Final Scientific Report

Section 1. Overall summary and statistics

- *Cover Page* (Appendix G6a)
- *Publication Summaries* (Appendix G6b)
- *Abstract* (one page maximum, single spaced)
- *Achievements* (three pages maximum, 1.5 spaced, font not smaller than 12)
- *Details of cooperation*
- *List of Publications: Include only reviewed publications reporting on work at least partially supported by BARD and which includes an acknowledgement to BARD.*

Final Scientific Report

Cover Page

BARD Project Number: US-4423-11

Date of Submission of the report: April 30, 2015

Project Title: Characterization of the *Or* regulatory network in melon for carotenoid biofortification in food crops

Investigators

Principal Investigator (PI):

Li Li

Co-Principal Investigator (Co-PI):

Joseph Burger

Collaborating Investigators:

Nurit Katzir

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Institutions

USDA-ARS

ARO, Min. Ag

ARO, Min. Ag

ARO, Min. Ag

ARO, Min. Ag

USDA-ARS

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

Chromoplast; Fruit; Provitamin A; Bulk segregant transcriptome analysis; Single nucleotide polymorphism

Abbreviations commonly used in the report, in alphabetical order:

BSRseq, RNA-Seq, SNP

Budget: IS: \$143,000

US: \$157,000

Total: \$300,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Final Scientific Report

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	2	1		3
Submitted, in review, in preparation	2			2
Invited review papers				
Book chapters	1			1
Books				
Master theses				
Ph.D. theses				
Abstracts	4			4
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.

Dr. Hui Yuan - 708 734 289

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1	3	5
Longer Visits (Sabbaticals)				

Description Cooperation:

Our collaborative research has been truly synergistic that bring different expertise and resources together from two countries. In addition to meet in face with all collaborators via short visits and meetings, we routinely exchange data and ideas through emails, skype and phone calls. As a result, our collaboration is productive and contribute greatly to advance our general understanding carotenoid accumulation in plants. Moreover, effective genetic tools and molecular markers have been developed for carotenoid biofortification in food crops.

Patent Summary (numbers)

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

Abstract

The general goals of the BARD research grant US-4423-11 are to understand how *Or* regulates carotenoid accumulation and to reveal novel strategies for breeding agricultural crops with enhanced β -carotene level. The original objectives are: 1) to identify the genes and proteins in the *Or* regulatory network in melon; 2) to genetically and molecularly characterize the candidate genes; and 3) to define genetic and functional allelic variation of these genes in a representative germplasm collection of the *C. melo* species.

Or was found by the US group to causes provitamin A accumulation in chromoplasts in cauliflower. Preliminary genetic study from the Israeli group revealed that the melon *Or* gene (*CmOr*) completely co-segregated with fruit flesh color in a segregating mapping population and in a wide melon germplasm collection, which set the stage for the funded research.

Major conclusions and achievements include:

- 1). *CmOr* is proved to be the gene that controls melon fruit flesh color and represents the previously described *gf* locus in melon.
- 2). Genetic and molecular analyses of *CmOr* identify and confirm a single SNP that is responsible for the orange and non-orange phenotypes in melon fruit.
- 3). Alteration of the evolutionarily conserved arginine in an OR protein to both histidine or alanine greatly enhances its ability to promote carotenoid accumulation.
- 4). OR promotes massive carotenoid accumulation due to its dual functions in regulating both chromoplast biogenesis and carotenoid biosynthesis.
- 5). A bulk segregant transcriptome (BSRseq) analysis identifies a list of genes associated with the *CmOr* regulatory network.
- 6). BSRseq is proved to be an effective approach for gene discovery.
- 7). Screening of an EMS mutation library identifies a *low β* mutant, which contains low level of carotenoids due to a mutation in *CmOr* to produce a truncated form of OR protein.
- 8). *low β* exhibits lower germination rate and slow growth under salt stress condition.
- 9). Postharvest storage of fruit enhances carotenoid accumulation, which is associated with chromoplast development.

Our research uncovers the molecular mechanisms underlying the *Or*-regulated high level of carotenoid accumulation via regulating carotenoid biosynthetic capacity and storage sink strength. The findings provide mechanistic insights into how carotenoid accumulation is controlled in plants. Our research also provides general and reliable molecular markers for melon-breeding programs to select orange varieties, and offers effective genetic tools for pro-vitamin A enrichment in other important crops via the rapidly developed genome editing technology. The newly discovered *low β* mutant could lead to a better understanding of the *Or* gene function and its association with stress response, which may explain the high conservation of the *Or* gene among various plant species.

