Final Scientific Report

Cover Page

BARD Project Number: IS-3764-05

Date of Submission of the report: February, 1st 2009

Project Title: "Nuclear encoded RNA splicing factors in plant mitochondria"

Investigators

Institutions

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Collaborating Investigators:

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

Group-II introns, CRM, maturases

Abbreviations (commonly used in the report, in alphabetical order):

CRM, Chloroplast RNA Maturation domain / nMat, nuclear-encoded maturases

Budget: IS: \$170,000

US: \$128,000

Total: \$298,000

Signature
Principal Investigator

State of Israel/Ministry of Agriculture
Agricultural Research Organization
The Volcani Center

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			1	1
Submitted, in review, in preparation	1 (in prep.)	1 (in prep.)	1 (submitted)	3
Invited review papers				
Book chapters				
Books				
Master theses			1	
Ph.D. theses			1	
Abstracts		1	6	7
Not refereed (proceedings, reports, etc.)			3	

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(*):

(* Listed within the report file)

Patent Summary (numbers)

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

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Title: “Nuclear encoded RNA splicing factors in plant mitochondria”

Short introduction

Mitochondria are the site of respiration and numerous other metabolic processes required for plant growth and development. Increased demands for metabolic energy are observed during different stages in the plants life cycle, but are particularly ample during germination and reproductive organ development. These activities are dependent upon the tight regulation of the expression and accumulation of various organellar proteins. Plant mitochondria contain their own genomes (mtDNA), which encode for a small number of genes required in organellar genome expression and respiration. Yet, the vast majority of the organellar proteins are encoded by nuclear genes, thus necessitating complex mechanisms to coordinate the expression and accumulation of proteins encoded by the two remote genomes. Many organellar genes are interrupted by intervening sequences (introns), which are removed from the primary presequences via splicing. According to conserved features of their sequences these introns are all classified as “group-II”. Their splicing is necessary for organellar activity and is dependent upon nuclear-encoded RNA-binding cofactors. However, to-date, only a tiny fraction of the proteins expected to be involved in these activities have been identified. Accordingly, this project aimed to identify nuclear-encoded proteins required for mitochondrial RNA splicing in plants, and to analyze their specific roles in the splicing of group-II intron RNAs.

In non-plant systems, group-II intron splicing is mediated by proteins encoded within the introns themselves, known as maturases, which act specifically in the splicing of the introns in which they are encoded. Only one mitochondrial intron in plants has retained its maturase ORF (*matR*), but its roles in organellar intron splicing are unknown. Clues to other proteins required for organellar intron splicing are scarce, but these are likely encoded in the nucleus as there are no other obvious candidates among the remaining ORFs within the mtDNA. Through genetic screens in maize, the Barkan lab identified numerous nuclear genes that are required for the splicing of many of the introns within the plastid genome. Several of these genes are related to one another (i.e. *crs1*, *caf1*, *caf2*, and *cfm2*) in that they share a previously uncharacterized domain of archaeal origin, the CRM domain. The *Arabidopsis* genome contains 16 CRM-related genes, which contain between one and four repeats of the domain. Several of these are predicted to the mitochondria and are thus postulated to act in the splicing of group-II introns in the organelle(s) to which they are localized. In addition, plant genomes also harbor several genes that are closely related to group-II intron-encoded maturases (nMats), which exist in the nucleus as 'self-standing' ORFs, out of the context of their cognate "host" group-II introns and are predicted to reside within the mitochondria.

The similarity with known group-II intron splicing factors identified in other systems and their predicted localization to mitochondria in plants suggest that nuclear-encoded CRM and nMat related proteins may function in the splicing of mitochondrial-encoded introns. In this proposal we proposed to (i) establish the intracellular locations of several CRM and nMat proteins; (ii) to test whether mutations in their genes impairs the splicing of mitochondrial introns; and to (iii) determine whether these proteins are bound to the mitochondrial introns *in vivo*.

