BARD

FINAL REPORT

PROJECT NO. US-3135-99

Plant Adaptation to Light Stress: Genetic Regulatory Mechanisms

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BARD Final Scientific Report

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Cooperating Investigators: None

Continuation of (Related to) Previous BARD Project:

Yes ☒ No ☐ Number:

Keywords: Chloroplast, protein disulfide isomerase, redox, translation, RNA-binding proteins

Abbreviations in report: PDI (protein disulfide isomerase),
psbA (chloroplast gene for D1 protein of photosystem II),
5' UTR (5 untranslated leader of *psbA* RNA)
RB60, RB47, RB38 (*psbA* RNA 5' UTR binding proteins),

Budget: IS: \$149,000 US: \$127,000 **Total: \$ 276,000**

Signature
Principle Investigator

Signature, Director of Research Services
Office of Research Services, University of Hawaii

BARD Final Scientific Report

Publication Summary (numbers of publications)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	2	0	0	2
Submitted, in review, in preparation		0	0	0
Invited review papers	0	0	0	0
Book chapters		0	0	0
Books		0	0	0
Master theses	0	1	0	1
Ph.D. theses	0	0	0	0
Abstracts - conferences	2	2	2	6
Not refereed (proceedings, reports)	0	0	0	0

Post-doctoral Training. Names and social security/identity numbers of all postdocs who received more than 50% funding from this grant:

University of Hawaii none
Weizmann Institute of Science none

Patent Summary (numbers of patents)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued	0	0	0	0
Licensed	0	0	0	0

BARD Final Scientific Report

Cooperation Summary (numbers of PI/co-PI meetings)

	From US to Israel	From Israel to US	Together, in US	Total
Short Visits & Meetings	0	1	1	2
Longer Visits (Sabbaticals)	0	0	0	0

Cooperation: (briefly explain).

In 1999, Dr. Christopher used travel funds (USDA-NRI grant) to visit Dr. Danon in Israel. During this visit, the idea for the BARD project was hatched and the PI's made plans to write a BARD research proposal together. Once funded, Drs. Danon and Christopher met (July, 2000), in New London, Connecticut, USA at the Gordon Research Conference on Mitochondria and Chloroplasts. They had several synergistic meetings in which they reviewed preliminary data together, outlined priorities and divided experiments between them for achieving the research objectives. The PI's exchanged protein samples, immunoblots, antibodies, cDNAs, methods and data. Dr. Danon visited Dr. Christopher's lab in May, 2002, and presented a seminar on his research. Drs. Christopher and Danon discussed their preliminary results and research strategies, division of labor and assignments for the coming year. The University of Hawaii supported the BARD cooperation by pre-paying for half of the hotel expenses for Dr. Danon, thus allowing him to receive a 50% discount rate. In 2003, cooperation was limited to a few brief emails.

Final Scientific Report - Abstract:

Original Objectives: 1. Purify and biochemically characterize RB60 orthologs in higher plant chloroplasts; 2. Clone the gene(s) encoding plant RB60 orthologs and determine their structure and expression; 3. Manipulate the expression of RB60; 4. Assay the effects of altered RB60 expression on thylakoid biogenesis and photosynthetic function in plants exposed to different light conditions. In addition, we also examined the gene structure and expression of RB60 orthologs in the non-vascular plant, *Physcomitrella patens* and cloned the poly(A)-binding protein orthologue (43 kDa RB47-like protein). This protein is believed to be a partner that interacts with RB60 to bind to the *psbA* 5' UTR. Thus, to obtain a comprehensive view of RB60 function requires analysis of its biochemical partners such as RB43.

