Final Scientific/Technical Report

DOE Award No. **DE-FG02-04ER15544**

Recipient: University of California at Santa Barbara

Project Title: ABI8: Prototype of a novel signaling factor

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Executive Summary:

The *Arabidopsis thaliana* *ABSCISIC ACID-INSENSITIVE8* locus encodes a highly conserved plant-specific protein that mediates abscisic acid (ABA) and sugar responses essential for growth. Although initial database comparisons revealed no domains of predictable function, it has recently been re-annotated as a member of the Glycosyltransferase family A. However, this function has not been demonstrated experimentally and no specific substrates have been identified. Mutations affecting ABI8 are near-lethal due to pleiotropic yet specific effects including altered ABA signaling, sugar transport, cell wall synthesis, root meristem maintenance, vascular patterning, and male sterility. Because the predicted sequence initially provided no clues, we used a “guilt by association” strategy to address function of this protein by determining its subcellular localization and identifying interacting proteins. Our studies showed that ABI8 is localized to the endomembrane system and may interact with proteins implicated in Golgi trafficking, lignification, and stress signaling. We found that the root meristem arrest reflects decreased auxin accumulation and resulting decreases in regulators required for meristem identity, all of which can be rescued by added glucose. Further studies showed that this glucose-dependence reflects reduced glucose uptake as well as the decreased expression of sugar-mobilizing enzymes. This work suggests that ABI8 may regulate trafficking of membrane proteins such as auxin transporters and cellulose synthase, but this hypothesis has not yet been tested. The altered gene expression is likely to be a secondary or later effect of this pleiotropic mutation.

Comparison of accomplishments with the goals and objectives of the project:

The specific aims of the funded proposal were to:

- 1) test whether ABA, sugar and possibly other stresses affect ABI8 protein accumulation (which is much more limited than the transcript accumulation)
- 2) test whether Glc-dependent growth of the *abi8* mutants reflects defects in sugar signaling and/or transport
- 3) analyze the mechanism of opposing effects of *ABI8* and its closest homolog (initial characterization of insertion lines for the homolog indicated these were hypersensitive to ABA)
- 4) determine whether *ABI8* affects shoot growth via a (novel) long-distance signaling mechanism or very low level expression in shoots
- 5) analyze physiological mechanism of ABI8 action by identifying interacting proteins

Our accomplishments, in relation to these aims, were:

For Aim 1, we tested and rejected the hypothesis that ABI8 expression might be subject to post-transcriptional regulation by uORFs that might be differentially present in stressed vs. control plants. Furthermore, ABA and stresses had no effect on ABI8 protein accumulation. Although we found limited evidence of proteasomal regulation, this was not sufficient to explain the discrepancy between transcript levels and GUS activity.

Surprisingly, tests of the effects of various sugars and sugar analogs showed that sugar analogs or tunicamycin promoted much greater accumulation of the ABI8-GUS fusion, suggesting that accumulation is somehow regulated by N-glycosylation, consistent with both a role in endomembrane trafficking and its recently proposed glycosyltransferase function

For Aim 2, we found that both sugar signaling and sugar transport appear defective: *abi8* seedlings appear slightly mannose resistant and have roughly two-fold reduced Glc uptake.

For Aim 3, although preliminary studies had suggested that insertional mutations in the homolog resulted in ABA hypersensitivity, that phenotype did not persist. Similarly, overexpression of the homolog did not affect any features regulated by ABI8. However, attempted overexpression of ABI8 also has no phenotype due to tight post-transcriptional regulation, and the homologs might be subject to similar regulation.

For Aim 4, we identified a strong root tip-specific promoter to drive ABI8-GUS and found that it does not appear to complement the mutant, suggesting that ABI8 is not acting by long-distance signaling, but that very low level expression is sufficient for ABI8 function in shoots. This result emphasizes the importance of understanding mechanism(s) of post-transcriptional regulation in shoot tissue.

For Aim 5, we tried multiple variants of yeast two-hybrid screening as approaches toward identifying interacting factors. The tight post-transcriptional regulation precluded our ability to identify interactors based on co-purification with an epitope-tagged ABI8. A GAL4-based yeast two-hybrid screen using truncations lacking the membrane attachment domain as “bait” identified many potential interactors, but the interactions were very weak (sufficient to complement growth auxotrophies, but not significantly increase expression of a beta-galactosidase reporter). These putative interactors represented all compartments of the cell, possibly consistent with a role in trafficking, and included proteins implicated in Golgi trafficking, lignification, and stress signaling. Because the GAL4-based system is not well-suited for analyzing interactions involving membrane-associated proteins, we tried to use the split-ubiquitin system, which relies on activity of a soluble reporter produced following interaction of membrane localized Nub- and Cub-fusion proteins, to retest these interactions. However, ABI8 fusions appeared to auto-activate this system, such that it was not informative. The last variation we tried was the RAS recruitment system, but this also did not give a clean enough background to be useful.

