be involved in eugenol and methyl eugenol biosynthesis. These genes have augmented the still small repertoire of genes that are available to modify the aroma of agricultural produce by genetic engineering.

manipulating the aroma. The work with transgenic plants up to the first generation was done in collaboration with Calgene, Inc., USA.

In addition to the successful manipulation of the early terpenoid pathway in tomato fruits, and within the framework of this project, we have started an investigation concerning the enzymatic and molecular mechanisms by which volatile phenylpropanoid compounds known to contribute to flavor in tomatoes are produced. Using flowers of *Clarkia breweri*, and glandular trichomes of sweet basil, as model systems that produce copious quantities of these compounds, we have identified several of the enzymes and genes involved in the production of allyl phenols such as eugenol and methyl eugenol. Eugenol and methyl eugenol are derived from phenylalanine, but their exact biosynthetic pathway is still unknown. In basil, these compounds are formed in anatomically specialized glandular trichomes. By preparing an EST library from mRNA obtained from isolated glandular trichomes, we have been able to partially identify and characterize about 1,200 EST's. Many of these EST's are involved in the production of the phenylpropanoid and other aroma compounds found in basil leaves. Several genes coding for O-methyltransferases involved in the formation of ferulic acid, estragole and methyl eugenol have been functionally expressed in bacterial cells, and thus biochemically identified. The availability of these and other genes that influence the formation of the tomato and other aromas, coupled to other biotechnological advances, will permit in the future to obtain superior agricultural produce with improved aroma characteristics.

Description of the cooperation.

The collaborating partners communicate regularly by E-mail, and discuss the results obtained in this and other collaborative projects. E. Pichersky visited Newe Ya'ar and the Technion on several occasions during the duration of this project and E. Lewinsohn visited the University of Michigan several times also during the duration of the project. During these mutual visits, transgenic tomato samples were jointly analyzed, and also relevant information was exchanged to jointly address research problems and also planned for the future. These interactions have greatly contributed specifically to the progress made in this project and promoted the successful

Achievements

Much of the research in fruit and vegetable breeding and improvement carried out during the last decades has been focused on obtaining desirable agronomical characteristics such as resistance to environmental stresses, pests and pathogens, controlling sugar/acid ratios and improving texture and storage characteristics of the products. Breeding towards improving the aroma of tomatoes has been limited. The lack of selection specifically aimed for aroma and flavor traits has been especially problematic because selection for fruit traits such as size and color often has often resulted for reasons that are not completely clear in the loss of these traits, a problem that genetic engineering is ideally suited to solve. We have looked into the possibility of improving the aroma and flavor of tomato fruits by first identifying genes that code for enzymes responsible for the formation of aroma compounds. Overexpression of selected genes coding for such key enzymes could divert the normal metabolic flow in the fruits to overproduce aroma chemicals, with the end result of improving taste. The genes examined originated from *Clarkia breweri* flowers (Onagraceae), an annual plant native to California, and included linalool synthase (LIS), a gene that codes for an enzyme that catalyzes the conversion of the central terpenoid precursor geranyl diphosphate (GPP) to the formation of *S*-linalool. The coding region of the LIS gene was fused to a tomato fruit-late-ripening-specific promoter (E8). Constructs were mobilized into *Agrobacterium* and tomato plants were transformed. Transgenic tomato plants were detected by the polymerase chain reaction using primers specific for LIS sequences. The volatiles present in the fruits of LIS -transformed tomatoes were analyzed, and found to contain elevated levels of linalool in all cases. Only the *S*-enantiomer of linalool was found in fruits. The transgenic fruits not only accumulate *S*-linalool, but novel compounds, such as 1-hydroxylinalool were found. LIS-transgenic fruits, probably derived by allylic hydroxylation of the linalool formed in ripening fruits. *S*-Linalool and 1-hydroxylinalool were absent in fruits from non-transformed controls. Apart from the difference in volatiles, no other phenotypic alterations were noted, including the levels of endogenous terpenoids such as γ - and α -tocopherols, lycopene, β -carotene and lutein. The trait was inherited at least for three generations. Our studies indicate that it is possible to enhance the levels of monoterpenes in ripening fruits by metabolic engineering, with the aim of

completion of the project, as planned. Due in a great part to BARD funding, the partners have established a lasting long-term relationship accompanied by many fruitful collaborative projects. In fact, E. Pichersky is currently in E. Lewinsohn's laboratory as a Sabbatical fellow, and future collaborations are being planned and put forward. The contribution of Prof. S. Gepstein has been material both in theoretical and practical issues of the work. A joint M.Sc. student and a postdoctoral research associate from the department of Biology in the Technion were involved in the research, greatly contributing to the project's success.

List of publications reporting on work supported by this grant

- Wang, J., Dudareva, N., Kish, C.M., Simon, J.E., Lewinsohn, E., and Pichersky, E. (1999) Nucleotide sequences of two cDNAs encoding caffeic acid *O*-methyltransferases (accession nos. AF154917 and AF154918) from sweet basil. Plant Physiol 120: 1205. (*Plant Gene Register* 99-105).
- Lewinsohn, E., Ziv-Raz, I., Dudai, N., Tadmor, Y., Lastochkin, E., Larkov, O., Chaimovitch, D., Ravid, U., Putievsky, E., Pichersky, E., and Shoham, Y. (2000) Biosynthesis of estragole and methyl-eugenol in sweet basil (*Ocimum basilicum* L.). Developmental and chemotypic association of allylphenol *O*-methyltransferase activities. Plant Science 160: 27-35.
- Gang, D.R., Wang, J., Dudareva, N., Nam, K.H., Simon, J., Lewinsohn, E., and Pichersky, E. (2001) An investigation of the storage and biosynthesis of phenylpropenes in sweet basil (*Ocimum basilicum* L.). Plant Physiology 125: 539-555.
- Lewinsohn, E., Schalechet F., Wilkinson, J., Matsui, K., Tadmor, Y., Nam, K.H., Amar, O., Lastochkin, E., Larkov, O., Ravid, U., Hiatt, W., Gepstein, S., Pichersky, E. (2001) Enhanced levels of the aroma and flavor compound *S*-linalool by metabolically engineering the terpenoid pathway in tomato fruits. Plant Physiol (*Submitted*).

Appendix

Enclosed are three copies of each of the publications stated above, and of the cover and abstract of the Master of Science's thesis by Fernand Shalechet, under the joint guidance of Prof. S. Gepstein and Dr. E. Lewinsohn. The thesis has been submitted and approved by the Technion's program in Biotechnology and is available upon request.