Background & Achievements: High levels of sunlight reduce photosynthesis in plants by damaging the photosystem II reaction center (PSII) subunits, such as D1 (encoded by the chloroplast *psbA* gene). When the rate of D1 synthesis is less than the rate of photodamage, photoinhibition occurs and plant growth is decreased. Plants use light-activated translation and enhanced *psbA* mRNA stability to maintain D1 synthesis and replace the photodamaged D1. Despite the importance to photosynthetic capacity, these mechanisms are poorly understood in plants. One intriguing model derived from the algal chloroplast system, *Chlamydomonas*, implicates the role of three proteins (RB60, RB47, RB38) that bind to the *psbA* mRNA 5' untranslated leader (5' UTR) in the light to activate translation or enhance mRNA stability. RB60 is the key enzyme, protein disulfide isomerase (PDI), that regulates the *psbA*-RNA binding proteins (RB's) by way of light-mediated redox potentials generated by the photosystems. However, proteins with these functions have not been described from higher plants. We provided compelling evidence for the existence of RB60, RB47 and RB38 orthologs in the vascular plant, *Arabidopsis*. Using gel mobility shift, Rnase protection and UV-crosslinking assays, we have shown that a dithiol redox mechanism which resembles a PDI (RB60) activity regulates the interaction of 43- and 30-kDa proteins with a thermolabile stem-loop in the 5' UTR of the *psbA* mRNA from *Arabidopsis*. We discovered, in *Arabidopsis*, the PDI gene family consists of 11 members that differ in polypeptide length from 361 to 566 amino acids, presence of signal peptides, KDEL motifs, and the number and positions of thioredoxin domains. PDIs catalyze the reversible formation and isomerization of disulfide bonds necessary for the proper folding, assembly, activity, and secretion of numerous enzymes and structural proteins. PDIs have also evolved novel cellular redox functions, as single enzymes and as subunits of protein complexes in organelles. We provide evidence that at least one PDI is localized to the chloroplast. We have used PDI-specific polyclonal and monoclonal antisera to characterize the PDI (55 kDa) in the chloroplast that is unevenly distributed between the stroma and pellet (containing membranes, DNA, polysomes, starch), being three-fold more abundant in the pellet phase. PDI-55 levels increase with light intensity and it assembles into a high molecular weight complex of ~230 kDa as determined on native blue gels. In vitro translation of all 11 different PDI's followed by microsomal membrane processing reactions were used to differentiate among PDI's localized in the endoplasmic reticulum or other organelles. These results will provide insights into redox regulatory mechanisms involved in adaptation of the photosynthetic apparatus to light stress. Elucidating the genetic mechanisms and factors regulating chloroplast photosynthetic genes is important for developing strategies to improve photosynthetic efficiency, crop productivity and adaptation to high light environments.

Achievements: The first three objectives were achieved and some progress has been made on objective four. The following specific accomplishments are highlighted:

(1) We mapped the major 5'-end of the *psbA* 5' UTR mRNA (Columbia ecotype of *Arabidopsis*) at 77 nt upstream from the translational start codon. Two clusters of much lower abundance 5'-ends were detected. The sequence of the *Arabidopsis psbA* 5' UTR is highly conserved with the corresponding 5' UTR's from other plants, but not with the *C. reinhardtii* sequence.

(2) We determined that a thermolabile secondary structure (predicted $\Delta G'$ of -6.8 kcal/mole) is required for protein binding to the *Arabidopsis psbA* 5' UTR. We identified a 35 nt RNA cis element that was crucial for protein binding and verified the protein/RNA complex using RNase A protection assays.

(3) UV crosslinking and SDS-PAGE were used to characterize a major protein of ~43 kDa, and a minor protein of ~30 kDa, binding to the *psbA* mRNA 5' UTR in *Arabidopsis* chloroplasts.

(4) We observed that oxidizing conditions (DTNB) abolished the association of proteins with the 5' UTR, while RNA-binding activity was recovered upon incubation with reductant (DTT). We proposed the involvement of a dithiol-redox, PDI-based mechanism of protein-RNA interaction.

(5) The Danon lab raised a number of high affinity monoclonal antibodies (mAbs) specific to RB60-PDI. Dr. Danon sent three of the mAbs to Dr. Christopher's group who found that the mAbs strongly cross-reacted with 55- and 60- kDa proteins. The 55 kDa protein was 5X more abundant in the chloroplast pellet subfraction containing thylakoids, envelope, starch, and DNA than in the stromal fraction. Further sub-fractionation is underway.

(6) Native blue gels were used to show that the RB60-PDI of *Arabidopsis* chloroplasts exists in a high molecular weight complex of 230 kDa.

