University of Arizona

October 24, 2013

Final Report

University of Arizona
School of Plant Sciences

DOE/Energy Biosciences Grant – Final Report Submitted October 24, 2013

Project Title:	Calcium-Mediated Regulation of Proton-Coupled Sodium Transport
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DOE Award Number:	DE-FG02-04ER15616
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The long-term goal of our experiments was to understand mechanisms that regulate energy coupling by ion currents in plants. Activities of living organisms require chemical, mechanical, osmotic or electrical work, the energy for which is supplied by metabolism. Adenosine triphosphate (ATP) has long been recognized as the universal energy currency, with metabolism supporting the synthesis of ATP and the hydrolysis of ATP being used for the subsequent work. However, ATP is not the only energy currency in living organisms. A second and very different energy currency links metabolism to work by the movement of ions passing from one side of a membrane to the other. These ion currents play a major role in energy capture and they support a range of physiological processes from the active transport of nutrients to the spatial control of growth and development.

In *Arabidopsis thaliana* (Arabidopsis), the activity of a plasma membrane Na^+/H^+ exchanger, SALT OVERLY SENSITIVE1 (SOS1), is essential for regulation of sodium ion homeostasis during plant growth in saline conditions. Mutations in *SOS1* result in severely reduced seedling growth in the presence of salt compared to the growth of wild type. SOS1 is a secondary active transporter coupling movement of sodium ions out of the cell using energy stored in the trans-plasma membrane proton gradient, thereby preventing the build-up of toxic levels of sodium in the cytosol. SOS1 is regulated by complexes containing the SOS2 and CALCINEURIN B-LIKE10 (CBL10) or SOS3 proteins. CBL10 and SOS3 (also identified as CBL4) encode EF-hand calcium sensors that interact physically with and activate SOS2, a serine/threonine protein kinase. The CBL10/SOS2 or SOS3/SOS2 complexes then activate SOS1 Na^+/H^+ exchange activity.

We completed our studies to understand how SOS1 activity is regulated. Specifically, we asked: (1) how does CBL10 regulate SOS1 activity? (2) What role do two putative CBL10-interacting proteins play in SOS1 regulation? (3) Are there differences in the regulation and/or activity of SOS1 in plants differing in their adaptation to salinity? We report our research findings below.

1. How does CBL10 regulate SOS1 activity?

Our studies indicate that CBL10 regulates SOS1 activity through post-transcriptional and post-translational regulation of *CBL10*.

Alternative splicing of *CBL10* regulates SOS1 activity through modulation of the ratio of *CBL10* transcript variants present during growth in salt. When we monitored *CBL10* expression in wild-type Arabidopsis grown in control conditions (no NaCl), an additional band appeared

above the expected size of the CBL10 transcript. The two bands were cloned, sequenced, and found to represent the characterized *CBL10* transcript and an alternatively spliced form, *CBL10L*A, with a retained intron. The *CBL10LA* transcript likely encodes a truncated protein due to a premature stop codon within the retained intron. When seedlings were grown in the presence of salt, accumulation of the *CBL10LA* transcript was reduced. Yeast two-hybrid and *in vitro* pull-down assays revealed that both *CBL10* and *CBL10LA* can interact with the SOS2 kinase. These results, in combination with the response of wild-type Arabidopsis to growth in salt and the salt-sensitive phenotype of the *cbl10* mutant led to the following working model. In control conditions, *CBL10* and *CBL10LA* are transcribed and translated. In response to increased cytosolic sodium, levels of *CBL10LA*, a negative regulator, are reduced so that CBL10 interacts with SOS2, activating SOS1.

