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BARD Project Number: IS-4033-07

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Project Title: Micro RNA targeted transcription factors for fruit quality improvement

Investigators

Principal Investigator (PI):

Tzahi Arazi

Co-Principal Investigator (Co-PI):

Vivian Irish

Collaborating Investigators:

Asaph Aharoni

Institutions

ARO, Volcani Center

Yale University

Weizmann Institute

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

DICER, miRNA

Abbreviations commonly used in the report, in alphabetical order: miRNA –micro RNA; siRNA – short interfering RNA

Budget: IS: \$74,000

US: \$36,000

Total: \$110,000

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) BARD support acknowledged				
Submitted, in review, in preparation				
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts				
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				
Longer Visits (Sabbaticals)				

Description of cooperation:

The US and IS collaborators communicated results and research strategies regularly by phone and email. The Arazi and Irish groups focused on detailed expression analysis of selected miRNA targets previously validated by us. The Aharoni group focused on the cloning, expression analysis and down regulation of tomato DCL1 in the fruit. These are parallel and complementary studies to identify pathways and miRNA targets that are important for fruit development.

Patent Summary (numbers)

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

Abstract

Fruits are unique to flowering plants and represent an important component of human and animal diets. Development and maturation of tomato fruit is a well-programmed process, and yet, only a limited number of factors involved in its regulation have been characterized. Micro-RNAs (miRNAs) are small, endogenous RNAs that regulate gene expression in animals and plants. Plant miRNAs have a vital role in the generation of plant forms through post-transcriptional regulation of the accumulation of developmental regulators, especially transcription factors. Recently, we and others have demonstrated that miRNAs and other type of small RNAs are expressed in tomato fruit, and target putative transcription factors during its development and maturation. The original objectives of the approved proposal were: **1. To identify fruit miRNA transcription factor target genes through a bioinformatic approach. 2. To identify fruit miRNA transcription factor target genes up-regulated in tomato Dicer-like 1 silenced fruit. 3. To establish the biological functions of selected transcription factors and examine their utility for improving fleshy fruit quality trait.** This project was approved by BARD as a feasibility study to allow initial experiments to peruse objective 2 as described above in order to provide initial evidence that miRNAs do play a role in fruit development. The approach planned to achieve objective 2, namely to identify miRNA transcription factor targets was to clone and silence the expression of a tomato DCL1 homolog in different stages of fruit development and examine alterations to gene expression in such a fruit in order to identify pathways and target genes that are regulated by miRNA via DCL1. In parallel, we characterized two transcription factors that are regulated by miRNAs in the fruit.

We report here on the cloning of tomato DCL1 homolog, characterization of its expression in fruit flesh and peel of wild type and ripening mutants and generation of transgenic plants that silence SIDCL1 specifically in the fruit. Our results suggest that the tomato homolog of DCL1, which is the major plant enzyme involved in miRNA biogenesis, is present in fruit flesh and peel and differentially expressed during various stages of fruit development. In addition, its expression is altered in ripening mutants.

We also report on the cloning and expression analysis of SI_SBP and SI_ARF transcription factors, which serve as targets of miR157 and miR160, respectively. Our data suggest that SI_SBP levels are highest during fruit ripening supporting a role for this gene in that process. On the other hand SI_ARF is strongly expressed in green fruit up to breaker indicating a role for that gene at pre-ripening stage which is consistent with preliminary *in_situ* analyses that suggest expression in ovules of immature green fruit.

The results of this feasibility study together with our previous results that miRNAs are expressed in the fruit indeed provide initial evidence that these regulators and their targets play roles in fruit development and ripening. These genes are expected to provide novel means for genetic improvement of tomato fleshy fruit.

Achievements

Isolation of a full-length DCL1 homolog gene from Tomato (Asaph Aharoni)

The gene *DCL1* was identified and sequenced in *Arabidopsis* (gi|145334933), rice (gi|115450390) and partially in corn (gi|21207970). An alignment search in the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the TIGR database (<http://www.tigr.org/index.shtml>) gave the CK714874.1 fragment in tomato, with homology of 74% to the *AtDCL1* gene. Based on this sequence, a Random Amplification of cDNA Ends (5' and 3' RACE) was used for cloning the rest of the sequence. Amino acid sequence alignment gave 71% identity to the *Arabidopsis* gene and 69% to the rice one, with e-value of zero. Indeed, a phylogenetic tree that was built based on different DICER and DICER-LIKE proteins indicates that the cloned *SIDCL1* is most similar to the *Arabidopsis* *DCL1* (not shown).

