The Pennsylvania State University

The Graduate School

Intercollege Graduate Degree Program in Plant Biology

# HETEROTRIMERIC G PROTEIN REGULATION OF TRANSPIRATION EFFICIENCY, STOMATAL DENSITY, AND PHENOTYPIC PLASTICITY IN *ARABIDOPSIS THALIANA*

A Dissertation in

Plant Biology

by

Sarah Elisabeth Nilson



Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2009

The thesis of Sarah Elisabeth Nilson was reviewed and approved* by the following:

Sarah M. Assmann
Waller Professor of Biology
Dissertation Advisor
Chair of Committee

Paula McSteen
Assistant Professor of Biology

Andrew G. Stephenson
Professor of Biology

David M. Eissenstat
Professor of Woody Plant Physiology, Department of Horticulture

Tei-hui Kao
Professor of Molecular Biology & Biochemistry
Chair, Intercollege Graduate Degree Program in Plant Biology

*Signatures are on file in the Graduate School

## ABSTRACT

Heterotrimeric G proteins are GTP-binding proteins that function in signal transduction in eukaryotes. Despite the relative paucity of genes which encode G protein subunits in plant compared to animal genomes, analysis of *Arabidopsis thaliana* and rice heterotrimeric G proteins mutants have revealed multiple and diverse roles for G proteins in plant physiology. These functions include environmental and hormonal signaling, cell division, development, and stomatal aperture regulation. The functions of heterotrimeric G proteins in Arabidopsis have been previously explored primarily via cell and molecular biology approaches with little emphasis on how G proteins contribute to whole plant water status and plant fitness. This work in this thesis investigates G protein function at the whole-plant level in attempt to understand this missing component of our knowledge of G protein function in plants.

Chapters 1-3 are introductory chapters that explain in more depth the rational and organization of this thesis (Chapter 1) and review literature on the regulation of transpiration in Arabidopsis (Chapter 2) and G protein regulation of stomatal movements (Chapter 3). Chapter 4 identifies the sole Gα subunit in Arabidopsis, GPA1, as a positive regulator of transpiration efficiency, despite stomatal ABA sensitivities of *gpa1* mutants that would suggest the opposite. GPA1 regulates transpiration efficiency in part via stomatal density control which modulates whole-leaf stomatal conductance. Chapter 5 is my attempt at understanding the molecular mechanism behind GPA1 regulation of stomatal density and development, by exploring potential biochemical interactions between GPA1 and previously identified proteins which function in stomatal development. Chapter 6 addresses the question, how do G proteins contribute to plant fitness? This chapter identifies the gene which encodes the sole Gβ subunit in Arabidopsis, *AGB1*, as a plasticity gene. AGB1 regulates phenotypic plasticity, the ability for one genotype to display different phenotypes under different environmental conditions, in response to water availability for a number of fecundity-related traits. Chapter 7 investigates whether G proteins also function in the regulation of cross-

generational phenotypic plasticity, when the environment of the parent affects the phenotype of the offspring. In this study I found significant genetic variation for cross-generational among G protein mutants and wild type plants and that *gpa1* and *agb1* mutants had reduced, if not abolished, cross-generational plasticity for these traits compared to wild type. Chapter 8 discusses how this thesis contributes to plant biology and also suggests additional avenues of inquiry to further elucidate the results of the previous chapters.

# TABLE OF CONTENTS

## LIST OF FIGURES

# LIST OF TABLES

# PREFACE

The research described in this thesis was performed in entirety by me and there are no additional coauthors on any of the research chapters (Chapter 4-7) aside from my research advisor Dr. Sarah M. Assmann (SMA) who funded the research and contributed to the design of experiments and analysis and interpretation of the data. Chapters 1 and 4-8 were also written by me with helpful comments, critique, and editing by SMA. Chapter 2 is a review that was published in *Plant Physiology* (published by the American Society of Plant Biologists which grants permission to authors for republication by the author: http://www.aspb.org/publications/permission.cfm) that I co-wrote with SMA. I authored the following sections of Chapter 2: Introduction, Genetic approaches toward the control of transpiration, Regulation of transpiration by ion channels and transporters and Control of transpiration via modulators of gene expression. Additionally, I made the table, conducted research for all sections of the article, and contributed to editing of the entire article. The remaining sections of Chapter 2 were written by SMA who also edited all sections of the article. Chapter 3 is an invited book chapter in Integrated G Protein Signaling in Plants (submitted) that I co-wrote with Dr. Wei Zhang (WZ). All text and figures of Chapter 3 appear in this thesis with kind permission of Springer Science and Business Media (additional citation information is not yet available because the chapter is pending publication). WZ contributed toward the researching of this article, citing of appropriate references, and made the figure. My contributions to Chapter 3 included research, writing the chapter, and compiling the table. Chapter 3 editing was done by WZ, SMA, and me.

## ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Sarah M. Assmann, for her support during the research and writing of this thesis and for allowing me to develop and pursue my own research projects in her lab. I am grateful for all the helpful feedback and suggestions of my committee members and would like to thank Drs. Paula McSteen, Andy Stephenson, and David Eissenstat for their time and attention. Current and former Assmann lab members were critical to my success by providing me with materials, technical assistance and knowledge. In particular I would like to thank Tim Gookin, Dr. Wei Zhang, Dr. Zhixin Zhao, Dr. Biswa Acharya, William Monsell, and especially, Liza Wilson. I had the pleasure of working with multiple undergraduate students during my time in the Assmann lab, and I would like to acknowledge Jordyn Rivell, Derrick Galligher, and Jennifer Sees for assisting me in setting up experiments and data collection. Thank you to Jennelle Malcos, Dr. Amelia Henry, and Dr. Andrea Skirpan for their assistance with experiments and their friendship and support. Funding for this thesis research came from the Huck Institute of Life Sciences, NASA, and USDA. This thesis would not have materialized without the support of my friends and family. Lastly, I would like to thank my husband, David Hermenau, for all the late night lab pick-ups, pot filling, seed planting and plant measuring he did not to mention the dinners and washed dishes. I could not have done this without him.

# DEDICATION

This thesis is dedicated to my father, Eric L. Nilson, for always challenging me, encouraging me to always ask questions and be skeptical, helping with my chemistry homework, and for filling my childhood with everyday science lessons. Thanks Dad!

# Chapter 1: Introduction

## HETEROTRIMERIC G PROTEIN SIGNALING

Heterotrimeric G proteins are eukaryotic nucleotide-binding proteins that function in signal transduction. According to the classical model of G protein signaling developed from animal studies, the protein exists as an inactive trimer consisting of a Gα subunit bound to GDP, a Gβ subunit, and a Gγ subunit (Assmann, 2002; McCudden *et al.*, 2005). G protein complexes are found at the cell membrane associated with G protein coupled-receptors (GPCRs) which have a characteristic seven transmembrane domain topology. When a ligand binds to the associated GPCR a conformational changes occurs in the G protein and Gα exchanges GDP for GTP and the trimer dissociates into a Gα-GTP unit and a Gβγ dimer. The activated subunits are then free to interact with downstream effectors of G protein signaling including ion channels, phospholipases, and adenylate cyclase. The G proteins stay in the active configuration until the intrinsic GTPase activity of Gα results in the hydrolysis of GTP to GDP and the reassociation of the heterotrimer (Assmann, 2002; McCudden *et al.*, 2005).

