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BARD

FINAL REPORT

PROJECT NO. US-2743-96

**Regulation of Arabidopsis Glutamate
Decarboxylase in Response to Heat
Stress: Modulation of Enzyme Activity**

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Cover Page**

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**Project Title: Regulation of Arabidopsis Glutamate Decarboxylase in Response to Heat
Stress: Modulation of Enzyme Activity**

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

GABA accumulation, calcium signaling, calmodulin,

Abbreviations commonly used in the report, in alphabetical order:

GAD, glutamate decarboxylase; GABA, γ -aminobutyric acid = 4-amino butyric acid

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TOTOL

\$300,000

Signature
Principal Investigator

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Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	1	2	1	5
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Invited review papers				
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Master theses				
Ph.D. theses		1 (Partial Bard supp)	1	1.5
Abstracts		5	4	9
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Shaw-Jye Wu, US SS# 422-37-4758

Cooperation Summary (numbers)

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

Description of Cooperation: The investigators met for discussions about the project at the 1997 ASPP Meeting in Vancouver, BC & at the 1998 ASPP Meeting in Madison, WI. The Israeli PI, planned to come to Auburn around the Madison Meeting, but was unable to schedule this, and the US PI planned to go to Israel in the third year, but the Israeli PI moved to England and a time could not be arranged. Much discussion and coordination of the project took place via email.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted	None	None	None	None
Issued (allowed)	None	None	None	None
Licensed	None	None	None	None

ABSTRACT

Most plants accumulate the nonprotein amino acid, γ -aminobutyric acid (GABA), in response to heat stress. GABA is made from glutamate in a reaction catalyzed by glutamate decarboxylase (GAD), an enzyme that has been shown by the Israeli PI to be a calmodulin (CaM) binding protein whose activity is regulated *in vitro* by calcium and CaM. In *Arabidopsis* there are at least 5 GAD genes, two isoforms of GAD, GAD1 and GAD2, are known to be expressed, both of which appear to be calmodulin-binding proteins. The role of GABA accumulation in stress tolerance remains unclear, and thus the objectives of the proposed work are intended to clarify the possible roles of GABA in stress tolerance by studying the factors which regulate the activity of GAD *in vivo*. Our intent was to demonstrate the factors that mediate the expression of GAD activity by analyzing the promoters of the GAD1 and GAD2 genes, to determine the role of stress induced calcium signaling in the regulation of GAD activity, to investigate the role of phosphorylation of the CaM-binding domain in the regulation of GAD activity, and to investigate whether ABA signaling could be involved in GAD regulation via the following set of original Project Objectives:

1. Construction of chimeric GAD1 and GAD2 promoter/reporter gene fusions and their utilization for determining cell-specific expression of GAD genes in *Arabidopsis*.
2. Utilizing transgenic plants harboring chimeric GAD1 promoter-luciferase constructs for isolating mutants in genes controlling GAD1 gene activation in response to heat shock.
3. Assess the role of Ca^{2+} /CaM in the regulation of GAD activity *in vivo* in *Arabidopsis*.
4. Study the possible phosphorylation of GAD as a means of regulation of GAD activity.
5. Utilize ABA mutants of *Arabidopsis* to assess the involvement of this phytohormone in GAD activation by stress stimuli.

The major conclusions of Objective 1 was that GAD1 was strongly expressed in the elongating region of the root, while GAD2 was mainly expressed along the phloem in both roots and shoots. In addition, GAD activity was found not to be transcriptionally regulated in response to heat stress. Subsequently, The Israeli side obtained a GAD1 knockout mutation, and in light of the objective 1 results it was determined that characterization of this knockout mutation would contribute more to the project than the proposed Objective 2. The major conclusion of Objective 3 is that heat-stress-induced changes in GAD activity can be explained by heat-stress-induced changes in cytosolic calcium levels. No evidence that GAD activity was transcriptionally or translationally regulated or that protein phosphorylation was involved in GAD regulation (objective 4) was obtained. Previously published data by others showing that in wheat roots ABA regulated GABA accumulation proved not to be the case in *Arabidopsis* (Objective 5). Consequently, we put the remaining effort in the project into the selection of mutants related to temperature adaptation and GABA utilization and attempting to characterize events resulting from GABA accumulation. A set of 3 heat sensitive mutants that appear to have GABA related mutations have been isolated and partially characterized, and a study linking GABA accumulation to growth stimulation and altered nitrate assimilation were conducted.

