Final Technical Report:

1) DOE Grant DE-FG02-99 ER20338, University of Arizona;
transferred to DE-SC0006646, University of Massachusetts

2) Title: Hsp100/ClpB Chaperone Function and Mechanism
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3) DE-FG02-99 ER20338: 9/15/2008 - 7/31/2011;
DE-SC0006646: 8/01/2011-7/31/2012

4) Accomplishments:

The supported research investigated the mechanism of action of a unique class of molecular chaperones in higher plants, the Hsp100/ClpB proteins, with the ultimate goal of defining how these chaperones influence plant growth, development, stress tolerance and productivity. Molecular chaperones are essential effectors of cellular "protein quality control", which comprises processes that ensure the proper folding, localization, activation and turnover of proteins. Hsp100/ClpB proteins are required for temperature acclimation in plants, optimal seed yield, and proper chloroplast development. The model plant *Arabidopsis thaliana* and genetic and molecular approaches were used to investigate two of the three members of the Hsp100/ClpB proteins in plants, cytosolic AtHsp101 and chloroplast-localized AtClpB-p. Investigating the chaperone activity of the Hsp100/ClpB proteins addresses DOE goals in that this activity impacts how "*plants generate and assemble components*" as well as "*allowing for their self repair*". Additionally, Hsp100/ClpB protein function in plants is directly required for optimal "*utilization of biological energy*" and is involved in "*mechanisms that control the architecture of energy transduction systems*".

Major outcomes of the funded research are outlined below.

Uncovering the function of Hsp101 protein domains.

Plants are the only higher eukaryotes that express Hsp101/ClpB proteins, which are otherwise restricted to bacteria, yeasts and parasitic protozoans. To determine the function of the unique N- and C-terminal domains of Hsp101 in plants, we generated multiple, independent Arabidopsis transgenic lines engineered to express full-length, N- or C-terminally truncated versions of AtHsp101 in the background of an Hsp101 protein null mutation. These plants are being analyzed for phenotypic changes at the physiological and biochemical level.

We first tested the transgenics for the ability to acquire heat tolerance. The full-length and C-terminally truncated Hsp101 transgenics behave like wild type in assays of thermotolerance, indicating the C-terminal domain is dispensable for this function. In contrast, plants carrying the N-terminally truncated Hsp101 respond essentially like Hsp101 null mutants, although all plants produced high levels of Hsp101 after heat stress. We are now testing whether the N-terminal domain truncation mutant assembles correctly and if the plants show other molecular phenotypes characteristic of an Hsp101 null mutant. Previous studies indicate the N-terminus is not required for oligomer assembly, therefore we hypothesize it could be important for substrate or co-factor interactions. This work is being continued toward publication.

In collaboration with Dr. Stephen Tonsor (U. Pittsburgh) we performed experiments to determine how levels of Hsp101 affect whole plant physiology and seed yield. This experiment

utilized the Hsp101 null mutant as well as ten different Arabidopsis accessions that expressed different levels of Hsp101 during heat stress. Leaf photosynthesis, transpiration, dry weight, nitrogen content, time to flowering and seed yield have been measured under control conditions, as well as using plants that were heat stressed every other day for a week before flowering. Results of these studies were published in Molecular Ecology (2008), and showed that the absence of Hsp101 changed the root shoot ratio to an increase in root mass and decreased seed yield, even in the absence of stress. However, there was no evidence for a greater loss in fecundity after heat stress related to decreased Hsp101 expression. Analogous experiments have now been performed with the transgenics carrying the N- and C-terminal truncations. Results of this multi-factorial design experiment are currently being analyzed.

In vivo pull down assays suggest that the N-terminal domain of Hsp101 may interact with the cellular proteolytic machinery.

We generated transgenic Arabidopsis lines in which the Hsp101 null mutation was complemented by a C-terminally StrepII affinity-tagged Hsp101 gene driven by the native promoter. These plants were used to capture proteins associated with Hsp101 after heat stress. Associated proteins were identified by mass spectrometry. As anticipated, the chaperone Hsp70 was recovered, as it is required for optimal protein disaggregation activity of Hsp101. However, in addition, multiple subunits of the 19S cap of the proteasome were also identified. We have been able to confirm the association of the Rpn1 subunit of the 19S cap by western analysis of pull downs. Work is in progress to confirm the interactions of other subunits and to determine the functional significance of this potential Hsp101-proteasome interaction. We hypothesize that Hsp101 may disaggregate proteins not only for refolding, but also to facilitate proteolysis. Consistent with this hypothesis, a Hsp101 null mutant accumulates more insoluble ubiquitinated proteins than wild type plants.

A ClpB-p null mutant was also complemented with a C-terminally StrepII-tagged ClpB-p gene. However, a limited number of proteins were recovered and identified by mass spectrometry, and none could be confirmed with subsequent western analysis.