(7) Chloroplast protein extracts were probed with polyclonal antisera generated against the *C. reinhardtii* proteins RB60 and RB47. The antisera against *C. reinhardtii* RB47 cross-reacted with proteins of approximately 43-47 kDa. High salt fractionation of the *Arabidopsis* chloroplast proteins removed some of the other cross-reacting proteins, leaving a prominent protein of ~45

kDa. The antisera against *C. reinhardtii* RB60 cross-reacted with numerous immuno-related proteins ranging in size from ~30- to 55 kDa.

(8) Full-length cDNAs for four different PDIs and two poly(A)-binding proteins (RB47) were initially cloned from an *Arabidopsis* leaf cDNA library, sequenced and characterized. We have subsequently cloned and analyzed the primary structure of all 11 PDI orthologues from *Arabidopsis* via RT-PCR. The sequences of the cDNAs were analyzed with various software to predict their putative protein products and presence of chloroplast transit signals, redox responsive disulfide bridge sites and RNA binding motifs. We have found that relying on such computer analysis to predict cleavable transit peptides has its pitfalls. For example, we have shown that the *C. reinhardtii* RB60 is imported into chloroplasts without cleavage of a transit peptide. The *Arabidopsis* RB60 may be imported by a similar mechanism.

(9) Chloroplast import assays were conducted using several of the PDI-GFP fusions in a transient expression system.

(10) Using RT-PCR and Northern blot hybridization analyses, we have analyzed the expression of the PDI genes during light-induced plant development, and found some PDI members to be light-induced, and others to have significantly different expression patterns, therefore indicating they have unique roles in the plant..

(11) In vitro translation of 11 different PDI's followed by microsomal membrane processing assays differentiated among PDI's localized in the endoplasmic reticulum.

(12) We have obtained from the Arabidopsis Biological Resource Center, nine *A. thaliana* lines each containing a T-DNA insertion on a different PDI-like gene identified through the Salk Institute Genome Analysis Laboratory (SIGnAL). The authenticity and position of the T-DNA of eight lines was verified. We are concurrently producing *A. thaliana* plants that contain double and triple PDI-like gene knock outs to manipulate the expression of PDI orthologs.

(13) Because the Moss *Physcomitrella patens* is the only plant system that is amenable to directed gene knockouts via homologous recombination, we expanded our experimental approach and included a new objective of studying RB60 orthologs in *P. patens*. To date, we

cloned nine PDI-like genes from *P. patens*, showing that the size of the PDI gene family in *P. patens* is similar to that of higher plants. We found that the expression of the different PDI-like genes varies dramatically, suggesting that each has a unique function. We have produced 4 knock-out mutants, each specific to a single PDI gene, demonstrating the high efficiency and feasibility of KO production in *P. patens*. Thus far, we have yet to detect a gross phenotype associated with any of the KO mutations. We developed a novel screening technique that enabled us to identify KO mutants that resulted from a single and precise homologous integration of the antibiotic gene. This protocol will allow us to produce a mutant *P. patens* plant in which a single unique wt PDI-like gene is replaced with a mutated form.

Agricultural or Economic Impacts: Elucidating the genetic mechanisms and PDI's regulating protein folding, in general, and chloroplast photosynthetic genes, in particular, is important for developing strategies to improve photosynthetic efficiency, crop productivity and adaptation to light stress. The specific agricultural impacts of this basic research are unknown at this time.

Description of the Cooperation: The two PI's cooperated in multiple tasks directly related to the research objectives. These tasks involved the cloning of PDI cDNAs, the analysis of the *Arabidopsis* protein extracts for the RB60 and RB47, characterizing their biochemical properties, and sharing of protocols. Drs. Danon and Christopher co-authored two papers resulting from studies funded by this grant.

Peer-Reviewed Manuscripts:

Shen, X., Danon, A., and Christopher, D.A. 2001. RNA binding-proteins interact specifically with the *Arabidopsis* chloroplast *psbA* mRNA 5' untranslated region in a redox-dependent manner. *Plant & Cell Physiol.* 42:1071-1078.

Meiri, E., Levitan, A., Guo, F., Christopher, D.A., Schaefer, D.G., Zryd, J-P., and Danon, A. 2002. Characterization of three PDI-like genes in *Physcomitrella patens* and construction of knock-out mutants. *Mol. Genet. Genomics* 267:231-240

Appendix: (attached 2 publications)