Several predictions arose from this model: (1) the CBL10LA protein is not functional during salt stress, (2) only CBL10 is needed to initiate a response to salt, and 3) down-regulation of CBL10LA is critical for a salt stress response. We completed the following experiments, aimed at testing these predictions. (1) Described in our 2009 Plant Cell manuscript (Lin et al., see publication list, Appendix 2, below) was the first evidence that CBL10LA does not encode a functional protein as it is missing a phosphorylation site that is critical for CBL10 function. Subsequently, we provided the following additional evidence to support this conclusion. (a) An early response of Arabidopsis to salinity is a transient increase in cytosolic calcium. This calcium binds to and activates CBL10 which subsequently activates the SOS2 kinase and ultimately, SOS1. As a result, the ability to bind calcium is critical for CBL10 function. The C-terminal truncation in CBL10LA results in the loss of a canonical calcium-binding (EF-hand) domain. When we used recombinant CBL10 protein in calcium binding assays, calcium binding was observed; however, no binding was found with the CBL10LA protein. These results indicated that CBL10 protein can be activated by calcium during a response to salinity and that CBL10LA does not function in this response. (b) Additional evidence that the CBL10LA protein does not function during salt stress came from studies in which we transformed the *cbl10* mutant with the *CBL10LA* cDNA driven by either the native *CBL10* promoter or the Cauliflower Mosaic virus *35S* promoter. Neither construct was able to complement the salt-sensitive phenotype of the mutant. (2) To test the second prediction (that only CBL10 is needed to initiate a response to salt), we transformed the *cbl10* mutant with the *CBL10* cDNA (driven by the *CBL10* and *35S* promoters). Both constructs were able to complement the mutant phenotype and restore growth on salt to wild-type levels, demonstrating that only CBL10 is needed to initiate a response to salt. (3) Experimental support for the third prediction, that CBL10LA must be down-regulated for a response to salt, came from experiments in which we over-expressed the *CBL10LA* cDNA in wild type. We predicted that over-expression of *CBL10LA* would result in the cell's inability to reduce CBL10LA levels and would produce plants sensitive to salt. Analysis of

multiple lines demonstrated that down-regulation of *CBL10LA* is critical for a salt stress response; over-expression of *CBL10LA* in wild type resulted in salt sensitivity similar to that of the *cbl10* mutant. Together, these results demonstrate that alternative splicing of *CBL10* regulates SOS1 activity through modulation of the ratio of *CBL10* transcript variants present during growth in salt. A manuscript describing our findings is in preparation.

2. What role do two putative CBL10-interacting proteins play in SOS1 regulation?

To identify additional proteins that interact with CBL10 and potentially regulate SOS1, we used the FISL domain (the sequence of amino acids in SOS2 which interact with CBL10 and SOS3) in a bioinformatic search to find proteins with a similar domain. Two proteins were identified: REDUCED EPIDERMAL FLUORESCENCE4 (REF4) and REF4-RELATED1 (RFR1). We monitored *in vitro* interaction between REF4 and RFR1 and the CBL proteins using yeast two-hybrid assays. Both proteins strongly interacted with CBL10 and another calcium sensor family member, CBL7; no interaction was seen for either REF4 or RFR1 with SOS3 or the other members of the CBL family. To determine if REF4 and RFR1 interact with CBL10 or CBL7 in a specific developmental process, we generated a *ref4/rfr1* double mutant. The double mutant, like the *cbl10* mutant, is sensitive to salt, having decreased leaf size and chlorosis relative to the growth of wild type. However, the *ref4/rfr1* double mutant is sensitive to chloride while *cbl10* is sensitive to sodium. Relative to the growth of wild type, we observed no phenotypic differences for double mutants on media with increasing concentrations of mannitol or sucrose indicating that reductions in growth in the presence of NaCl represent an ionic and not an osmotic defect. In addition to the salt-sensitive phenotype, we found that the double mutant senesces earlier than wild type, especially under short day growth conditions. Subsequent studies pointed to novel functions for these proteins. REF4 and RFR1 were identified in pull down experiments with proteins that are part of the Mediator complex – a large complex of proteins involved in processes that regulate transcription in plants and in animals. We carried out experiments to mechanistically link these proteins (as a part of the Mediator complex) with the chloride phenotype we observe in the *ref4/rfr1* (now referred to as *med33a/med33b*) double mutant. Measurements of chloride in plant cells indicate that cytoplasmic levels of this ion increase when plants are grown in the presence of NaCl or KCl and there is a greater accumulation of chloride in leaves versus roots. This higher leaf accumulation of chloride could explain our observation that sensitivity to chloride in the *med33* double mutant is manifested in leaves. One recently reported effect of chloride is increased genotoxic stress. Wild-type Arabidopsis grown in the presence of NaCl, KCl and $MgCl_2$ but not Na_2SO_4 or $MgSO_4$ had higher levels of double stranded breaks in DNA and higher rates of recombination. In addition, plants that were treated with high levels of chloride had elevated transcript levels of enzymes involved in the homologous recombination pathway that functions to repair breaks in DNA. One model to explain the increased sensitivity of the