SIDCL1 expression analysis (Asaph Aharoni)

Quantitative real-time PCR (qRT) assays were carried out in order to characterize *SIDCL1* expression in different tissues and stages of fruit development. The *SIDCL1* gene expression level was found to be higher in the fruit peel than in fruit flesh in all stages of development. The highest expression level of the gene was in the immature green stage and the lowest in breaker and orange stages (Fig 1). These results indicate that *SIDCL1* is actively transcribed in fruit and suggest higher activity of miRNA pathway in immature green stage and red ripe fruit. In addition the levels of *SIDCL1* were tested in fruit mutants altered in their development and ripening process (Fig. 2). Tested fruit was 42, 44, 48 and 50 days post anthesis (dpa) *rin* and *nor* (equivalent of mature green, breaker, orange and red visible stages in wt fruit). In the mature green and orange stages the levels of the gene were higher in both *rin* and *nor* mutants than in wt, in the breaker stage the level was higher in *rin* mutant but lower in *nor* mutants and an opposite pattern in the red stage- higher in *nor* mutant and lower in the *rin*. The *rin* mutant exhibit a more severe ripening delay than the *nor* mutant, and is in correlation with the difference of the *SIDCL1* expression levels found in our analysis. Together these data suggest that the expression level of the *SIDCL1* fluctuates during the development and ripening process of tomato fruit and is also altered in some ripening mutants.

Fruit specific silencing of SIDCL1 in transgenic tomato plants (Asaph Aharoni)

In this study *SIDCL1* expression was reduced by using the RNAi technology, which is taking advantage of the siRNA pathway not dependent on DCL1 (1). The RNAi fragment was created by amplifying inverted repeats (IR) of the *SIDCL1* non-conserved region between the DUF283 and the PAZ domain. The IR was cloned under the fruit specific promoters: PTPRP, Crabs Claw (CRC) (expression at immature green), 2a11, and E8 (expression during ripening) mobilized into a binary vector and transformed to micro-tom tomato plants. T₀ transgenic plants were identified using PCR

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amplification of the promoter-gene region. To date no visible phenotypes were identified in regenerating T₀ transgenic plants. We are now in the process of generating additional transgenic lines in parallel to analyzing the expression level of *SIDCL1* in the T₁ progeny of generated lines.

Fig 1. *SIDCL1* levels in tomato plants Relative expression level of the *SIDCL1* gene in tomato fruit during development and ripening process was analyzed in flesh and peel fruit by qRT. The expression level of *SIDCL1* is higher in the peel than in the flesh in all of the fruit development stages. The highest expression level was detected in the immature green stage, declining until the orange stage and increasing in the red stage. Stages: IG- immature green, MG- mature green, Br- breaker, Or- orange.

Fig 2. *SIDCL1* levels in aberrant development and ripening tomato fruits. *SIDCL1* relative expression level in tomato fruit during development and ripening process in (A) 1-MCP treated fruits (three biological repeats) and in (B) *rin* and *nor* mutants. Stages: MG- mature green, Br- breaker, Or- orange, R- red; wt- wild type.

Expression analysis of candidate miR157 and miR160 targets in fruit (Tzahi Arazi)

In parallel to the silencing of tomato DCL1 gene, the expression of two fruit ESTs, *SGN-U223167* and *SGN-U332548* was studied. Both encoded mRNAs, have already been predicted and experimentally validated by us to serve as miR157 (*SGN-U223167*) and miR160 (*SGN-U332548*) targets (also see BARD proposal Fig. 2). Firstly, the full length cDNAs of *SGN-U223167* and *SGN-U332548* was cloned by 5' and 3' RACE. BLASTP search and phylogenetic analysis (not shown) indicates that *SGN-U223167* encodes for a squamosa binding protein (Sl_SBP) that is closely related to *Arabidopsis* SPL6, and *SGN-U332548* encode for a tomato auxin response factor (Sl_ARF) closely related to *Arabidopsis* ARF10 and ARF16.

