Cover Page

BARD Project Number: US-4228-09

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Project Title: The role of FtsH11 protease in chloroplast biogenesis and maintenance at elevated temperatures in model and crop plants

Investigators
Principal Investigator (PI): Junping Chen

Institutions
USDA-ARS

Co-Principal Investigator (Co-PI): Zach Adam

Hebrew Univ

Collaborating Investigators: Arie Admon

Technion-Israel Institute of Tech

Keywords: high temperature stress, thermotolerance, chloroplast thermostability, ARC6, CPN60, Suppressor

Abbreviations commonly used in the report, in alphabetical order: ARC, accumulation and replication of chloroplasts; BC, backcross; E-line, enhancer line; HT, high temperature; KO, knockout; OE, overexpression; MS, mass spectrometry; PNP, polyribonucleotide nucleotidyltransferase; PS, photosystem; S-line, suppressor line; TEM, transmission electron microscope; WT, wild type

Budget: IS: $166,000 US: $134,000 Total: $300,000

Signature Principal Investigator

Signature Authorizing Official, Principal Institution

Appendix G6a
**Publication Summary (numbers)**

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**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)**

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**Description Cooperation:**

A synergistic collaboration between Labs of PI and Co-PI in US and Israel was well established at the starting of the project. Progresses made in both labs were shared and discussed among the PI, Co-PI and Dr. Admon, a collaborator on the proteomic aspect of the project. Plant materials, assay protocols, experiments procedures and results generated in Chen’s lab were shared with Dr. Adam’s lab to endure the generation of HA-tagged transgenic lines for the need of his lab’s work. Likewise, the material and information generated by the Israeli labs were shared with Dr. Chen and further characterize in the US lab to complement and facilitate rationale interpretation of results obtained in the genetic studies. Dr. Chen served as the primary PI for genetic and functional genomic studies proposed in objectives No 3 and 4. Her lab performed screening, characterization and gene identification of FtsH11 enhancer/supressor lines that showed modification of thermosensitive phenotypes of fshH11 mutant; and evaluated the roles of FtsH11 homologs in other plant species in thermostability of photosynthetic machinery. Dr. Adam served as the primary PI for biochemical and proteomic studies proposed in objectives No 1 and 2. Transgenic plants expressing tagged FtsH11 complexes were generated and characterized in his lab. The proteomic studies were conducted in Dr. Admon’s lab collaboratively among 3 labs.

**Patent Summary (numbers)**

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Abstract

**The specific objectives of this proposal** were to: 1) determine the location, topology, and oligomerization of FtsH11 protease; 2) identify the substrate/s of FtsH11 and the downstream components involved in maintaining thermostability of chloroplasts; 3) identify new elements involved in FtsH11 protease regulatory network related to HT adaptation processes in chloroplast; 4) Study the role of FtsH11 homologs from crop species in HT tolerance

**Background to the topic:** HT-tolerant varieties that maintain high photosynthetic efficiency at HT, and cope better with daily and seasonal temperature fluctuations are in great need to alleviate the effect of global warming on food production. Photosynthesis is a very complex process requiring accurate coordination of many complex systems and constant adjustments to the changing environments. Proteolytic activities mediated by various proteases in chloroplast are essential part of this process and critical for maintaining normal chloroplast functions under HT. However, little is known about mechanisms that contribute to adaptation of photosynthetic processes to HT. Our study has shown that a chloroplast-targeted Arabidopsis FtsH11 protease plays an essential and specific role in maintaining thermostability of thylakoids and normal photosynthesis at moderate HT. We hypothesized that FtsH11 homologs recently identified in other plant species might have roles similarly to that of AtFtsH1. Thus, dissecting the underlying mechanisms of FtsH11 in the adaptation mechanisms in chloroplasts to HT stress and other elements involved will aid our effort to produce more agricultural products in less favorable environments.