med33 double mutant to chloride is that the Med33 proteins, as components of Mediator, play a role in co-activating the transcription of genes involved in DNA repair. We reasoned that, if the Med33 Mediator has a general role in the transcriptional regulation of DNA repair genes, then the double mutant would likely be sensitive to multiple DNA damaging agents. We analyzed the double mutant for sensitivity to genotoxic agents including UV light and the chemicals methyl methane sulfonate and hydroxyurea. The double mutant was hypersensitive to all of these agents suggesting that the Med33 proteins are involved in DNA damage repair. We are working with a collaborator in China to complete the experiments needed to publish our findings. Specifically, experiments are underway to: (1) monitor DNA damage and recombination rates in the double mutant compared to wild type during chloride stress, (2) analyze *in vitro* DNA repair in wild type and the double mutant in response to chloride, and (3) profile transcription of genes involved in DNA repair pathways in wild type and the double mutant. Our goal is to submit a manuscript describing our findings in 2014.

3. Are there differences in the regulation and/or activity of SOS1 in plants differing in their adaptation to salinity?

Recently *Eutrema salsugineum* (Eutrema, formally called *Thellungiella halophila*), a salt-tolerant relative of both Arabidopsis and the agriculturally important Brassica species, was identified in the saline environments of coastal China and the Canadian prairies. Eutrema is emerging as a key model plant for understanding adaptation to plant growth in salt. Eutrema is able to survive in levels of salt as high as 500 mM NaCl (soil treatment), compared to Arabidopsis which is unable to survive at concentrations as low as 75 mM NaCl. Salt tolerance in Eutrema appears to be a function of regulatory changes in basic biochemical and physiological mechanisms also present in salt-sensitive plants. Using RNAi technology, we generated Eutrema *SOS1* knock-down (*Essos1*) lines and, as was found for the Arabidopsis *sos1* mutant (*Atsos1*), *Essos1* shows severely reduced growth in the presence of NaCl suggesting a similar function for SOS1 in both species. We carried out comparative studies of SOS1 regulation in the two species; the specific questions we asked are: (1) does *EsSOS1* confer increased salt tolerance relative to its putative Arabidopsis homolog? (2) Is there differential regulation of SOS1 by salicylic acid in the two species?

We tested the contribution of *EsSOS1* (promoter and coding sequences) to Eutrema's greater salt tolerance by expressing it in *Atsos1*. For these studies, four constructs were generated: *EsSOS1* promoter (*EsSOS1p*) driving expression of the *EsSOS1* coding sequence (cDNA), *EsSOS1p:AtSOS1cDNA*, *AtSOS1p:AtSOS1cDNA*, and *AtSOS1p:EsSOS1cDNA*. We found that the two constructs with the Eutrema *SOS1* promoter rescued the salt-sensitivity of the Arabidopsis mutant, while the two constructs with the Arabidopsis *SOS1* promoter did not. To understand

why the *AtSOS1p* constructs did not rescue the mutant phenotype, we generated additional constructs including the *AtSOS1* introns or the 3'UTR. Our results from these studies demonstrate that: (1) introns are necessary for the Arabidopsis constructs to complement the *Atsos1* mutant phenotype (likely required for transcript synthesis or stability) and (2) the Eutrema *SOS1* promoter is stronger (drives expression of the Eutrema and Arabidopsis *SOS1* cDNAs without introns) than the *AtSOS1* promoter. We have identified the first three introns as necessary for *AtSOS1* expression and determined that introns also increase the expression of *EsSOS1* and its ability to confer salt tolerance.