Research achievements

I. Characterization of the roles of individual CRS1-CRM domains in *atpF* intron RNA binding activity. Detailed biochemical analysis of CRS1, a triple CRM domain protein, demonstrated its high-affinity and specific binding to the single group II intron whose splicing it facilitates, the plastid-encoded *atpF* intron RNA (Ostersetzer et al., 2005). To understand how multiple CRM domains cooperate to achieve high-affinity sequence-specific binding to RNA, we analyzed the RNA-binding activities associated with individual CRS1 CRM domains as recombinant GST-CRM fusion proteins, and assayed their RNA binding activity *in vitro* (Keren et al., 2008). While the third domain (CRM3) binds tightly to RNA, CRM1 associated specifically with a unique region in the *atpF* intron. CRM2, which demonstrated only low binding affinity, may function in protein binding. We further showed that CRM domains share structural similarities and RNA-binding characteristics with the well-known RRM motif (Keren et al., 2008).

II. Intracellular locations of CRM and maturase related proteins in

Arabidopsis. The nuclear genome of *Arabidopsis* encodes several CRM and maturase related genes, which are predicted to reside within the mitochondria (Table 1). However, none of the CRM or maturase paralogs are found among the numerous proteins identified by mass spectrometry analyses of

Table 1: Proposed nuclear-encoded mitochondrial splicing factors in *Arabidopsis*.

Name	AtGene identifier ⁽¹⁾	T-DNA lines	Homozygote Phenotypes
At-mtCAF1	At5g54890 (Mit)	SALK_081851 (5'-UTR) SALK_052460 (5'-UTR) SALK_000750 (3'-UTR)	?
At-mtCAF2	At4g31010 (Mit)	SALK_86774 (exon)	Slightly Pale
At-CFM3a	At3g23070 (Clp & Mit)	SAIL_595_F03 (exon) SALK_125653 (exon)	Poor germination. Very slow growing <i>nad1-int2</i> splicing defect
At-CFM6	At4g13070 (Mit)	SALK_081355 (exon) GABI_267F06 (exon)	Poor germination. Very slow growing <i>nad3-int4</i> splicing defect
At-nMat1	At1g30010 (Mit)	SAIL_171_D06 (exon) SAIL_821_D08 (exon)	moderate growth defects ⁽²⁾
At-nMat2	At5g46920 (Mit)	SALK_064667 (exon)	low germination; slow growth; hardly fertile <i>nad7-int2</i> and <i>cox</i> intron splicing defects
		SALK_064659 (exon)	late flowering; partial male and female fertile <i>nad1-int2</i> , <i>nad7-int2</i> and <i>cox</i> intron splicing defects
At-nMat3	At5g04050 (Mit)	SALK_011307 (promoter) SALK_139392 (promoter) SALK_144082 (3'-UTR)	?
At-nMat4	At1g74350 (Clp)	SALK_015975 (exon)	embryo-lethal (?) ⁽³⁾

- (1) Brackets indicate intracellular locations based on the transient expressions of GFP-fusion proteins.
(2) Analysis of At-nMat1 mutants by Nakagawa and Sakurai (2006) Plant Cell Physiol. 47, 772–783.
(3) Microscopic analysis of heterozygote SALK_015975 siliques.

organellar extracts in plants, and therefore each localization has to be established experimentally. For this purpose, the N-terminal region of each protein, (about 150 amino acids, which should contain plastidic and/or mitochondrial targeting pre-sequences), was fused in-frame to *GFP* and introduced into

tobacco SC cell line protoplasts via biolistic transformation. The localization of the GFP-fusion proteins were determined by confocal microscopy. The GFP-localization analyses have localized the four CRMs and three of the maturases (i.e. At-nMat 1, 2 & 3) to mitochondria, as their GFP signals colocalized with those of MitoTracker, while the forth maturase paralog (At-nMat4) was localized to the chloroplasts. At-CFM3, a CRS1 paralog, was localized to both mitochondria and chloroplasts.

III. Identify whether decreased expression of the CRM and maturase related gene disrupts splicing and organellar activities in Arabidopsis mitochondria. To analyze the roles of the mitochondrial-localized CRM and nMat proteins in organellar splicing, we examined the phenotypes associated with available T-DNA insertions lines within and around each of the *Arabidopsis* genes (Table 1). Insertions within the promoter or untranslated regions in At-mtCAF1 or At-nMat3 had no effect on the expression of the genes, as observed by semi-quantitative RT-PCR analyses and thus we could not draw any firm conclusions regarding the putative roles of these proteins in organellar intron splicing (see Table 1). Thus, other methods are required for the analysis of the putative roles of these putative organellar splicing factors in the splicing of organellar introns, including RNAi (expressed under either constitutive or inducible promoters) approach to reduce the expression of each gene (Ostersetzer's group) or "RIP-chip" analysis of native organellar extracts (Barkan's group), in order to identify specific RNA targets of each maturase protein in vivo. Homozygote At-mtCAF2 (At4g31010) mutant was slightly pale and analysis of the respiratory activity of wild-type and SALK line 086774 suggested that mitochondrial activity was impaired in the mutant. We are now in the process of establishing whether (i) the T-DNA insertions indeed reduced the expression of At4g31010 gene and (ii) whether its reduced expression would affect the splicing of individual group-II introns in the organelles.