Achievements

1) Identify and characterize a golden SNP in *CmOr* that governs melon fruit flesh color (Published in *The Plant Journal*)

We have defined *CmOr* as the single gene that controls fruit flesh color in melon. It is also the gene as the previously described *gf* locus in melon. We demonstrate that a single SNP (single nucleotide polymorphism) in *CmOr* is responsible for the orange and non-orange phenotypes in melon fruit. *CmOr* with the “golden” SNP can serve as a novel and critical molecular tool to investigate the molecular mechanisms underlying the *Or*-induced carotenoid accumulation in plants. The research findings are agriculturally important in 1) providing general and reliable molecular markers for melon-breeding programs to select orange varieties, and 2) offering an effective genetic tool for pro-vitamin A enrichment in other important crops. Since the allelic variation of *CmOr* is limited to a single SNP, it is possible to create functional alleles of *Or* in many agriculturally important crops via the rapidly developed genome editing technology.

2) Discover the dual functions of *Or* and the effectiveness of the single amino acid substitution in an *Or* protein to confer carotenoid accumulation in plants (Under revision for *Plant Physiology*)

We demonstrate the effectiveness of alteration of a single amino acid in any wild type OR protein in promoting high levels of carotenoid accumulation. The molecular basis for the *Or*-induced massive carotenoid accumulation is due to its dual functions in regulating phytoene synthase activity to increase biosynthetic capacity and in triggering chromoplasts differentiation to enhance storage sink strength. Furthermore, during the study we have established an effective Arabidopsis callus system for functional study of genes involved in chromoplast biogenesis and carotenoid accumulation. The findings not only further strengthens our melon discovery for *OR* as a powerful tool for carotenoid enrichment in plants, but also provide new insights into the mechanisms underlying carotenoid accumulation in plants. The new genetic tools identified and knowledge developed here will have broad applications for nutritional biofortification in many important food crops.

3) Identify genes in the melon *Or* regulatory network via a bulk segregant transcriptome (BSRseq) analysis and functionally analyze them (Manuscript to be submitted to *BMC Plant Biology*)

The metabolic and cellular processes associated with *CmOR* allelic variation and a list of genes that participate in these processes were identified via BSRseq analysis of large families of orange- and green-flesh fruit bulks. Moreover, the effectiveness of the bulk approach for gene discovery was also demonstrated in this study. Sequence variations in some of the identified genes may be involved in the quantitative regulation of carotenoid accumulation in melon fruit and could be applied as new genetic tools for breeding high carotenoid content melons. The BSRseq approach identified an increasing fruit sucrose effect that is associated with *CmOr* green allele, probably due to a genetic linkage to an acid invertase gene.

4) Identification of *low β* mutant

Visual screening of an EMS mutation library of 'CEZ', a 'Charantais' type (orange flesh) melon, identified an M₂ family that segregates recessively for a green flesh phenotype. We self-pollinated one of these plants to develop a homozygous green flesh *low β* mutant line. A complementation test for allelism indicates that *low β* is mutated in *CmOr*. Quantitative RT-PCR analysis of *CmOr* mRNA levels during fruit development indicates that *CmOr* expression is dramatically reduced in *low β*. We sequenced the region of His¹⁰⁸ and found that the mutant still carries His¹⁰⁸. Whole gene sequence analysis reveals a transition of A⁴⁸⁷ to T that alters CmOr protein Lys¹⁶³ to a premature 'Stop' codon (AAG to TAG). This mutant and its wild type NIL progenitor could serve as an invaluable tool for future characterization of *Or* gene function. Preliminary results indicate that *low β* is more sensitive to salt stress than its wildtype NIL, indicating a possible role of OR in plant stress response.

5) Other related achievements (Published in *Planta*, *Archives of Biochemistry and Biophysics*, and in *Plant Biotechnology: Experience and Future Prospects*)

We extend fruit carotenoid research during ripening into postharvest and discover that storage of a winter squash leads to a continuous increase of carotenoid level likely through regulating chromoplast development. Postharvest loss of provitamin A in staple crops is a serious problem. The discovery of chromoplasts as stable storage organelles for carotenoids from this

and our previous *Or* research implies the potential of creating proper metabolic sink for carotenoid biofortification in major staple crops. In addition, we provide the research community the recent progress toward our understanding of various processes of chromoplast biogenesis and an integrated view of chromoplast biogenesis and carotenoid accumulation in plants, as well as approaches for vegetable and fruit quality trait improvement via novel gene discovery with natural variation in germplasm of fruit and vegetables.