In our comparative analysis of the SOS1 sequences from Eutrema and Arabidopsis (described in our 2009 Genomics manuscript listed in publications, below) three Simple Sequence Repeats (SSRs), (TCA)8, (CTT)18, and (TA)12, were identified within 540 bp upstream of the *EsSOS1* putative translational start site while no distinct SSRs were identified in the corresponding region in *AtSOS1*. The (CTT)n repeat contains sequence similar to the TCA-element (TCATCTTCTT) which has been identified as a putative salicylic acid (SA) responsive element. It has been reported that 70-80% of CTT/GAA-associated genes in Arabidopsis are regulated by SA. We hypothesized that these repeats may serve as cis-acting elements affecting the synthesis of the *EsSOS1* transcript and salt tolerance in the two species. We carried out experiments to link SA in the regulation of SOS1 by: (1) monitoring *SOS1* expression in the two species in the presence of SA, (2) comparing expression of the Arabidopsis and Eutrema *SOS1* genes in response to SA with constructs expressed in *Atsos1*, and (3) determining if the Eutrema SSRs increase the activity of a minimal promoter in response to SA. We were unable to link SA to the regulation of SOS1.

APPENDIX 1: GRADUATE STUDENTS RECEIVING Ph.D. DEGREES WITHIN THE DOE GRANT RENEWAL PERIOD

Student	Date
Shea Monihan	December 2011
David E. Jarvis	December 2013
Courtney Magness	May 2014

APPENDIX 2: PUBLICATIONS IIN THE LAST RENEWAL PERIOD

Lin H, Yang Y, Quan R, Mendoza I, Wu Y, Du W, Zhao S, Schumaker KS, Pardo JM, Guo Y. 2009. SOS2 phosphorylation of SCaBP8 stabilizes the SCaBP8-SOS2 complex and enhances salt tolerance in Arabidopsis. Plant Cell 21, 1607-1619.

Nah G, Pagliarulo CL, Mohr PG, Luo M, Sisneros N, Yu Y, Collura K, Currie J, Goicoechea JL, Wing RA, Schumaker KS. 2009. Comparative Sequence Analysis of the SALT OVERLY SENSITIVE1 Orthologous Region in *Thellungiella halophila* and *Arabidopsis thaliana*. Genomics 94, 196-203.

Yang Y, Qin Y, Xie C, Zhao F, Zhao J, Liu D, Chen S, Fuglsang AT, Palmgren MG, Schumaker KS, Deng XW, Guo Y. 2010. The Arabidopsis chaperone J3 regulates the plasma membrane H^+-ATPase through interaction with the PKS5 kinase. Plant Cell 22, 1313-1332.

Zhang C, Guo H, Zhang J, Guo G, Schumaker KS, Guo Y. 2010. Arabidopsis Cockayne Syndrome A-Like proteins 1A and 1B proteins form a complex with CULLIN4 and Damage DNA Binding Protein 1A and regulate the response to UV irradiation. Plant Cell 22, 2353-2369.

Zheng Y, Schumaker KS, Guo Y. 2012. Sumoylation of transcription factor MYB30 by the small ubiquitin-like modifier E3 ligase SIZ1 mediates abscisic acid response in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA, 109, 12822–12827.

Zhou H, Zhao J, Yang Y, Chen C, Liu Y, Jin X, Chen L, Li X, Deng X-W, Schumaker KS, Guo Y. 2012. UBIQUITIN-SPECIFIC PROTEASE 16 modulates salt tolerance in Arabidopsis by regulating Na^+/H^+ antiport activity and serine hydroxymethyltransferase stability. Plant Cell 24, 5106-5122.

Haudry A, Platts AE, Vello E, Hoen D, Leclercq M, Williamson R, Forczek E, Joly-Lopez Z, Steffen JG, Hazzouri KM, Dewar K, Stinchcombe JR, Schoen DJ, Wang X, Schmutz J, Town CD, Edger PP, Pires JC, Schumaker KS, Jarvis DE, Mandáková T, Lysak MA, van den Bergh E, Schranz ME, Harrison PM, Moses AM, Bureau TE, Wright SI, Blanchette M. 2013. An atlas of over 90,000 conserved noncoding sequences provides insight into crucifer regulatory regions. Nature Genetics 45, 891–898.

Yang R, Jarvis DE, Chen H, Beilstein MA, Grimwood J, Jenkins J, Shu SQ, Prochnik S, Xin M, Ma C, Schmutz J, Wing RA, Mitchell-Olds T, Schumaker KS, Wang X. 2013. The reference genome of the halophytic plant *Eutrema salsugineum*. Frontiers in Plant Science (doi: 10.3389/fpls.2013.00046).