The expression of *Sl_SBP* and *Sl_ARF* and their corresponding miRNAs was analyzed in tomato (cultivar MP1) in seedlings, leaves, flowers at anthesis and in the fruit pericarp during its development, maturation and ripening. This analysis demonstrated that *Sl_ARF* mRNA levels were very low in seedlings, increased slightly in leaves and flowers, were higher in developing and ripening fruit with a peak in the mature green and breaker stages suggesting a role for *Sl_ARF* in fruit development (Fig. 3A). Northern analysis of miR160 showed that it was maximal in flowers and young green fruit, in which relatively lower levels of *Sl_ARF* were detected, weaker in mature green and barley detectable in ripening fruit consistent with its role as a negative regulator of *Sl_ARF* (Fig.

3B). RT-PCR demonstrated that *Sl_SBP* mRNA was present in all tissues tested with lowest expression in seedlings and highest levels in ripening fruit supporting a role for this gene during fruit ripening (Fig. 3C). Conversely, miR157 was weakly expressed during fruit ripening and maximal in seedlings consistent with its role as negative regulator of *Sl_SBP* (Fig. 3D).

prepared from tomato two weeks old seedlings, second leaf from top (leaves), flowers at anthesis (Anthesis) and the pericarp of young green fruit (IG), mature green fruit (MG), breaker fruit (Br), orange fruit (Or), red ripe fruit (Re). (A) *Sl_ARF* transcript accumulation, P- a plasmid containing *Sl_ARF* cDNA clone. gDNA-genomic DNA. NTC-non template control. (C) *Sl_SBP* transcript accumulation. Genomic DNA and NTC controls are not shown. In each experiment *ubi3* amplification was used as a control. The number of cycles of amplification is denoted in parentheses. RNA gel blot analysis of miR160 (B) and miR157 (D) accumulation in tomato. Total RNA from (20 µg) the samples analyzed in A and C was separated on polyacrylamide gel, blotted and probed with oligonucleotides complementary to indicated miRNAs. Hybridization to miR159 served as a loading control.

Figure 3. Expression of *Sl_ARF* and *Sl_SBP* and their corresponding miRNAs in tomato. Transcript level was measured by semiquantitative RT-PCR in 500 ng total RNA

In situ hybridization of *Sl_ARF* (Vivian Irish)

To investigate the spatial patterns of expression of *Sl_ARF*, a 322 nt probe was designed against the 5' UTR and a 265 nt probe specific for the 3' coding region. Using both probes, we obtained preliminary data that indicate that this gene is expressed in a spatially localized manner in fruit and also in flowers (Fig 4). In young green fruit, expression can be detected in the developing ovules in the outer cell layers of the funiculus (Fig 1A,B). Although little is known of the regulatory pathways controlling funiculus development, analyses in Arabidopsis suggest that funiculus development is closely linked with outer integument development (Brambilla et al 2007; Ito et al 2004). Thus, it is possible that the pattern of expression we observe in ovules may reflect polarity in the ovule. In flowers, low levels of expression are detected in petal, stamen and carpel primordia; in sepal primordia, it appears that expression may be more prominent on the adaxial side (Fig 1C). These observations suggest that *Sl_ARF* is expressed in a spatially localized manner, and present the possibility that this gene is required for establishing adaxial/abaxial polarity in lateral organs and ovules.

Fig. 4. Expression patterns of *Sl_ARF*. (A) Expression in ovule of young green fruit; (B) close up of one ovule showing expression in outer cell layers of the funiculus. (C) Expression in young flowers. Se=sepal, p=petal, st=stamen, c=carpel primordia.

References:

- Brambilla et al. (2007) *Plant Cell* 19, 2544-2456.
- Dunoyer P, Himber C, & Voinnet O (2005) *Nat Genet* 37, 1356-1360.
- Ito et al. (2004) *Nature* 430, 356-360.

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List of publications and patents to date (* indicates corresponding author)

None

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Appendix:

Abstracts in meeting and workshop:

None