While animal genomes contain large numbers of genes which encode heterotrimeric G protein subunits and GPCRs (Fredriksson and Schioth, 2005; McCudden et al., 2005), G protein signaling-related genes in plant genomes are relatively rare. The Arabidopsis genome contains single canonical Gα (Ma *et al.*, 1991) and Gβ (Weiss *et al.*, 1994) subunits (encoded by *GPA1* and *AGB1*, respectively) and two known Gγ subunits (*AGG1* and *AGG2*) (Mason and Botella, 2000, 2001). AGG1 and AGG2 were both identified by interaction with Gβs. While there are conserved structural features among known Gγs, there is little amino acid homology (Mason and Botella, 2000, 2001). Therefore, additional Arabidopsis Gγs may be identified in the future. Approximately a dozen putative GPCRs have been predicted for Arabidopsis based on bioinformatics (Moriyama *et al.*, 2006; Gookin *et al.*, 2008) and coupling to GPA1 in yeast-based assays of protein-protein interaction (Gookin *et al.*, 2008) but the ligands of these putative receptors have not yet been identified. Of the predicted GPCRs in

Arabidopsis, only one, GCR1, has been functionally characterized (Pandey and Assmann, 2004).

## JUSTIFICATION OF THESIS TOPICS

Despite the limited diversity in the Arabidopsis genome for heterotrimeric G protein genes, phenotypic analyses of Arabidopsis and rice mutants that do not have functional G protein subunits have suggested that G proteins play diverse roles in plant physiology and development (Perfus-Barbeoch *et al.*, 2004). One of the earliest identified and well-characterized functions of G proteins is in the regulation of stomatal movements (reviewed extensively in Chapter 3). However, the role of G proteins in stomatal aperture regulation has been mainly studied at the single stomate or guard cell level in epidermal peel and electrophysiological assays. Therefore, there was a need to address how G proteins function in the regulation of whole-leaf and whole-plant water status and how G proteins contribute to fitness under both drought stress and "optimal" growth conditions. These are the central questions of my thesis.

## STUDY SYSTEM AND HETEROTRIMERIC G PROTEIN MUTANTS USED IN THESIS RESEARCH

*Arabidopsis thaliana*, common name mouse-eared cress or thale cress, is a member of the family Brassicaceae and has become a model plant species in plant molecular genetics (Pigliucci, 2002). Arabidopsis is an annual plant that, depending on the ecotype, can display either a summer or winter annual lifecycle (Pigliucci, 2002). The most commonly studied wild type ecotype of Arabidopsis (and the wild type used for all research described in this thesis) is Columbia (Col), which is a rapid cycling summer annual. Arabidopsis reproduces primarily via self fertilization and is a colonizing species found throughout the world particularly in disturbed habitats (Al-Shehbaz and O'Kane, 2002; Pigliucci, 2002). Arabidopsis has a completely sequenced genome and large collections of mutant lines are available for research purposes (Scholl *et al.*, 2003). The majority of these mutants are generated via Agrobacterium-mediated mutagenesis which

occurs when a portion of the tumor-inducing plasmid of Agrobacterium, the T-DNA, is randomly inserted into the plant genome (Alonso and Stepanova, 2003). The T-DNA contains a gene conferring antibiotic resistance allowing for the isolation of transformants which contain a T-DNA insert (Alonso and Stepanova, 2003). PCR-based techniques are then used to identify the plant genomic DNA flanking the insert which allows for the inserts to be mapped to the specific gene. Two wild type ecotypes of Arabidopsis are commonly used to generate T-DNA mutant lines, Col and Wassilewskija (Ws).

In order to examine the function of G protein subunits at the whole-plant level and the contributions of G proteins to phenotypic plasticity and plant fitness I used *gpa1*, *agb1*, and *gcr1* mutants in my research (*agg1* and *agg2* alleles have only recently become available). I used two independent mutant alleles of each gene for all studies (the alleles are caused by independent mutational events) and all the alleles are in the Col background. The mutants I used were backcrossed twice in the laboratory of Dr. Alan Jones and backcrossed once by me. All the mutants were identified, characterized, and published on prior to my using them. *gpa1-3*, *gpa1-4*, *agb1-2*, *gcr1-1*, and *gcr1-2* are the result of T-DNA insertional mutagenesis whereas *agb1-1* is the result of a point mutation. The mutant alleles are summarized in Table 1 with information concerning their status as null alleles and the original reference for each allele.

## ORGANIZATION OF THESIS CHAPTERS

The organization of this thesis is roughly based on biological complexity. Chapters 2 and 3 are introductory chapters that review the regulation of transpiration in Arabidopsis and G protein regulation of stomatal movements, respectively. Chapter 4 addresses how GPA1 contributes to whole leaf and whole plant water status. Chapter 5 is my attempt to better understand the molecular biology behind GPA1 regulation of stomatal development which is first identified in Chapter 4. Chapters 6 and 7 address how G proteins contribute to phenotypic plasticity (both within and between generations) and plant fitness. Since the research in Chapters 6 and 7 have evolutionary and ecological implications they are the final research chapters of my thesis. Chapter 8 is the concluding chapter in which I discuss how this work contributes to the plant biology

body of knowledge and I suggest future research directions that would expand on the discoveries described in this thesis.

## LITERATURE CITED


**Al-Shehbaz I, O'Kane JS** (2002) Taxonomy and phylogeny of *Arabidopsis* (Brassicaceae): September 30, 2002. In The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD

**Alonso JM, Stepanova AN** (2003) T-DNA mutagenesis in *Arabidopsis*. Methods Mol Biol **236:** 177-188

**Assmann SM** (2002) Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. Plant Cell **14 Suppl:** S355-373

**Chen JG, Pandey S, Huang J, Alonso JM, Ecker JR, Assmann SM, Jones AM** (2004) GCR1 can act independently of heterotrimeric G-protein in response to brassinosteroids and gibberellins in *Arabidopsis* seed germination. Plant Physiol **135:** 907-915

**Fredriksson R, Schioth HB** (2005) The repertoire of G-protein-coupled receptors in fully sequenced genomes. Mol Pharmacol **67:** 1414-1425

**Gookin TE, Kim J, Assmann SM** (2008) Whole proteome identification of plant candidate G-protein coupled receptors in *Arabidopsis*, rice, and poplar: computational prediction and *in-vivo* protein coupling. Genome Biol **9:** R120

**Jones AM, Ecker JR, Chen JG** (2003) A reevaluation of the role of the heterotrimeric G protein in coupling light responses in *Arabidopsis*. Plant Physiol **131:** 1623-1627

**Lease KA, Wen J, Li J, Doke JT, Liscum E, Walker JC** (2001) A mutant *Arabidopsis* heterotrimeric G-protein β subunit affects leaf, flower, and fruit development. Plant Cell **13:** 2631-2641

**Ma H, Yanofsky MF, Huang H** (1991) Isolation and sequence analysis of *TGA1* cDNAs encoding a tomato G protein α subunit. Gene **107:** 189-195

**Mason MG, Botella JR** (2000) Completing the heterotrimer: isolation and characterization of an Arabidopsis thaliana G protein γ-subunit cDNA. Proc Natl Acad Sci USA **97:** 14784-14788

**Mason MG, Botella JR** (2001) Isolation of a novel G-protein γ-subunit from *Arabidopsis thaliana* and its interaction with Gβ. Biochim Biophys Acta **1520:** 147-153

**McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS** (2005) G-protein signaling: back to the future. Cell Mol Life Sci **62:** 551-577