**Major conclusions, solutions, achievements**

- Identified the chloroplast inner envelope membrane localization of FtsH11.
- Revealed a specific association of FtsH11 with the α and β subunits of CPN60.
- Identified the involvement of ARC6, a protein coordinates chloroplast division machineries in plants, in FtsH11 mediated HT adaptation process in chloroplast.
- Reveal possible association of a polyribonucleotide nucleotidyltransferase (cpPNPase), coded by At3G03710, with FtsH11 mediated HT adaptation process in chloroplast.
- Mapped 4 additional loci in FtsH11 mediated HT adaptation network in chloroplast.
- Demonstrated importance of the proteolytic activity of FtsH11 for thermotolerance, in addition to the ATPase activity.
- Demonstrated a conserved role of plant FtsH11 proteases in chloroplast biogenesis and in maintaining structural and functional thermostability of chloroplast at elevated temperatures.

**Implications, both scientific and agricultural:** Three different components interacting with FtsH11 were identified during the course of this study. At present, it is not known whether these proteins are directly involved in FtsH11 mediated thermotolerance network in chloroplast and/or how these elements are interrelated. Studies aiming to connect the dot among biological functions of these networks are underway in both labs. Nevertheless, in bacteria where it was first studied, FtsH functions in heat shock response by regulating transcription level of σ32, a heat chock factor regulates HSPs expression. FtsH also involves in control of biosynthesis of membrane components and quality control of membrane proteins etc. In plants, both Arc6 and CPN60 identified in this study are essential in chloroplast division and developments as mutation of either one impairs chloroplast division in Arabidopsis. The facts that we have found the specific association of both α and β CPN60 with FtsH11 protein biochemically, the suppression/ enhancement of ftsh11 thermosensitive phenotype by arc6/pnp allele genetically, implicate inter-connection of these networks via FtsH11 mediated network(s) in regulating the dynamic adaptation processes of chloroplast to temperature increases at transcriptional, translational and post-translational levels. The conserved role of FtsH11 proteases in maintaining thermostability of chloroplast at HT demonstrated here provides a foundation for improving crop photosynthetic performance at high temperatures.
The works proposed in this project were conducted as planned in both labs and research goals set for all 4 objectives were achieved. It should be noted first that almost all our major findings came from researches conducted in the last few months of grand period. As originally proposed, a major part of the efforts spent during the proposal period was to generate and characterize the research materials essential for achieving the goals of this project. Currently, both labs are actively pursuing the leads in a collaborative manner. We strongly believe that the new findings of this project may well lead us to uncover the connections of thermodynamic adaption mechanisms of chloroplast mediated by FtsH11 with 3 other networks: 1) chloroplast division and development networks mediated by ARC 6; 2) maintenance of protein homeostasis in chloroplast as temperature rises above optimum mediated by type I chaperonin CPN60 and possibly other CPN co-chaperonins; 3) regulation of dynamic balance of RNAs in chloroplast mediated by cpPNP.

**Research materials essential for the project were generated and genetically characterized.**

Four types of plant materials were generated for various aspects of the research work. 1) The transgenic lines containing either active or inactive (E621Q) variants of HA-tagged FtsH11 in both WT and *Ftsh11* KO background were generated for biochemical and proteomic study of FtsH11 complex in chloroplast. The HA-tagged lines became indispensable for the work since the FtsH11 antibodies from all sources no longer worked during the course of our study. 2) EMS mutation population of 40 pools was generated in *Ftsh11* KO background and screened for enhancers and suppressors (E/S) for their enhancement/ suppression of thermosensitive phenotype of KO. More than 20 confirmed E/S lines were isolated and advanced to M6. Genetic analysis of 10 E/S lines indicate that a) two S traits are recessive and one is dominant; b) the enhanced thermosensitivities in 7 E-lines are recessive traits; & c) traits in 10 E/S lines are controlled by a single locus. 3) Seven mapping populations of *Ler x E-lines* were generated first and analyzed for the association of *FtsH11* mutation with enhancer phenotype in F2 plants used to differentiate the false enhancers from true ones. One false E line was identified. Nine mapping populations were generated for 9 true E/S by crossing M6 plants to *atts405*, a different allele of *ftsh11* mutant in WS background. 4) Transgenic lines containing a WT FL-cDNA of *FtsH11* homologs from *Populus trichocarpa* (*PtFtsH11*), *Ricinus communis* (*RcFtsH11*) or *Pisum sativum* (*PsFtsH11*) were generated in *AtFtsH11* KO mutant background and genetically characterized. The attempts to clone FL-cDNAs of other *FtsH11* homologs from rice, sorghum, grape and tomato in-house and commercially failed due to complicated secondary structures at the N-termini of these genes.
Both ATPase and proteolytic activities of FtsH11 are essential for HT tolerance.
Aforementioned lines allowed us to answer the question whether the proteolytic activity of FtsH11 is essential for its role in conferring thermotolerance, or its ATPase activity is sufficient. For this purpose, we compared the behavior of WT, FtsH11 KO, and the KO line expressing either an active or inactive AtFtsH11 under the control of its endogenous promoter at different temperatures. All genotypes grew nicely at optimal growth temperature. At elevated temperatures, two different lines of the KO complemented with active FtsH11 behaved like WT. However, the E621Q line remained thermosensitive. The results suggest that it is the proteolytic activity of FtsH11 that is important for thermotolerance, and not only the ATPase activity.