Summary of project activities for the entire period of funding:

We isolated the *abi8* mutant in a screen for decreased ABA response, so initially focused on the role of *ABI8* in ABA signaling. As has been described for several other ABA response mutants, the *abi8* mutants also had defects in response to sugars. Although the near-lethal highly pleiotropic nature of the *abi8* mutants suggested at most minimal redundancy with any homologous proteins, preliminary studies of T-DNA insertion lines disrupting its closest homologs indicated that one of these appeared hypersensitive to ABA and sugars. We proposed to address the role of *ABI8* and its closest homolog in ABA and sugar signaling by extending our characterization of the mutants, analyzing overexpression lines, characterizing genetic interactions with other ABA signaling components, analyzing regulation of expression and localization, and identifying interacting factors. Our progress toward these goals is described in the previous section. The initial characterization of the *abi8* mutants, interactions with other ABA signaling components, and localization data was published just prior to the beginning of the award period in Brocard-Gifford et al. (2004) *Plant Cell* 16: 406–421.

Concurrent with our characterization of the *abi8* mutant, two other groups had identified mutations in the same locus on the basis of dwarf and cellulose deficient phenotypes. Their localization data, using overexpressed N-terminal and C-terminal GFP fusions, placed the ABI8 protein in the plasma membrane and cell wall, respectively. To resolve the discrepancies between these reports and our earlier finding of punctate cytoplasmic localization, we constructed another GFP fusion under control of the *ABI8* promoter and demonstrated its correct function by complementation of the mutant. Time-lapse confocal images, co-localization and inhibitor studies indicate that it is actually Golgi-localized; this work is not yet published.

As described above, the closest homologs from Arabidopsis did not appear to have a similar function, despite having a nearly 70% identical protein sequence. However, mutations in a close homolog (75% identical) from *Nicotiana plumbaginifolia*, the *SDL-1* (seedling lethal) gene, produced a similar phenotype. To determine whether this reflected a conserved function, we tested the *sdl-1* mutant for defects in ABA and sugar sensing and attempted to complement the *abi8* mutation with the *SDL-1* cDNA. Although *SDL-1* rescued the seedling lethality of the *abi8* mutant, the *sdl-1* mutant did not display defects in ABA and sugar response. We identified N-terminal and C-terminal regions of 50-100 amino acids that contained almost all the differences between these proteins to use in domain swapping experiments to identify the region(s) required for ABA and/or sugar response, but ran out of funding before these experiments were completed.

Investigation of the root meristem arrest led us to explore other signals regulating root meristem identity, such as auxin and the *PLETHORA* (*PLT*) family of transcription factors. We obtained the auxin-responsive promoter *DR5-GUS* and *PLTpro-GUS* marker lines and crossed these into the *abi8* background to determine whether the meristem arrest, and rescue by exogenous glucose, correlated with presence of the auxin maximum and corresponding localized *PLT* expression. Our results have led us to the hypothesis that ABI8 may be involved in trafficking of auxin transporters such as the PIN proteins. We attempted to determine whether PIN1-GFP fusions were underexpressed or mislocalized in the mutant background, but the autofluorescence of the ectopically deposited suberin in the mutant produced too much background to adequately score the PIN1-GFP. Much of this work has been done, with sporadic undergraduate assistance, after the termination of the funding for this project.

The view that ABI8 may be involved in trafficking of membrane proteins also suggested the possibility that the defects in cellulose synthesis might reflect defects in delivery of cellulose synthase(s) to the plasma membrane. We attempted to establish collaborations with groups that had reported use of fluorescently tagged cellulose synthases but, despite their apparent interest, have yet to receive any of the relevant lines or constructs.

We encountered two major types of problems in this project. The first was that the ABI8 protein was incompatible with every type of analysis used to investigate its biochemical function: no homology with domains of known function identified during the period of funding, very low activity in GAL4-based yeast two-hybrid assays resulting in many options but no strong leads, very high background in two different types of membrane-localized two-hybrid assays, and tightly post-transcriptionally regulated thereby inhibiting co-purification of interacting proteins. The other major problem was personnel: the major project participant was on extended maternity leave for ~3 months in the first year of the project, returned on only a part-time basis in the second year, and then left the position permanently. She was replaced in the third year by a new Postdoctoral Fellow with extensive experience in plant molecular biology and biochemistry. However, when it became clear that the funding was not being renewed because all possible leads were considered too risky, he left for a faculty position after only 8 months. At that point, there were insufficient funds remaining to recruit anyone new, so the remaining experiments were carried out by a part-time technician, assorted

undergraduates, and myself (between teaching and administrative duties), working frugally over 2 years of no-cost extensions. Consequently, we still have many interesting, but unpublished results from this project waiting for someone (another undergraduate with a tiny supply budget from the college?) to have time to complete these stories.

PATENT CERTIFICATION

The Regents of the University of California

Contractor

DE-FG02-04ER15544

DOE Prime and/or Subcontract Nos.

☐ Interim Certification
☒ Final Certification

Contractor hereby certifies that:

1. All procedures for identifying and disclosing subject inventions as required by the patent clause of the contract have been followed throughout the reporting period.
2. There were no subcontracts or purchase orders involving research, development, and demonstration except as follows: [State none when applicable.] NONE
3. No inventions or discoveries were made or conceived in the course of or under this contract other than the following (Certification includes ☒ , does not include ☐ all subordinates): NONE

[State none when applicable.]

TITLE

INVENTOR

DATE REPORTED

DOE "S" NO.*

NONE

4. The completion date of this contract is as follows: 7/14/2009

5. The following period is covered by this certification:

JULY 15, 2004

to

JULY 14, 2009

Month Day Year

The Regents of the University of California

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Date of Certification

* Also include Subcontract No. If available