**Moriyama E, Strope P, Opiyo S, Chen Z, Jones A** (2006) Mining the *Arabidopsis thaliana* genome for highly-divergent seven transmembrane receptors. Genome Biol **7:** R96

**Pandey S, Assmann SM** (2004) The *Arabidopsis* putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signaling. Plant Cell **16:** 1616-1632

**Perfus-Barbeoch L, Jones AM, Assmann SM** (2004) Plant heterotrimeric G protein function: insights from *Arabidopsis* and rice mutants. Curr Opin Plant Biol **7:** 719-731

**Pigliucci M** (2002) Ecology and evolutionary biology of *Arabidopsis*: April 4, 2002. In The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD

**Scholl R, Sachs MM, Ware D** (2003) Maintaining collections of mutants for plant functional genomics. Methods Mol Biol **236:** 311-326

**Ullah H, Chen JG, Temple B, Boyes DC, Alonso JM, Davis KR, Ecker JR, Jones AM** (2003) The β-subunit of the *Arabidopsis* G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell **15:** 393-409

**Weiss CA, Garnaat CW, Mukai K, Hu Y, Ma H** (1994) Isolation of cDNAs encoding guanine nucleotide-binding protein β-subunit homologues from maize (ZGB1) and *Arabidopsis* (AGB1). Proc Natl Acad Sci USA **91:** 9554-9558

**Table 1.** Mutation details of the heterotrimeric G protein mutants used in thesis research.

| Allele | Background ecotype | Type of mutation | Location of mutation | Null status | Reference |
|---|---|---|---|---|---|
| *gpa1-3* | Col | T-DNA | 9th intron of coding region | Full length transcript and protein null | (Jones *et al.*, 2003) |
| *gpa1-4* | Col | T-DNA | 12th exon of coding region | Full length transcript and protein null | (Jones *et al.*, 2003) |
| *agb1-1* | Col | Point mutation | Splicing donor site at the 5' end of the 1st intron | mRNA is less abundant and larger in size than wild type mRNA<br><br>Translation prediction results in a frameshift and addition of 44 novel amino acids before a premature stop codon | (Lease *et al.*, 2001) |
| *agb1-2* | Col | T-DNA | 4th exon of coding sequence | Full length transcript null | (Ullah *et al.*, 2003) |
| *gcr1-1* | Col | T-DNA | 8th intron of coding sequence | Full length transcript null | (Chen *et al.*, 2004) |
| *gcr1-2* | Col | T-DNA | 6th exon of coding sequence | Full length transcript null | (Chen *et al.*, 2004) |

# The Control of Transpiration. Insights from Arabidopsis[1]

**Sarah E. Nilson and Sarah M. Assmann***

Biology Department, Penn State University, University Park, Pennsylvania 16802

Stomatal complexes in the epidermes of aerial plant parts are critical sites for the regulation of gas exchange between the plant and the atmosphere. Stomata consist of microscopic pores, each flanked by a pair of guard cells. Guard cells can increase or decrease the size of the pore via changes in their turgor status, hence regulating both $CO_2$ entry into the leaf and transpiration, or the loss of water from the leaf. This *Update* focuses on recent progress in our understanding of the regulation of transpiration and drought tolerance that has been garnered through the use of Arabidopsis (*Arabidopsis thaliana*) as a model experimental system.

The coordinated regulation of gas exchange is integral to land plant survival because $CO_2$ must be able to penetrate the leaf to allow photosynthesis, yet water loss (transpiration) must be minimized to prevent desiccation, drought stress, and plant death. Transpiration also provides the driving force for the transport of water and nutrients from the roots to the aerial tissues, and the evaporation of water from the substomatal cavity cools the plant (Lambers et al., 1998). While a number of morphological traits can contribute to the overall level of leaf gas exchange (e.g. the density and distribution of stomata, leaf epidermal structure and internal organization, cuticle thickness), the regulation of stomatal aperture size is unique in that it is a dynamic and reversible process by which water loss and $CO_2$ influx can be rapidly fine tuned in response to a number of environmental and intrinsic signals, such as light, $CO_2$, and the plant stress hormone abscisic acid (ABA; Schroeder et al., 2001). Because guard cells integrate and respond to a plethora of signals, they have become a model cell type in the field of plant cell signaling (Blatt, 2000; Schroeder et al., 2001; Roelfsema and Hedrich, 2005).

This *Update* highlights recent research reports on the guard cell physiology of Arabidopsis that include some quantitative measure of stomatal function. These measures include transpiration, stomatal conductance (stomatal conductance is defined as stomatal transpiration divided by the vapor pressure difference between the leaf and the air, and increases with increasing stomatal aperture), leaf water status, and water-use efficiency/transpiration efficiency (the ratio of photosynthetic assimilation to transpiration). By focusing the article in this manner, we hope to promote the synthesis of ideas and approaches between whole-plant physiologists and molecular biologists/geneticists. The former typically measure stomatal regulation of gas exchange and its impact on whole-plant physiology, and may treat the cellular and molecular biology of guard cells as a "black box" that receives and reacts to inputs. The latter typically use model plant species to investigate cell and molecular regulation of guard cell function, and may employ gene expression, stomatal aperture, or a specific guard cell parameter, such as ion fluxes, as a "readout," without quantifying alterations in gas exchange and concomitant whole-plant impacts. Our premise is that Arabidopsis is an excellent reference plant in which these complementary approaches can be readily combined, and that such an integrated approach has great potential to yield new insights into the biology of transpiration in $C_3$ angiosperms.

## GENETIC APPROACHES TOWARD THE CONTROL OF TRANSPIRATION

Arabidopsis is a powerful biological tool for the identification and characterization of the molecular regulators of transpiration because it has a small, sequenced genome and is easy to transform. These characteristics allow researchers to experimentally modulate the levels of candidate regulatory molecules via techniques such as RNA interference, insertional mutagenesis, or genetic overexpression, and many studies that employ such tools are discussed in the following sections. Additionally, the availability of collections of genetic mutants allows for large-scale screens for potential regulators of transpiration and for functional analyses of candidate regulators. For example, one such screen used infrared thermography to detect differences in leaf temperature, a correlate of transpiration, among a collection of Arabidopsis mutants (Merlot et al., 2002; Wang et al., 2004). The screen identified two novel mutations in stomatal regulation, *ost1* and *ost2*; *OST1* has been cloned and identified as encoding an Arabidopsis homolog of an ABA-activated protein kinase first identified in *Vicia faba* and is discussed further below (Li et al., 2000; Mustilli et al., 2002).

[1] This work was supported by the U.S. Department of Agriculture (grant no. 2006–35100–17254 to S.M.A.).

* Corresponding author; e-mail sma3@psu.edu; fax 814–865–9131.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Sarah M. Assmann (sma3@psu.edu).

www.plantphysiol.org/cgi/doi/10.1104/pp.106.093161

Quantitative trait loci (QTL) analysis is an alternative to mutant analysis that harnesses naturally occurring variation within a species to identify putative genes and genomic regions involved in the regulation of quantitative traits such as transpiration (Alonso-Blanco and Koornneef, 2000). QTL mapping involves the generation of a segregating population for a particular trait, often either an $F_2$ population or a population of homozygous recombinant inbred lines. The population is then phenotyped for the traits of interest and genotyped using molecular markers. Statistical techniques are then employed to link specific genotypes to traits, which allows for the mapping of traits to particular chromosomal regions. Arabidopsis is a useful species for QTL analysis because of its small size and rapid life cycle; large mapping populations can be grown in a small space and recombinant inbred lines can be generated relatively quickly compared to other species (Alonso-Blanco and Koornneef, 2000). Additionally, once candidate genes of interest are identified, they can be further characterized using the molecular techniques mentioned above.