Two other members of the CRM family, CFM 3 and 6, were found to be required for the splicing of mitochondrial introns. The mitochondrial-localized isoform of At-CFM3 (Asakura et al., 2008) was found to facilitate the splicing of *nad1*-intron 2 while CFM6, a single-domain CRM protein, is involved in the splicing of *nad5*-intron 4 (manuscript in preparation), .

In addition to CRM proteins, our results also provide the first detailed analysis of the roles of a nuclear-encoded maturase protein in the splicing of organellar-encoded group-II introns in plants (Keren et al, 2008, submitted). We demonstrated that At-nMat2 is localized to the mitochondria, where it facilitates the splicing of several group-II introns. Analysis of the respiration activity and protein profiles of wild-type and *nMat2* plants suggest that complex I activity and/or assembly are altered in the mutants, possibly due to the lower availability of specific organelle-encoded subunits, where

splicing defects were observed. *nMat2* plants demonstrated late-flowering phenotypes, which are likely associated with impaired complex I activity in mutant mitochondria.

nMat2 plants demonstrated late flowering and reduced fertility phenotypes, with no other growth defects observed during the vegetative growth phase, thus allowing us to obtain large quantities of plant material required for the analysis of their associated organellar phenotypes. To determine whether At-*nMat2* facilitates the splicing of mitochondrial RNAs, the ratio of mature RNA (mRNA, marked with asterisks) to unspliced transcripts (pre-RNA, marked by arrows), from different loci encoding mitochondrial group II introns, was assayed in wild-type and *nMat2* plants by RNase protection and Northern blot analyses. These assays demonstrated that *nMat2* is involved in the splicing of at least three introns, including the second intron of *nad1*, *nad7*-intron2, and *cox2* intron, where the mRNA levels were in the mutant were significantly reduced compare with the wild-type plants.

Antibodies to mitochondrial COX2, SHMT and NAD9 proteins were used in immunoblot assays to determine whether reduced splicing affects the accumulation of organellar proteins in *nMat2* mutants. While no differences in protein levels were observed for the SHMT and COX2, whose splicing was altered in the mutant, the level of NAD9 was largely reduced in the mutant. In addition, we also analyzed the respiratory activity in wild-type and *nMat2* mutants. *nMat2* demonstrated significantly reduced activity, suggesting that oxygenic respiration in mitochondria was altered in *nMat2* mutants. The addition of rotenone, an inhibitor of complex I, had a profound effect on the respiration of wild-type mitochondria, but had only a minor effect on the average O₂ uptake of *nMat2* plants, further suggesting that complex I activity was impaired in the mutant.

Description of the cooperation

By taking advantage of the expertise offered by each of our groups, we were able to establish the roles of at least three nuclear-encoded RNA-binding cofactors in the splicing of several mitochondrial group II introns. Ostersetzer laboratory analyzed the intracellular locations of putative nuclear-encoded mitochondrial splicing factors that are related to either CRM or maturase proteins (Table 1), by assays that are based on the transient expressions of GFP protein fused to the N-termini of each protein. Using *Arabidopsis* as a genetic system, we established the roles of proteins which were proved to be localized to the organelle, either from the CRM family (i.e. *At-cfm6* and *At-cfm3*; Barkan group) or maturase-related ORF (*At-nMat2*; Ostersetzer group) in the splicing of mitochondrial introns, by analyzing available T-DNA lines within each of these genes.

The Barkan lab, which developed a co-IP / microarray assay (RIP-chip) for identifying RNA and proteins associated with plastidic RNA-binding proteins, will further expand upon this approach by applying it to the mitochondrially-localized splicing factors. In addition, we are currently characterizing the protein profiles (A. Barkan) and organellar respiration activities (O. Ostersetzer) in the CRM and maturase mutants, and will shortly submit these results for publication in a paper that includes authors from both the Barkan and Ostersetzer groups.

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