Yeast two-hybrid analysis was also performed by Hybrigenics (http://www.hybrigenics-services.com/) with both the N-terminal segment of cytosolic Hsp101 and of the chloroplast ClpB-p. A very limited set of putative interacting proteins were identified in both screens. Interestingly, one of the proteins identified as interacting with Hsp101 was CSN6, a subunit of the Cop9 signalosome, which has homology to RPN8, one of the proteasome subunits identified using our in vivo pull down assays. This lends further support to the hypothesis that Hsp101 and the ATP-dependent proteolytic machinery in the cell are coordinated. However, to date we have been unable to confirm any of the putative interacting partners of ClpB-p or Hsp101 identified in the yeast two-hybrid analysis.

A mutant reducing oxidative stress can suppress loss of Hsp101 function

A most exciting result was the successful map-based cloning of an extragenic suppressor of an Hsp101 dominant-negative allele. The mutation was found to be in an mTERF (mitochondrial transcription termination factor) gene, which we named SHOT1 (suppressor of *hot1).* mTERFs are a large family of understudied proteins that most likely regulate organelle gene expression in plants as well as metazoans. Arabidopsis has 35 mTERF genes, *Populus* 55, maize 28, *Physcomitrella* 13, and *Ostreococcus* three, while metazoans have at most four. In plants mTERFs function in both chloroplasts and mitochondria, and possibly other sites in the cell, but

in Arabidopsis the larger proportion of mTERFs are targeted to mitochondria. We showed that SHOT1 is targeted to mitochondria and was, therefore, the first known mutant of this class of proteins in plants. We subsequently discovered that plants with a strong allele of *shot1* (*shot1-2*) were more tolerant to heat stress than wild type plants, and that *shot1-2* could suppress the heat sensitivity of other mutants. However, the *shot1* mutants showed reduced growth rate compared to wild type, suggesting a tradeoff between growth and stress tolerance.

In experiments directed toward determining the basis of the enhanced heat tolerance of *shot1*, we found that mutant seedlings exhibited reduced production of reactive oxygen species, and reduced oxidative damage to proteins and lipids compared to wt plants. Analysis of changes in mitochondrial proteins revealed that the mutant had higher levels of mitochondrial heat shock proteins (HSPs) as well as increased alternative oxidase (AOX). The level of transcripts encoding AOX, UCPS and an NDH was also increased. However, we ruled out the idea that AOX and the slow growth alone were responsible for heat tolerance, as other slow growing mitochondrial mutants were not heat tolerant, and plants with reduced AOX were not more sensitive to heat than wt plants. Also, other heat sensitive mitochondrial mutants accumulated mitochondrial HSPs. We hypothesize that *shot1* heat tolerance is due to primarily to the reduced ROS in *shot1*. These results also genetically separate the requirement for protein folding from the requirement for control of reactive oxygen during heat stress. Defining the targets of SHOT1 function should provide important insight into the control of plant energy balance, which is critical for the generation of our food, fuel and fiber. This work was published in The Plant Cell (2012) and was a major component of the PhD dissertation of Minsoo Kim (PhD awarded 2012). Continuation of our studies on mTERFs is currently being supported by the NSF.

5) Publications & Presentations

Kim, M., U. Lee, I. Small, C. des Francs-Small, E. Vierling. Mutations in a mitochondrial transcription termination factor (mTERF)-related protein enhance thermotolerance in the absence of the major molecular chaperone HSP101. Plant Cell, 24:3349-65 (2012). PMID: 22942382 PMCID: PMC3462636. Exclusively supported by DOE funds.

Tonsor, S.J., C. Scott, I. Boumanza, T.R. Liss, J.L. Brodsky, E. Vierling. Heat shock protein 101 effects in Arabidopsis thaliana: Genetic variation, fitness and pleiotropy in controlled environments. Mol. Ecol., 17: 1614-1626 (2008). Co-authors of this paper were also supported as follows: S.J.T. was supported by NSF 0130347, J.L.B. by NIH grant GM7506.

Seminars and meeting presentations covering DOE research during the granting period:

Seminar - University of Toronto, Scarborough Campus, Canada – March 2012
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International Symposium: Biotechnology for Better Crops, Energy and Health – Taipei, Taiwan, May 2008
Adaptation Potential in Plants: Gregor Mendel Institute, Vienna Austria, March 2009.
Keystone Conference: Plant Abiotic Stress Tolerance Mechanisms, Water and Global Agriculture, CO, Jan 2011.
Perspectives on Modern Plant Physiology Symposium: Frankfurt Germany, August 2011.
10th Anniversary Symposium of The Gregor Mendel Plant Research Institute (GMI): Vienna, Austria, November 2011.

Additional presentations specifically of mTERF studies supported by DOE:
EMBO Conference: The Biology of Molecular Chaperones. Pula, Sardinia, Italy. May 2013.
ARC Center of Excellence Plant Energy Biology Retreat. Partner investigator lecture. Canberra Australia, Sept 2014.