QTL analysis has led to the identification of a number of QTLs affecting transpiration efficiency in Arabidopsis (Juenger et al., 2005; Masle et al., 2005). It will be interesting to see to what extent these loci are found to encode known regulators of stomatal response, such as those discussed in subsequent sections, versus novel regulatory mechanisms. One example of the latter was provided by Masle and colleagues (Masle et al., 2005). Using QTL analysis, they identified one genetic locus, *ERECTA*, which encodes a Leu-rich repeat receptor-like kinase, as a genetic regulator of transpiration efficiency (Masle et al., 2005). Complementation of genotypes harboring mutations in *ERECTA* (including the common Arabidopsis ecotype Landsberg *erecta*) with the wild-type *ERECTA* allele results in increased transpiration efficiency and reduced stomatal conductance compared to *erecta* mutants.

## HORMONAL REGULATION OF TRANSPIRATION

When plants are drought stressed, the plant hormone ABA accumulates in the shoot, where it both inhibits stomatal opening and promotes stomatal closure, resulting in reduced water loss from the plant. ABA is a key regulator of plant water status and stomatal function, and ABA and drought responses are the focus of the majority of the studies discussed in this *Update*. It is important to note that the terms drought stress and drought tolerance are used in this review just as they were reported in the original references. In these references, it is usually the case that a plant is deemed drought tolerant if it survives a restricted watering regime. However, if the effect of, e.g. a genetic manipulation, is to reduce transpiration, then, under identical watering regimes, the mutant plant is actually experiencing less drought stress than the wild-type control plant.

Research on the effects of altered levels of ABA on transpiration spans several decades, starting with the discovery of the wilty *flacca* mutant of tomato (*Lycopersicon esculentum*), which is deficient in ABA synthesis (Tal, 1966). Recent research on this topic has taken advantage of the molecular genetic tools available in the Arabidopsis model system. Production of xanthoxin from epoxycarotenoids is a key step in ABA synthesis (Nambara and Marion-Poll, 2005). A family of seven 9-cis-epoxycarotenoid dioxygenase (*NCED*) genes is implicated in this process in Arabidopsis, of which *NCED3* is most strongly induced by drought (Iuchi et al., 2001). Iuchi and co-authors demonstrated that overexpression of *NCED3* resulted in elevated ABA levels, strong induction of the *RD29B* ABA reporter gene following drought onset, reduced transpiration under well-watered conditions, and improved drought survival. Antisense and T-DNA knockout lines exhibited the opposite phenotypes.

ABA levels in the plant reflect a balance between ABA synthesis and ABA catabolism into inactive forms by conjugation or oxidation. ABA oxidation to 8′-hydroxyl ABA (from which spontaneous isomerization to phaseic acid occurs) is catalyzed by four cytochrome P450 monooxygenases in Arabidopsis: CYP707A1 to 4. Of these, *CYP707A3* is most strongly induced by ABA during dehydration and rehydration (Umezawa et al., 2006). In a recent study, Shinozaki and colleagues characterized T-DNA insertional mutants and constitutive overexpressing lines of CYP707A3 (Umezawa et al., 2006). The T-DNA mutants exhibited greater ABA content under all conditions, more rapid expression of ''classic'' markers of ABA-induced gene expression (such as *RD29A* and *RAB18*), reduced transpiration, and improved survival after drought treatment. Conversely, *CYP707A3*-overexpressing lines exhibited lowered ABA content coupled with higher levels of the ABA metabolites phaesic acid and dihydrophaseic acid; these lines exhibited increased transpiration. Interestingly, transgenic alterations in levels of two RING-finger proteins, the RING-H2 protein XERICO and the R2R3-type MYB transcription factor HOS10, strongly affect *NCED3* transcript levels, with correlated effects on ABA levels, drought tolerance, and water loss (Zhu et al., 2005; Ko et al., 2006).

Numerous genetic mutants in Arabidopsis with alterations in production, sensing, or response to all the major plant hormones provide a wealth of resources with which to investigate hormonal regulation of transpiration. Tanaka and colleagues have used such tools to investigate hormonal cross talk between ABA, ethylene, cytokinins, and auxins in the regulation of stomatal apertures (Tanaka et al., 2005, 2006). When ethylene levels were increased, either via provision of exogenous ethylene or through use of the ethylene-overproducing mutant *eto1-1*, ABA-induced stomatal closure in epidermal peels was retarded, and greater rates of fresh weight decrease in excised shoots were observed. The effects seem specific to the ABA response, as no alterations in dark-induced stomatal

closure were seen. Treatment of epidermal peels with cytokinins (6-benzyladenine or kinetin) or auxins (naphthaleneacetic acid or indole-3-acetic acid) similarly opposed ABA-induced stomatal closure. Tanaka et al. hypothesize that these hormones act indirectly, through enhancement of ethylene production, since the repressive effects of 6-benzyladenine and naphthaleneacetic acid on ABA-induced stomatal closure were negated by genetic (use of the *ein3-1* ethylene-insensitive mutant) or pharmacological (application of 1-methylcyclopropene, a competitive inhibitor of ethylene-receptor binding) abrogation of ethylene signaling. These studies illustrate the interconnectivity of hormone signaling in plant systems, an emerging theme in phytohormone research (Gazzarrini and McCourt, 2003; Ko et al., 2006).

## REGULATION OF TRANSPIRATION BY ION CHANNELS AND TRANSPORTERS

Stomatal conductance is altered by the opening and closing of stomata, processes which in turn are mediated via changes in the turgor status of the adjacent guard cells. Changes in guard cell turgor result from water influx or efflux into the cell following changes in cell water potential, which arise from alterations in symplastic ion concentrations. Stomatal opening occurs when $K^+$, $Cl^-$, malate$^{2-}$, and Suc accumulate inside the cells, resulting in water entry into the guard cells and the outbowing and opening of the stomatal pore. Stomatal closure occurs following $K^+$ and anion efflux, resulting in loss of water from the cell, a reduction in cell turgor, and pore closure (Schroeder et al., 2001). Therefore, the channels and transporters responsible for ion transport across cell membranes are key regulators in the control of stomatal aperture and plant water loss.

Signals resulting in changes in stomatal aperture alter the activities of a number of ion channels and transporters. For example, ABA can promote stomatal closure and inhibit stomatal opening in part by stimulating an increase in cytosolic $Ca^{2+}$ levels via activation of plasma membrane and endomembrane $Ca^{2+}$-permeable channels (Sanders et al., 2002; Fan et al., 2004; Hetherington and Brownlee, 2004). The increase in cytosolic $Ca^{2+}$ is a signal that initiates anion efflux and consequent plasma membrane depolarization, which inhibits inward-rectifying $K^+$ channels and activates outward-rectifying $K^+$ channels (Schroeder et al., 2001). A net movement of ions out of the cell causes water efflux and closure of the stomatal pore.