By providing a better understanding of how GAD activity was and was not regulated *in vivo*, we have ruled out the use of certain genes for genetically engineering thermotolerance, and suggested other areas of endeavor related to the thrust of the project that may be more likely approaches to genetically engineering thermotolerance.

ACHIEVEMENTS

Significance of Main Scientific Achievements:

The Israeli side investigated the expression of GAD1 and GAD2 in Arabidopsis plants by northern and western blot analysis, and subsequently using reporter-gene, promoter fusions. The conclusion of this work was that GAD 1 was expressed in elongating zones of roots, while GAD2 was expressed in the phloem throughout the plant. However, there does not appear to be any significant transcriptional or translational up-regulation of either GAD1 or GAD2 activity in response to heat stress. The US side showed that heat stress leads to a significant increase in GABA accumulation (and confirmed that GAD1 and GAD2 protein levels do not change) in response to heat stress. They further showed that the heat-stress induced accumulation of GABA correlates with heat-stress alterations in cytosolic calcium levels, as does thermotolerance. Taken together these 2 findings show that GAD expression is tightly tissue-specifically regulated in and around the phloem in the shoot and in the phloem and throughout the elongating zone of the root. This suggests that GAD2, at least, may play a role in converting glutamate moving to or in the phloem into GABA which further suggests that the ratio of glutamate to GABA arriving at sink tissues in the phloem may be regulated through GAD by heat stress.

Most measurements of stimulus-mediated changes in cytosolic calcium show that such responses are mediated by calcium-specific, stimulus-gated channels either in the plasma membrane or an endomembrane within the cell. Since any increase in cytosolic calcium leads to activation of calcium ATPases that immediately pump the calcium from the cytosol, these responses typically consist of brief bursts of cytosolic calcium. Our measurements of the heat-induced changes in cytosolic calcium show that heat produces changes in the steady state level of calcium in the cytosol rather than bursts. Thus, our data show that heat regulates cytosolic calcium levels via a unique mechanism of regulation, that could result from an effect of heat on calcium channels, calcium/proton antiporters, and/or calcium-ATPases.

We also investigated the possibility that ABA mediated GABA accumulation in Arabidopsis, since others had shown that this occurred in rice. However, we were unable to demonstrate the ABA stimulated accumulation of GABA. Similarly, we were unable to collect any subsequent data that supported our preliminary data that GAD activity might be modulated by

protein phosphorylation. These studies were not continued in light of the other ongoing activities that yielded more productive avenues of subsequent investigation.

The best way to determine which of the mechanisms discussed above are involved in heat-induced calcium accumulation and to determine the role of GABA accumulation in thermotolerance involve the isolation of mutants with altered calcium accumulation, GABA accumulation, and thermotolerance. Three such mutants were isolated by the US side during the course of this work, one of which has been characterized, and it does appear to have altered GABA metabolism. Specifically, it is unable to grow on GABA as the sole carbon source, in addition to being more thermo- and osmo- sensitive than wild type plants. The remaining two thermosensitive mutations are being characterized at this time, and we know that these two mutants also demonstrate altered growth (extremely premature flowering) when grown on GABA as the sole nitrogen source. On media containing nitrate and ammonia as the nitrogen sources these mutants grow normally. Thus, we believe that these mutants will be useful in demonstrating that aspects of GAD regulation and GABA metabolism have significant impacts on plant growth both during periods of stress and in the absence of stress, and they may be useful in creating an understanding of how to regulate GABA metabolism to modulate thermotolerance. Additional mutants are being isolated, and will be characterized subsequently.

Agricultural Impacts of Research Findings:

The proposed research effort would be classified as basic research designed to determine the role of GABA accumulation in environmental stress tolerance. We have extended our knowledge of how GABA accumulation is regulated, and in fact data published as a result of this grant shows that modulation of heat-stress-induced calcium signaling can effect the thermotolerance of Arabidopsis seedlings and the accumulation of GABA. However, only by the use of GAD knockouts and transgenic plants overexpressing GAD can we be assured that changes in the expression of GAD can lead to thermotolerance in crop plants. These tools have been obtained as a result of this work, but only the GAD1 knockout, and the transgenic plants have been characterized. These data do not suggest that thermotolerance can be altered by modifications of GAD expression levels, but because the studies are not complete, this cannot be ruled out at this time.

The fact that after the initiation of our project, the genome sequencing project has shown that the Arabidopsis genome contains 5 genes that may code for GADs rather than just the 2 we have worked with, further complicate the ability to be incisive concerning the regulation of GAD activity and thermotolerance, although at present, there is no data to suggest that the 3 new GADs are expressed in any tissue in Arabidopsis.

The outcome of the work done on this project also suggests that simple transgenic experiments may not lead to thermotolerance. Overexpression using a constitutive promoter such as CaMV 35S promoter may not produce the necessary tissue specific expression required to gain beneficial effects of overexpression. Consequently, we need to acquire more precise information regarding where and how GABA is produced in the plant, where it accumulates, and what are the target tissues that respond to the GABA produced, and what are these tissue specific responses.

Details of Cooperation:

During the course of the project, the investigators met annually for the first 2 years of the project at the American Society of Plant Physiology Meetings, and exchanged emails to foster the collaborative work. The most critical area in which cooperation lead to work that would not have been possible without the cooperation concerned the transfer of isoform specific monoclonal antibodies from the Israeli lab to the US lab that were used to generate a publication with joint authorship from both laboratories. A US to Israel visit would have taken place in year 3 of the project, but the Israeli PI was in the act of moving to England from Israel, and a time when we could get together in Israel could not be worked out.

APPENDIX

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Published Papers:

Refereed research articles:

- Barbosa JM, Locy RD, Barger TW, Singh NK, and Cherry JH. (2000) GABA increases the rate of nitrate uptake and utilization in Arabidopsis roots. IN: Plant Tolerance to Abiotic Stresses in Agriculture: Role of Genetic Engineering. NATO Advanced Research Workshop Series in Cell Biology, J. H. Cherry, R.D. Locy, and A.Rychter, EDS. Kluwer Academic Publishers, Dordrecht, pp53-63.
- Locy RD, Wu SJ, Bisnette J, Barger TW, McNabb D, Zik M, Fromm H, Singh NK, and Cherry JH. (2000) The regulation of GABA accumulation of by heat stress in Arabidopsis. IN: Plant Tolerance to Abiotic Stresses in Agriculture: Role of Genetic Engineering. NATO Advanced Research Workshop Series in Cell Biology, J. H. Cherry, R.D. Locy, and A.Rychter, EDS. Kluwer Academic Publishers, Dordrecht, pp39-53.
- Wu SJ, Locy RD, Cherry JH, Singh NK, and Shaw J. (2000) Isolation and characterization of a mutant of Arabidopsis that has enhanced thermosensitivity. *J. Plant Physiol.* 157:543-547.
- Zik M, Arazi T, Snedden WA and Fromm H. (1998) Two isoforms of glutamate decarboxylase in *Arabidopsis* are regulated by calcium/calmodulin and differ in organ distribution. *Plant Mol. Biol.* 37, 967-975.

PhD Theses and Dissertations:

Zik M. (1999) Characterization of Calmodulin-Binding Isoforms of Glutamate Decarboxylase in *Arabidopsis thaliana*: Physiological Roles and Regulation. Thesis for the Degree of Doctor of Philosophy, The Weizmann Institute of Science, Rehovot, Israel.

Barger, T.W. (2000) Metabolic Responses of Plants Exposed to Abiotic Stresses. Dissertation for the Degree of Doctor of Philosophy, Auburn University, Auburn, AL

Abstracts:

Israeli Side:

H. Fromm, H. Takatsuji and WA Snedden. Metabolic regulation by calcium/calmodulin (CaM): a lesson from studies of glutamate decarboxylase (GAD): Annual meeting of the American Society of Plant Physiologist. August 2-August 6, 1997. Vancouver, Canada.

<http://www.rycomusa.com/aspp1997/25/0096.shtml>

Fridmann Y, Zik M, Yang T and Fromm H. Selective association of calmodulin isoforms with petunia glutamate decarboxylase and other target proteins. Annual meeting of the American Society of Plant Physiologists. August 2-August 6, 1997. Vancouver, Canada

<http://www.rycomusa.com/aspp1997/60/0094.shtml>

Zik M, Arazi T, Snedden WA and Fromm H. Two isoforms of glutamate decarboxylase in *Arabidopsis* are regulated by calcium/calmodulin and differ in organ distribution. IX International Congress on Plant Tissue Culture, June 14-19, 1998. Jerusalem, Israel

M. Zik, N. Bouché, A. Fait, D. Bouchez and H. Fromm Molecular-genetic approaches to decipher the function of plant glutamate decarboxylase Annual Meeting of the American Society of Plant Physiologists, July 24 - July 28, 1999, Baltimore, MD, USA

<http://www.rycomusa.com/aspp1999/public/cgi-bin/showabs.cgi?0120>

US Side:

Locy RD, Cherry JH, Singh NK. The effects of GABA on the growth of *Arabidopsis* seedlings. Annual meeting of the American Society of Plant Physiologist. August 2-August 6, 1997. Vancouver, Canada.

<http://www.rycomusa.com/aspp1997/45/1333.shtml>

Locy RD, Wu S-J, McNabb D, Cherry JH, Singh NK., Knight MR. The heat-induced

accumulation of GABA involves the generation of cytoplasmic calcium signal, and enhances the thermotolerance of *Arabidopsis* seedlings. Annual Meeting of the American Society of Plant Physiologists, June 27 - July 1, 1998. Madison, WI, USA

<http://www.rycomusa.com/aspp1998/31/0454.shtml>

Wu S-J, Locy RD, Shaw JJ, Cherry JH, Singh NK. Mutation in *Arabidopsis* *HIT1* locus causing heat and osmotic hypersensitivity. Annual Meeting of the American Society of Plant Physiologists, June 27 - July 1, 1998. Madison, WI, USA

<http://www.rycomusa.com/aspp1998/41/0479.shtml>

Barbosa JM, Locy RD, Singh NK, Cherry JH. GABA increases the rate of nitrate uptake and utilization in *Arabidopsis* roots. Annual Meeting of the American Society of Plant Physiologists, July 24 - July 28, 1999, Baltimore Md.

http://www.rycomusa.com/aspp1999/public/cgi_bin/showabs.cgi?0834

Locy RD, Wu S-J, Knight MR, Singh NK, Cherry JH. The heat-induced accumulation of GABA is correlated with heat-induced cytoplasmic calcium level in *Arabidopsis* seedlings. Annual Meeting of the American Society of Plant Physiologists, July 24 - July 28, 1999, Baltimore Md.

http://www.rycomusa.com/aspp1999/public/cgi_bin/showabs.cgi?0718

Barbosa JM, Singh NK, Cherry JH, Locy RD. GABA increases the rate of nitrate uptake and utilization in *Arabidopsis* roots. Annual Meeting of the American Society of Plant Physiologists, July 15 - July 19, 2000, San Diego, CA

<http://www.rycomusa.com/aspp2000/public/P38/0944.html>

Wu S-J, Cherry JH, Locy RD. Evidence of Heat-elevated Cytoplasmic Calcium Level in *Arabidopsis* Using Luminescent Aequorin. Annual Meeting of the American Society of Plant Physiologists, July 15 - July 19, 2000, San Diego, CA

<http://www.rycomusa.com/aspp2000/public/P33/0863.html>

Saurette J, Wu S-J, Cherry JH, Singh NK, Locy RD. Components of calcium signaling in *Arabidopsis thaliana* in response to heat, osmotic, and salinity stress. Annual Meeting of the American Society of Plant Physiologists, July 15 - July 19, 2000, San Diego, CA

<http://www.rycomusa.com/aspp2000/public/P21/0848.html>