Conserved role of FtsH11 proteases in HT tolerance of chloroplast were demonstrated.
Thermotolerance phenotyping of homozygous single insertion transgenic lines expressing a WT PtoFtsH11 or RcFtsH11 or PsFtsH11 gene show the ability of all 3 FtsH11 homologs to complement the thermosensitivity of Arabidopsis KO mutant, indicating a conserved role of FtsH11s in alleviating HT stress in plants. Chlorophyll fluorescence analysis showed that transgenic plants maintained normal photosynthetic activities as those of WT plants at 30°C. The thylakoids of chloroplast in true leaf of transgenic plants remained intact after 3 to 5 days at 30C comparing with disintegrated thylakoids in KO mutant. The results suggest the role of FtsH11s in the maintenance of normal chloroplast functions at HT is conserved in plant.

The localization of FtsH11 in chloroplast was determined and the association of FtsH11 with other FtsH members was examined. The inactive E621Q line was used for immunoblot analysis. Results from total leaf extract and isolated chloroplast and mitochondria suggested that most of the tagged FtsH11 resides in the chloroplast and not the mitochondria. In parallel, we have found in MS analysis that FtsH11 is localized to the chloroplast envelope membrane (Knopf et al. 2012). This was now further confirmed by immunoblot analysis, where FtsH11-HA was found in the envelope fraction and not in thylakoids. Together with the results from other proteomic studies, the chloroplast inner envelope membrane localization of FtsH11 is now confirmed. The findings corrected the thylakoid-targeted localization of FtsH11 as previously proposed.
Apart from FtsH11, chloroplast inner envelope membrane contains FtsH7, FtsH9, FtsH12, and 4 inactive FtsH proteins – FtsH1-4. It is interesting to note that well characterized proteolytic complexes, such as the 20S proteasome in the cytosol of eukaryotic cells or the Clp complex in the stroma of chloroplasts, contain proteolytically-inactive homologs of the active subunits as well.
Immuno-affinity purification experiments were performed to determine if FtsH11 associates with any other protein. Mass spectrometry analysis of the eluted material revealed, as expected, the presence of FtsH11. Nevertheless, none of the other seven FtsH and FtsHi proteins that reside in the envelope was found to be associated with FtsH11. The results suggest the non-heteromeric nature of FtsH11 with other FtsH protease, setting it apart from those characterized thylakoid-targeted FtsHs involved in protecting plant from light stress.

Specific association of cpCPN60 with FtsH11 was revealed. An unexpected finding was the presence of large amounts α and β of CPN60 in eluted material from the column along with FtsH11. As CPN60 is found mostly in the soluble stromal fraction, we interpret its association with FtsH11 as a specific one, although its functional significance is not yet clear to us. Less than 10% of the eluted material comprised of other chloroplast proteins that could be substrates trapped in the inactive protease complex. However, we consider that these proteins are contaminants as we could recover these proteins in control experiments.