The major outward-rectifying $K^+$ channel involved in guard cell closure in Arabidopsis is encoded by the *GORK* gene (Hosy et al., 2003). Functional analyses of *gork* mutants suggest that GORK plays an important role in the regulation of transpiration. *gork-1* T-DNA insertional mutants and *gork-dn1* dominant-negative mutants displayed reduced ABA- and dark-induced stomatal closure in isolated epidermal peels and increased water loss from excised rosettes compared to wild-type plants (Hosy et al., 2003). Whole-rosette gas-exchange analysis revealed that the *gork-1* mutants transpired more, especially under water-stressed conditions, and had slower reductions in transpiration when light-acclimated plants were placed in the dark (Hosy et al., 2003).

Because $K^+$ influx is critical for stomatal opening, inward-rectifying $K^+$ channels, such as KAT1, are also candidate transpiration regulators. Analysis of an Arabidopsis mutant harboring a transposon-induced mutation in *KAT1*, however, found no altered stomatal functioning or regulation of transpiration, suggesting genetic redundancy may exist for inward-rectifying $K^+$ channels in guard cells (Kwak et al., 2001; Szyroki et al., 2001). Indeed, a number of genes encoding inward-rectifying $K^+$ channels are expressed in guard cells of Arabidopsis, including *KAT1*, *KAT2*, *AKT1*, *AtKC1*, and *AKT2/3* (Szyroki et al., 2001). To avoid the confounding effects of likely functional redundancy among $K^+$ channels, Schroeder and colleagues used a dominant-negative approach to decrease the overall level of functional inward-rectifying $K^+$ channels in Arabidopsis (Kwak et al., 2001). Transgenic plants overexpressing a dominant-negative mutant form of KAT1 displayed reduced inward $K^+$ current and guard cell $K^+$ content (Kwak et al., 2001). These mutant *KAT1* lines also had reduced light-induced stomatal opening, reduced water loss from excised leaves, and increased water content in leaves following drought stress compared to empty-vector control lines, supporting a role for inward-rectifying $K^+$ channels in the regulation of transpiration.

In addition to functioning in cellular detoxification, two ATP-binding cassette transporters that are expressed in guard cells, AtMRP4 and AtMRP5, are also involved in the control of transpiration, possibly as regulators of ion channel activity (Leonhardt et al., 1997, 1999; Klein et al., 2003, 2004). *mrp5* mutants were insensitive to ABA promotion of stomatal closure but displayed reduced light-induced stomatal opening (Klein et al., 2003). Whole-plant and leaf gas-exchange measurements showed reduced transpiration in the *mrp5* mutant compared to control, concomitant with an approximately 20% increase in instantaneous water-use efficiency, and *mrp5* mutants had reduced water loss from excised leaves and were less wilty than wild-type plants under drought conditions (Klein et al., 2003). These data suggest that in the *mrp5* mutant, the reduction in light-induced stomatal opening and resultant decrease in transpiration are more important to maintaining whole-plant water status than any increase in water loss due to reduced ABA sensitivity of stomatal closure. Interestingly, *mrp4* mutants display phenotypes opposite to those of *mrp5*; *mrp4* mutants have larger stomatal apertures in both the light and the dark and exhibit increased water loss from excised leaves. Nevertheless, *mrp4* mutants retain ABA sensitivity of stomatal closure (Klein et al., 2004). Gas-exchange measurements reveal that *mrp4*

mutants have increased transpiration and reduced water-use efficiency, and wilt earlier than wild type when drought stressed (Klein et al., 2004).

To date, no genes encoding anion channels involved in stomatal movements have been definitively identified, although members of the ATP-binding cassette transporter family are being scrutinized as candidates. However, a guard cell-expressed $NO_3^-$ transporter, AtNRT1.1/CHL1, has been shown to function in $NO_3^-$-dependent stomatal opening and plant drought responses (Guo et al., 2003). *chl1* mutants show no altered sensitivity to ABA but show reduced $NO_3^-$ uptake and light-stimulated stomatal opening when $NO_3^-$ is the sole anion available, presumably because, under these conditions, $NO_3^-$ is the only anion available to serve as a counter ion for $K^+$ uptake. Replacement of $NO_3^-$ with $Cl^-$ eliminates altered stomatal opening in the mutant (Guo et al., 2003). Additionally, when grown in substrates containing $NO_3^-$, *chl1* mutants are more drought tolerant and have reduced transpiration compared to wild type (Guo et al., 2003). Interestingly, wild-type plants lost more water from excised leaves when $NO_3^-$ was present, suggesting that $NO_3^-$ availability allowed for wider apertures (Guo et al., 2003). Taken together, these data suggest that the amount of $NO_3^-$ in the soil can affect stomatal regulation and magnitude of transpiration, and this $NO_3^-$ effect is in part mediated by $NO_3^-$ uptake into guard cells via the $NO_3^-$ transporter CHL1.

In Arabidopsis, 20 Glu receptor-like (*GLR*) genes have been identified, and evidence is accumulating that suggests that the GLR proteins may function as nonselective cation channels (Davenport, 2002). One putative plant Glu receptor, AtGLR1.1, has recently been implicated in functioning in ABA biosynthesis, ABA signaling, and control of transpiration (Kang et al., 2004). Antisense *AtGLR1.1* lines had smaller stomatal apertures, reduced transpiration rates, and were more drought resistant than wild-type plants (Kang et al., 2004). Consistent with these results, these lines also had higher transcript levels of ABA biosynthetic genes and higher levels of ABA, as well as reduced expression of *ABI1* and *ABI2* genes, which encode negative regulators of ABA response. The mechanism by which an alteration in cation flux would influence gene expression remains unknown.

## CONTROL OF TRANSPIRATION BY CELLULAR SIGNALING MECHANISMS

The appropriate transduction of abiotic stress signals into cellular and developmental responses is of paramount importance in both natural and agroecosystems (J.Z. Zhang et al., 2004; Chaerle et al., 2005; Wang et al., 2005). Accordingly, the identification of intracellular second messengers for drought and ABA is a major area of research in plant biology (Rock, 2000). It is impossible to do justice to ABA signaling within the constraints of this article; for a more comprehensive discussion of this topic in the context of guard cell physiology, readers are pointed toward several excellent reviews (Blatt, 2000; Schroeder et al., 2001; Sheng, 2003; Roelfsema and Hedrich, 2005; Verslues and Zhu, 2005). Instead, in this section, we have chosen to exemplify the progress that is being made by focusing on just one second messenger of guard cell ABA signaling, ABI1, and the web of molecules with which it is being found to interact. ABI1 is chosen first because it is an important regulator of ABA responses and second because it is one of the best-studied second messengers in guard cells.

ABI1 is a type 2C protein phosphatase (PP2C). The first *ABI1* mutant to be characterized was the dominant-negative mutant *abi1-1* (Koornneef et al., 1989; Leung et al., 1994; Meyer et al., 1994). This mutant exhibits a strong ABA-insensitive, wilty phenotype (Koornneef et al., 1989), accompanied by elevated, ABA-insensitive stomatal conductance (Assmann et al., 2000). Subsequently, intragenic revertant recessive mutants and, more recently, T-DNA insertional mutants of *ABI1* were isolated (Gosti et al., 1999; Mishra et al., 2006; Saez et al., 2006). These mutants exhibit moderate ABA hypersensitivity in stomatal regulation, and this hypersensitivity is strongly enhanced when double mutants are created with the related PP2C genes *ABI2* (Merlot et al., 2001) or *HAB1* (Saez et al., 2004, 2006). Since loss of ABI1 results in ABA hypersensitivity, ABI1 is characterized as a negative regulator of ABA responses.