Details of cooperation

The USDA-ARS group has broad expertise in functional characterization of genes in plants and in bioinformatics research on cucurbit, and discovered the *Or* gene. The ARO team is one of the leading groups in the breeding, genetics, and genomics of melon with emphasis on melon fruit quality, and found the melon *Or* orthologous gene that co-segregates in melon segregating mapping populations and in a broad germplasm collection. The US scientists conducted the molecular, biochemical, and bioinformatics aspects of the reported achievements, which include functional characterization of melon *Or* and other plant *Or*, RNA-seq experiments, and bioinformatics analysis of the differentially expressed genes in the *Or* network. The Israeli scientists carried out genetic and some molecular aspects of the research, which include selection and growth of melon fruit materials, preparation of melon RNA and protein samples for BSRseq and biochemical analysis of OR in the US labs, validation of RNA-seq result following bioinformatics analysis from the US labs, analysis of candidate gene expression patterns during fruit development, and association of candidate genes in segregating mapping populations. All collaborators were meet in face via short visits and meetings. In addition, we routinely exchange data and ideas through emails, skype and phone calls. As a result, our collaboration is productive. Because of synergistic nature of our collaboration, the project objectives would not be achieved without such close collaboration that brings different expertise and resources together from two countries.

List of publications

Published:

1. Tzuri G*, Zhou X*, Chayut N*, Yuan H, Portnoy V, Meir A, Saar U, Baumkoler F, Yuan H, Mazourek H, Lewinsohn E, Fei Z, Schaffer AA, Li L, Burger J, Katzir N. Tadmor Y. (2015) A “golden” SNP in CmOr governs fruit flesh color in melon (*Cucumis melo*). *The Plant Journal* 82:267-279
2. Zhang MK, Zhang MP, Mazourek M, Tadmor Y, Li L (2014) Regulatory control of carotenoid accumulation in winter squash during storage. *Planta* 240:1063-1074
3. Li L, Tadmor Y, Xu Q (2014) Approaches for vegetable and fruit quality trait improvement. In *Plant Biotechnology – Experience and Future Prospects* (Edited by A. Richoch, S Chopra, S Fleischer). Springer, Chapter 18, pp227-243
4. Li L, Yuan H (2013) Chromoplast biogenesis and carotenoid accumulation. *Archives of Biochemistry and Biophysics* 539: 102-109

Under revision and ready to be submitted:

5. Yuan H, Owsiany K, Sheeja T, Zhou X, Rodriguez C, Li Y, Welsch R, Chayut N, Yang Y, Thannhauser TW, Partjasaratju MV, Xu Q, Deng X, Fei Z, Schaffer A, Katzir N, Burger J, Tadmor Y, Li L. (2015) A single amino acid substitution of the orange protein causes carotenoid accumulation in Arabidopsis. *Plant Physiology* (under revision)
6. Chayut N, Yuan H, Fei Z, Schaffer A, Katzir N, Burger J, Li L, Tadmor Y (2015) A bulk segregant transcriptome analysis spotlights a link identified metabolic and cellular processes associated with *CmOR* allelic variation (to be submitted to *BMC Plant Biology*)

Section 2. Appendix

- *Published papers.*
- *One copy of each ‘in press’, ‘accepted’ or ‘submitted’ papers.*

Published papers (4)

1. Tzuri G*, Zhou X*, Chayut N*, Yuan H, Portnoy V, Meir A, Saar U, Baumkoler F, Yuan H, Mazourek H, Lewinsohn E, Fei Z, Schaffer AA, Li L, Burger J, Katzir N. Tadmor Y. (2015) A “golden” SNP in CmOr governs fruit flesh color in melon (*Cucumis melo*). *The Plant Journal* 82:267-279
2. Zhang MK, Zhang MP, Mazourek M, Tadmor Y, Li L (2014) Regulatory control of carotenoid accumulation in winter squash during storage. *Planta* 240:1063-1074
3. Li L, Tadmor Y, Xu Q (2014) Approaches for vegetable and fruit quality trait improvement. In *Plant Biotechnology – Experience and Future Prospects* (Edited by A. Richoch, S Chopra, S Fleischer). Springer, Chapter 18, pp227-243
4. Li L, Yuan H (2013) Chromoplast biogenesis and carotenoid accumulation. *Archives of Biochemistry and Biophysics* 539: 102-109

Copy of “under revision” and “submitted” paper (2)

1. Yuan H, Owsiany K, Sheeja T, Zhou X, Rodriguez C, Li Y, Welsch R, Chayut N, Yang Y, Thannhauser TW, Partjasaratju MV, Xu Q, Deng X, Fei Z, Schaffer A, Katzir N, Burger J, Tadmor Y, Li L. (2015) A single amino acid substitution of the orange protein causes carotenoid accumulation in Arabidopsis. *Plant Physiology* (under revision)
2. Chayut N, Yuan H, Fei Z, Schaffer A, Katzir N, Burger J, Li L, Tadmor Y (2015) A bulk segregant transcriptome analysis spotlights a link identified metabolic and cellular processes associated with *CmOR* allelic variation (to be submitted to *BMC Plant Biology*)