Six loci involved in FtsH11 regulated network were mapped on chromosome regions, 2 genes were cloned and the association of FtsH11 with 2 networks mediated by Arc6 and PNP, respectively revealed. About 200 F3 families and 40-60 markers were used to map the mutation to a chromosome region for each mapping population. Additional 1500-3000 F2s and markers of all types within the mapped region were used to fine map the mutation to as small a region as it allowed. The E mutations were mapped on chr 1, 2, 3, & 5 while 2 E line mutations mapped on Chr. 1 & 5. Candidate genes from mapped regions of 3 lines were sequenced. Genes contain point mutations that resulted in amino acid changes were identified for 2 lines and further identified as P73L in Arc6 in S32 and G578D in PNP in E20C. ARC6 protein also located on chloroplast inner membrane. It contains a conserved heat shock DNAJ domain and is an essential part of regulatory networks mediating the chloroplast division and development, PSII assembly, and protein folding in chloroplast. ARC6 interacts specifically with FtsZ2 to tether FtsZ into a functional ring. It also interacts with PDV2 to coordinate the chloroplast division machineries. Although the exact connection between ARC6 and FtsH11 is not yet known, the ability of arc6 to suppress the thermosensitivity of fish11 in a dominant manner suggests a role of ARC6 in thermodynamic adaptation of chloroplast and/or a role of FtsH11 in maintain normal chloroplast division and differentiation at elevated temperatures. AT3G03710 encodes a chloroplast targeted PNP. Recent studies have shown the function of cpPNP in mRNA and rRNA 3’-end maturation and RNA degradation.

4b. Agricultural and/or economic impacts of the research findings, if known.
5. Details of cooperation: whether and how project objectives were promoted as a result of the cooperation.

A synergistic collaboration between Labs of PI and Co-PI in US and Israel was well established at the starting of the project. Progresses made in both labs were shared and discussed among the PI, Co-PI and Dr. Admon, a collaborator on the proteomic aspect of the project. Specifically: Plant materials, plasmid clones and other genetic materials generated in Chen’s lab were sent to Dr. Adam’s lab and related information was shared. The materials and information provided to Dr. Adam’s lab allowed his lab to initiate biochemical characterization of ftsh11 mutants and start the process into generating HA-tagged FtsH11 transgenic plants. The transgenic plant materials of active and inactive (E621Q) variants of HA-tagged FtsH11 generated in Dr. Adam’s lab were sent to PI’s lab where the thermotolerance of these transgenic lines were evaluated. As a result of this collaboration, 1) the essential role of ATPase and proteolytic activities of FtsH11 for HT tolerance was demonstrated; 2) plant materials indispensable for the proposed biochemical and proteomic work in objectives 1 and 2 were produced, especially when the commercially available FtsH11 antibodies from Agrisera, as well as from the lab where these were generated, no longer work during the course of our study.

The biochemical aspect of the work proposed in objective 1 and 2 were conducted mostly in Dr. Adam’s lab while the proteomic analyses were carried out by Dr. Admon’s lab. The localization of FtsH11 on inner envelope of chloroplast was revealed first by MS analysis and then further confirmed by immunoblot analysis in Adam’s lab (Objective 1). The immuno-affinity purification experiments were carried out biochemically in Adam’s lab and proteomically in Admon’s lab. Result from this work revealed 1) the non-heteromeric nature of FtsH11 with other FtsH protease (Obj. 1), and 2) specific association of αCPN60 and βCPN60 with FtsH11(obj. 2).

A comprehensive proteomic experiments to analyze the protein changes in WT and FtsH11 KO plants during a time course of high temperature treatments were carried out collaboratively among the three institutions. Dr. Chen’s lab produced enough N\textsuperscript{14} or N\textsuperscript{15} labeled seeds. Two independent time-course HT experiments were performed and plant samples collected were shipped to Dr. Admon’s lab where proteomic analysis was performed by Dr. Ziv. A Post-Doc in Dr. Adam’s lab participated in the detailed analysis of the MS data. Some plant samples became unusable due to lack of care by FedEx at Israeli port where the package were held for an extended period of time. Nevertheless, proteomic analysis has identified gene clusters that are either up- or
down regulated in FtsH11 mutants including the CPN60s and FtsZ2-2. Several selected leads are
being followed-up in both labs.

Although identified independently for their association with FtsH11, both Arc6 and CPN60s
have essential roles in chloroplast development division. The complementary results facilitate
rationale interpretation of our findings.

6. List of Publications: Include only reviewed publications reporting on work at least partially
supported by BARD and which includes an acknowledgement to BARD.

hexameric nature of a chloroplast AAA+ FtsH protease contributes to its thermodynamic
of photosynthetic membranes during plastid differentiation in the shoot apex of Arabidopsis.
Plant cell 24(3): 1143-1157.