Some of the signaling components functioning upstream (Guo et al., 2002) and downstream of ABI1 have been identified. Downstream, production of reactive oxygen species (ROS) is impaired in the dominant-negative *abi1-1* mutant (Murata et al., 2001). Production of ROS is also impaired in the aforementioned *ost1* mutant (Mustilli et al., 2002); thus, OST1 likely functions upstream of the NADPH oxidases that produce ROS in guard cells (Kwak et al., 2003). However, the relative positions of OST1 and ABI1 in the signaling cascade are still unclear. ROS inhibit ABI1 activity (Meinhard and Grill, 2001), suggesting that that ROS and thus OST1 may function upstream of ABI1. On the other hand, OST1 was recently shown to physically interact with ABI1 in a yeast two-hybrid assay and the *abi1-1* mutant form of ABI1 inhibits ABA activation of OST1 (Yoshida et al., 2006), suggesting that OST1 might function downstream of ABI1. It is also important to note that these two possibilities are not mutually exclusive, e.g. OST1 activation of ROS production could be a regulatory, negative feedback mechanism on ABI1 and thus also feedback regulate OST1 activity. In the dominant *abi1-1* mutant, activation of ROS-activated, $Ca^{2+}$-permeable channels at the plasma membrane is also impaired (Murata et al., 2001), as is elevation of cytosolic $Ca^{2+}$ (Allen et al., 1999). Activation of slow anion channels, which participate in the large anion efflux needed to drive stomatal closure, is likewise impaired in dominant-negative *abi1-1* plants because these channels are $Ca^{2+}$ activated

(Pei et al., 1997). Because all of these responses are inhibited in dominant-negative *abi1-1*-insensitive mutant plants, it is reasonable to hypothesize that they may be strengthened in recessive, ABA-hypersensitive *abi1* mutants. ABI1 also physically interacts with the transcription factor ATHB6 (Himmelbach et al., 2002). As discussed in the next section, overexpression studies show that ATHB6 is a negative regulator of ABA-induced gene expression, and perhaps it is activated by ABI1.

Given that ABI1 is a negative regulator of ABA action, one would expect that the net result of ABA activation of components functioning upstream of ABI1 would be to inhibit the activity of this PP2C phosphatase. One of the enzymes activated by ABA in guard cells is phospholipase D (PLD; Jacob et al., 1999), which hydrolyzes phospholipids, producing a headgroup and phosphatidic acid (PA). Interestingly, the lipid metabolite PA binds to ABI1 and inhibits its activity (W. Zhang et al., 2004). Knockdown of PLD$\alpha$1 in Arabidopsis by antisense methods increases stomatal conductance and impairs drought tolerance (Sang et al., 2001; W. Zhang et al., 2004), effects that would be consistent with loss of inhibition of ABI1 in the PLD$\alpha$1 antisense guard cells. Indeed, a mutant version of ABI1 that is unable to bind PA but has normal phosphatase activity also results in hyposensitivity of ABA-induced stomatal closure (Mishra et al., 2006).

PLD$\alpha$1 also has additional roles in modulation of ABA inhibition of inward $K^+$ channels and stomatal opening, through a pathway that involves the heterotrimeric G protein $\alpha$-subunit GPA1(Jacob et al., 1999; Wang et al., 2001; Coursol et al., 2003; Zhao and Wang, 2004; Mishra et al., 2006). Since the GPA1-dependent pathway is proposed to be ABI1 independent (Mishra et al., 2006), readers are referred to the cited references for further details.

The above summary has focused only on ABI1, and literally dozens of ABA-regulated secondary messengers have been identified in guard cells. A figure that summarizes the current guard cell signaling network for ABA-induced stomatal closure, including the portion described above, has recently been published (Li et al., 2006). Ultimately, the power of computational and systems biology approaches will be needed to derive comprehensive and predictive models of ABA signaling, and the paper by Li et al. describes one such approach (Li et al., 2006).

## CONTROL OF TRANSPIRATION VIA MODULATORS OF GENE EXPRESSION

Recent evidence suggests that, in addition to rapid cellular signaling events, gene expression changes also function in the regulation of stomatal aperture size and transpirational water loss in Arabidopsis. Table I summarizes names and functions of regulators of gene expression that have been implicated in the control of transpiration. Two R2R3-MYB domain transcription factors, *AtMYB60* and *AtMYB61*, both guard cell expressed, have been shown to play opposite roles in the regulation of diurnal stomatal movements (Cominelli et al., 2005; Liang et al., 2005). The *atmyb60-1* T-DNA insertional mutant displays reduced sensitivity toward light-induced stomatal opening, reduced water loss from excised leaves, and reduced transpirational water loss when drought stressed as measured by the relative water content of the rosette leaves (Cominelli et al., 2005). Conversely, *myb61* mutants display reduced dark-induced stomatal closure and increased stomatal conductance compared to wild type (Liang et al., 2005). The *atmyb60-1* and *atmyb61* mutants and overexpressing plants showed no altered sensitivities toward ABA (Cominelli et al., 2005; Liang et al., 2005). Therefore, it appears that AtMYB60 and AtMYB61 function specifically in the diurnal regulation of stomatal aperture and transpirational water loss.

Expression of a number of genes is controlled by ABA. Some of the ABA-induced genes serve protective functions in the plants, while others are regulatory in nature, such as protein kinases, protein phosphatases, and transcription factors (Rock, 2000). One method to identify potential regulators of ABA-modulated gene expression and thus of transpiration is to screen for proteins that bind to ABA-responsive cis-elements, such as ABREs, found in the promoters of a number of ABA up-regulated genes (Busk and Pages, 1998). ABF3 and ABF4 are basic Leu zipper (bZip) proteins that were identified via a yeast one-hybrid screen as ABRE-interacting proteins (Kang et al., 2002). Compared to wild type, transgenic lines overexpressing ABF3 or ABF4 exhibited drought tolerance and reduced water loss from excised rosette leaves (Kang et al., 2002). Conversely, *abf3* and *abf4* mutants are more susceptible to drought than wild type (Kim et al., 2004). Based on reporter gene analysis (Kang et al., 2002), both *ABF3* and *ABF4* are expressed in leaf tissues, including guard cells, suggesting that they may influence stomatal function in part through direct regulation of gene expression in guard cells. Consistent with this idea, transcripts of the *KAT1* and *KAT2* genes, which encode inward $K^+$ channels that mediate $K^+$ uptake during stomatal opening, are repressed in *ABF3*-overexpressing lines (Kang et al., 2002).

Another ABRE-binding protein, the bZip protein ABF2 (also known as AREB1), has been shown to confer drought tolerance when overexpressed (Kim et al., 2004). However, in this case, transgenics overexpressing a constitutively active form of ABF2 did not exhibit a reduction in water loss (Fujita et al., 2005; Furihata et al., 2006). Instead, drought tolerance may have been conferred because there was increased expression of a number of ABA-induced genes, including LATE EMBRYOGENESIS ABUNDANT class proteins, which are thought to serve protective functions. Thus, these experiments illustrate the fact that plants employ a diversity of mechanisms to achieve drought tolerance, only some of which involve alterations in stomatal regulation.

**Table I.** *Transcription factors, chromatin-remodeling factors, and RNA-processing proteins implicated in drought and ABA regulation of transpiration in Arabidopsis and discussed in this article*

In this table, "Mutant" refers to recessive underexpressing or null lines; "OEX" refers to overexpressing lines.

| Locus | Gene Name | Function/Putative Function | Type of Line: Whole-Plant Phenotype | Putative Role in Transpiration Regulation | References |
|---|---|---|---|---|---|
| At1g08810 | *AtMYB60* | R2R3-MYB transcription factor | Mutant: reduced water loss from excised and drought-stressed leaves | Function in diurnal regulation of transpiration | Cominelli et al. (2005) |
| At1g09540 | *AtMYB61* | R2R3-MYB transcription factor | Mutant: increased stomatal conductance<br>OEX: reduced stomatal conductance | Function in diurnal regulation of transpiration | Liang et al. (2005) |
| At1g35515 | *HOS10* | R2R3-MYB transcription factor | Mutant: increased water loss from excised shoots | Positive regulator of ABA biosynthetic gene, *NCED3*, and ABA levels | Zhu et al. (2005) |
| At4g34000 | *ABF3/AREB3* | ABRE-binding bZip transcription factor | Mutant: susceptible to drought stress<br>OEX: reduced water loss from excised leaves, drought tolerant | Positive regulator of ABA response | Kang et al. (2002), Kim et al. (2004) |
| At3g19290 | *ABF4/AREB2* | ABRE-binding bZip transcription factor | Mutant: susceptible to drought<br>OEX: reduced water loss from excised leaves, drought tolerant | Positive regulator of ABA response | Kang et al. (2002), Kim et al. (2004) |
| At2g22430 | *ATHB6* | HD-zip transcription factor | OEX: increased water loss from excised leaves | Negative regulator of ABA signaling | Himmelbach et al. (2002) |
| At3g20310 | *AtERF7* | AP2/EREBP-type transcription factor | OEX: increased water loss from excised leaves, susceptible to drought | Negative regulator of ABA signaling | Song et al. (2005) |
| At5g03740 | *AtHD2C/HDT3* | Histone deacetylase | OEX: reduced water loss from excised leaves | Tissue-specific regulator of ABA signaling | Sridha and Wu (2006) |
| At5g44200 | *CBP20* | Nuclear mRNA cap-binding protein | Mutant: reduced stomatal conductance, drought tolerant | Negative regulator of ABA signaling | Papp et al. (2004) |
| At2g13540 | *ABH1/CBP80* | Nuclear mRNA cap-binding protein | Mutant: reduced stomatal conductance, wilt tolerant | Negative regulator of ABA signaling | Hugouvieux et al. (2002) |

Transcription factors serving as negative regulators of ABA signaling may also play a role in the regulation of transpiration. One such repressor is ATHB6, a HD-zip protein that interacts with ABI1, a PP2C and known negative regulator of ABA responses (Himmelbach et al., 2002). Transgenic plants overexpressing ATHB6 exhibit increased water loss from excised leaves and reduced stomatal closure following leaf detachment compared to control plants (Himmelbach et al., 2002). A second transcriptional repressor of ABA response is AtERF7, an AP2/EREBP-type transcription factor that binds to the GCC-box ABRE and can be phosphorylated by protein kinase PKS3, a negative regulator of ABA signaling (Guo et al., 2002; Song et al., 2005). In lines overexpressing AtERF7, ABA-induced up-regulation of two genes containing GCC boxes in their promoters was shown to be eliminated. These lines also displayed increased water loss from excised leaves, decreased drought tolerance compared to wild type, and hyposensitivity toward ABA-induced stomatal closure compared to wild type (Song et al., 2005), leading to the conclusion that AtERF7 suppresses positive regulators of ABA response. Conversely, RNA interference lines that had reduced levels of AtERF7 displayed ABA hypersensitivity (Song et al., 2005).

Interestingly, in transient expression assays, repression of ABA-induced genes by AtERF7 is enhanced by the histone deacetylase HDA19 (Song et al., 2005). In addition, AtERF7 interacts with a transcriptional corepressor, AtSin3, which may interact with HDA19 (Song et al., 2005). This suggests a role for histone deacetylation and chromatin remodeling in ABA regulation of gene expression (Song et al., 2005).

AtHD2C, one of four plant-specific HD2-type histone deacetylases, is also implicated in ABA regulation of gene expression (Sridha and Wu, 2006). AtHD2C-overexpressing plants display up-regulation of the ABA-responsive genes *RD29B* and *RAB18*, and reduced transcript levels of *ABI2*, a negative regulator of ABA response. Consistent with these results, AtHD2C-overexpressing plants also display drought tolerance

and reduced water loss from excised leaves (Sridha and Wu, 2006). However, overexpression of AtHD2C also confers reduced sensitivity toward ABA in ABA inhibition of germination and root growth, indicating that the role of AtHD2C in ABA response may exhibit tissue and cell specificity.

Proteins involved in the posttranscriptional modifications of mRNAs also play a role in the regulation of stomatal movements. Plants harboring mutations in genes encoding two subunits of the nuclear cap-binding complex, CBP20 and ABH1/CBP80, display marked ABA hypersensitivity (Hugouvieux et al., 2001; Papp et al., 2004). *abh1* mutants are hypersensitive to ABA induction of cytosolic $Ca^{2+}$ elevation in guard cells and stomatal closure, and wilt less than wild type following drought stress (Hugouvieux et al., 2001). In the absence of exogenous ABA, *abh1* mutants exhibit reduced inward $K^+$ currents and enhanced anion efflux currents, responses that accord well with the reduced stomatal apertures and stomatal conductances seen under these conditions, and are consistent with hypersensitivity to endogenous ABA (Hugouvieux et al., 2002). *cbp20* mutants similarly display drought tolerance and have reduced stomatal conductance compared to wild type (Papp et al., 2004).

Although transcription factors have long been known to participate in ABA regulation of plant development, the studies cited above are providing new information on the roles of transcription factors in the dynamic regulation of stomatal movement (Rock, 2000). In addition, compelling new information on roles of chromatin-remodeling factors and RNA-processing proteins in ABA responses suggests that we have only scratched the surface with regard to the intricate mechanisms by which modulators of gene expression participate in the control of transpiration.

## CONCLUSIONS AND PERSPECTIVES

This *Update* has illustrated some of the recent progress that is being made in understanding the control of transpiration at the whole-plant, cellular, and molecular levels, using Arabidopsis as a model system. We hope that this brief review will encourage increased collaboration among researchers studying this phenomenon at disparate levels of biological organization.

Drought and ABA are two environmental signals that were discussed in depth in this article. Yet, guard cells respond to a wide diversity of environmental cues (Hetherington and Woodward, 2003). Studies that assess impacts of light (Kinoshita et al., 2001; Sothern et al., 2002), $CO_2$ (Hashimoto et al., 2006; Teng et al., 2006; Young et al., 2006), and humidity (Assmann et al., 2000; Yoshida et al., 2002; Xie et al., 2006) on transpiration, while not discussed here, are equally important to our knowledge of transpirational control. Finally, while this article has focused on levels ranging from the molecular to the whole plant, it is important to note that Arabidopsis is found in natural ecosystems (Pigliucci, 2002; Mitchell-Olds and Schmitt, 2006). Thus, Arabidopsis is also proving to be a valuable tool for ecophysiological and ecological studies of how plant populations in situ respond to water availability and other environmental signals that impact the control of gas exchange (McKay et al., 2003; Engelmann and Schlichting, 2005), topics that were not covered in this brief *Update*.

## ACKNOWLEDGMENT

We apologize to the many authors whose research was not covered owing to space constraints.

Received November 16, 2006; accepted November 23, 2006; published January 8, 2007.

## LITERATURE CITED

**Allen GJ, Kuchitsu K, Chu SP, Murata Y, Schroeder JI** (1999) Arabidopsis *abi1-1* and *abi2-1* phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. Plant Cell **11:** 1785–1798

**Alonso-Blanco C, Koornneef M** (2000) Naturally occurring variation in Arabidopsis: an underexploited resource for plant genetics. Trends Plant Sci **5:** 22–29

**Assmann SM, Snyder JA, Lee YRJ** (2000) ABA-deficient (*aba1*) and ABA-insensitive (*abi1-1, abi2-1*) mutants of Arabidopsis have a wild-type stomatal response to humidity. Plant Cell Environ **23:** 387–395

**Blatt MR** (2000) Cellular signaling and volume control in stomatal movements in plants. Annu Rev Cell Dev Biol **16:** 221–241

**Busk PK, Pages M** (1998) Regulation of abscisic acid-induced transcription. Plant Mol Biol **37:** 425–435

**Chaerle L, Saibo N, Van der Straeten D** (2005) Tuning the pores: towards engineering plants for improved water use efficiency. Trends Biotechnol **23:** 308–315

**Cominelli E, Galbiati M, Vavasseur A, Conti L, Sala T, Vuylsteke M, Leonhardt N, Dellaporta SL, Tonelli C** (2005) A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. Curr Biol **15:** 1196–1200

**Coursol S, Fan LM, Le Stunff H, Spiegel S, Gilroy S, Assmann SM** (2003) Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. Nature **423:** 651–654

**Davenport R** (2002) Glutamate receptors in plants. Ann Bot (Lond) **90:** 549–557

**Engelmann KE, Schlichting CD** (2005) Coarse- versus fine-grained water stress in *Arabidopsis thaliana* (Brassicaceae). Am J Bot **92:** 101–106

**Fan LM, Zhao ZX, Assmann SM** (2004) Guard cells: a dynamic signaling model. Curr Opin Plant Biol **7:** 537–546

**Gazzarrini S, McCourt P** (2003) Cross-talk in plant hormone signalling: what Arabidopsis mutants are telling us. Ann Bot (Lond) **91:** 605–612

**Gosti F, Beaudoin N, Serizet C, Webb AA, Vartanian N, Giraudat J** (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. Plant Cell **11:** 1897–1910

**Guo FO, Young J, Crawford NM** (2003) The nitrate transporter AtNRT1.1 (CHL1) functions in stomatal opening and contributes to drought susceptibility in Arabidopsis. Plant Cell **15:** 107–117

**Guo Y, Xiong L, Song CP, Gong D, Halfter U, Zhu JK** (2002) A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in Arabidopsis. Dev Cell **3:** 233–244

**Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki KM** (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. Plant Cell **17:** 3470–3488

**Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K** (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. Proc Natl Acad Sci USA **103:** 1988–1993

**Hashimoto M, Negi J, Young J, Israelsson M, Schroeder JI, Iba K** (2006) Arabidopsis HT1 kinase controls stomatal movements in response to $CO_2$. Nat Cell Biol **8:** 391–397

**Hetherington AM, Brownlee C** (2004) The generation of $Ca^{2+}$ signals in plants. Annu Rev Plant Biol **55:** 401–427

**Hetherington AM, Woodward FI** (2003) The role of stomata in sensing and driving environmental change. Nature **424:** 901–908

**Himmelbach A, Hoffmann T, Leube M, Hohener B, Grill E** (2002) Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis. EMBO J **21:** 3029–3038

**Hosy E, Vavasseur A, Mouline K, Dreyer I, Gaymard F, Poree F, Boucherez J, Lebaudy A, Bouchez D, Very AA, et al** (2003) The Arabidopsis outward $K^+$ channel GORK is involved in regulation of stomatal movements and plant transpiration. Proc Natl Acad Sci USA **100:** 5549–5554

**Hugouvieux V, Kwak JM, Schroeder JI** (2001) An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in Arabidopsis. Cell **106:** 477–487

**Hugouvieux V, Murata Y, Young JJ, Kwak JM, Mackesy DZ, Schroeder JI** (2002) Localization, ion channel regulation, and genetic interactions during abscisic acid signaling of the nuclear mRNA cap-binding protein, ABH1. Plant Physiol **130:** 1276–1287

**Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K** (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. Plant J **27:** 325–333

**Jacob T, Ritchie S, Assmann SM, Gilroy S** (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. Proc Natl Acad Sci USA **96:** 12192–12197

**Juenger TE, McKay JK, Hausmann N, Keurentjes JJB, Sen S, Stowe KA, Dawson TE, Simms EL, Richards JH** (2005) Identification and characterization of QTL underlying whole-plant physiology in *Arabidopsis thaliana*: $\delta^{13}C$, stomatal conductance and transpiration efficiency. Plant Cell Environ **28:** 697–708

**Kang JM, Mehta S, Turano FJ** (2004) The putative glutamate receptor 1.1 (AtGLR1.1) in *Arabidopsis thaliana* regulates abscisic acid biosynthesis and signaling to control development and water loss. Plant Cell Physiol **45:** 1380–1389

**Kang JY, Choi HI, Im MY, Kim SY** (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14:** 343–357

**Kim S, Kang JY, Cho DI, Park JH, Kim SY** (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. Plant J **40:** 75–87

**Kinoshita T, Doi M, Suetsugu N, Kagawa T, Wada M, Shimazaki K** (2001) phot1 and phot2 mediate blue light regulation of stomatal opening. Nature **414:** 656–660

**Klein M, Geisler M, Suh SJ, Kolukisaoglu HU, Azevedo L, Plaza S, Curtis MD, Richter A, Weder B, Schulz B, et al** (2004) Disruption of AtMRP4, a guard cell plasma membrane ABCC-type ABC transporter, leads to deregulation of stomatal opening and increased drought susceptibility. Plant J **39:** 219–236

**Klein M, Perfus-Barbeoch L, Frelet A, Gaedeke N, Reinhardt D, Mueller-Roeber B, Martinoia E, Forestier C** (2003) The plant multidrug resistance ABC transporter AtMRP5 is involved in guard cell hormonal signalling and water use. Plant J **33:** 119–129

**Ko JH, Yang SH, Han KH** (2006) Upregulation of an Arabidopsis RING-H2 gene, *XERICO*, confers drought tolerance through increased abscisic acid biosynthesis. Plant J **47:** 343–355

**Koornneef M, Hanhart CJ, Hilhorst HW, Karssen CM** (1989) In vivo inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. Plant Physiol **90:** 463–469

**Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JD, Schroeder JI** (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO J **22:** 2623–2633

**Kwak JM, Murata Y, Baizabal-Aguirre VM, Merrill J, Wang M, Kemper A, Hawke SD, Tallman G, Schroeder JI** (2001) Dominant negative guard cell $K^+$ channel mutants reduce inward-rectifying $K^+$ currents and light-induced stomatal opening in Arabidopsis. Plant Physiol **127:** 473–485

**Lambers H, Chapin FS, Pons TL** (1998) Plant Physiological Ecology. Springer-Verlag, New York

**Leonhardt N, Marin E, Vavasseur A, Forestier C** (1997) Evidence for the existence of a sulfonylurea-receptor-like protein in plants: modulation of stomatal movements and guard cell potassium channels by sulfonylureas and potassium channel openers. