BCHM 421/422 Project – 2023-24

Project Outline: Glutamate decarboxylase (**GAD**) is a Ca^{2+}-calmodulin activated cytosolic enzyme that produces γ-aminobutyrate (**GABA**) as the 1st committed step of the GABA shunt. This pathway bypasses the 2-oxoglutarate to succinate reactions of the Krebs' cycle and is upregulated to help plants acclimate to certain (a)biotic stresses. AtGAD1 is a root-specific GAD isozyme that was *in vivo* hyperphosphorylated at multiple, conserved serine residues located near its N-terminus 48 h following orthophosphate (**Pi**, H_2PO_4) resupply to Pi-starved (**–Pi**) cell cultures of the model plant *Arabidopsis thaliana* (Mehta *et al.* 2021). Preliminary evidence indicates that these phosphorylation events downregulate AtGAD1 activity and GABA shunt flux in unstressed (i.e. Pi-fertilized = **+Pi**), relative to –Pi *Arabidopsis* (Raytek, 2022). Benidickson *et al.* (2022) demonstrated that AtGAD1 and the GABA shunt play important roles in –Pi *Arabidopsis*, as the loss of AtGAD1 in roots of *atgad1* 'knockout' mutants was very deleterious to development of –Pi, but not +Pi plants. *However, nothing is known about the identity and biochemical properties of protein kinase(s) (or upstream signaling pathways) responsible for plant GAD phosphorylation.* This aim of project is to initiate steps needed to bridge this knowledge gap.
Project relevance: Studies of how the remarkable flexibility of plant metabolism and bioenergetics contribute to the survival of –Pi plants are enabling the development of a broad range of innovative strategies for engineering P-efficient crops. Such cultivars are urgently needed to reduce inputs of unsustainable, polluting, and non-renewable Pi fertilizers for long-term global food security and ecosystem preservation.

Supervisor: William Plaxton

Project Title: What protein kinase catalyzes regulatory phosphorylation of glutamate decarboxylase-1 in Pi-resupplied *Arabidopsis thaliana?*

Project Goals: Our ultimate goal is to: (i) identify which of the approximate 1,100 protein kinase isozymes encoded by the *Arabidopsis* genome catalyzes *in vivo* AtGAD1 phosphorylation in Pi-resupplied Arabidopsis, and (ii) eventually characterize its physical and kinetic/regulatory properties.

Experimental Approaches: A non-radioactive, semi-quantitative immunological based assay will initially be used to detect AtGAD1 protein kinase (**AtGAD1-K**) activity in desalted root and cell culture extracts of Pi resupplied *Arabidopsis*. This parallels similar 'protein kinase fishing' projects conducted by Hill et al. (2014), Ying et al. (2014) and Fedosejevs et al. (2017) and exploits a rabbit phospho-site specific antibody that we have raised to the pSer8 phosphosite of phospho-AtGAD1 (**anti-pSer8**), along with purified recombinant dephospho-AtGAD1 and a synthetic AtGAD1 dephosphopeptide as substrates (Raytek, 2022). Immunoblotting with anti-pSer8 will be used in time course assays to detect ATP and Mg^{2+}-dependent phosphoryl group incorporation into purified dephospho-AtGAD1 at Ser8 by desalted root and cell culture extracts of Pi resupplied *Arabidopsis*. Parallel immunoblots probed with rabbit anti-AtGAD1 (i.e. a non-phosphosite specific AtGAD1 antibody) that we also raised provides 'loading' controls. Part 2 of this project involves using this assay to monitor AtGAD1-K activity during its purification from Pi-resupplied cell cultures (using an AKTA FPLC located in the 'Plax-lab'). LC-MS/MS of highly enriched AtGAD1-K can then be used to possibly identify the gene encoding AtGAD1-K (i.e. as done by Ying et al. 2017 and Fedosejevs et al. 2016 for the respective protein kinases that they purified and characterized). Longer term studies could include: (i) the use of quantitative [γ-^{32}P]ATP-based assays for accurate kinetic studies of purified AtGAD1-K, & (ii) cloning and heterologous expression/purification of recombinant AtGAD1-K in *E. coli.*

References:

Mehta *et al.* (2021) Phosphate and phosphite differentially impact the proteome and phosphoproteome of *Arabidopsis* suspension cell cultures. *Plant J* 105: 924-941

Raytek (2022) What is the role of phosphorylation of the Ca^{2+}/calmodulin-dependent glutamate decarboxylase isozyme, AtGAD1, in response to phosphate nutrition of *Arabidopsis thaliana*? *MSc Thesis,* Queen's Biology

Benidickson *et al.* (2022) The root-specific glutamate decarboxylase-1 is essential for efficient acclimation of *Arabidopsis thaliana* to nutritional phosphorus deprivation. *New Phytologist* (submitted for publication)

Hill *et al.* (2014) Phosphorylation of bacterial-type PEP carboxylase by a Ca^{2+}-dependent protein kinase suggests a link between Ca^{2+}-signaling and anaplerotic pathway control in developing castor oil seeds. *Biochem J* 458: 109-118

Ying *et al.* (2017) Regulatory phosphorylation of bacterial-type PEP carboxylase by the Ca^{2+}-dependent protein kinase RcCDPK1 in developing castor oilseeds. *Plant Physiol* 174: 1012-1027.

Fedosejevs *et al*. (2016) The Ca^{2+}-dependent protein kinase RcCDPK2 phosphorylates sucrose synthase at Ser11 in developing castor oil seeds. *Biochem J* 473: 3667–3682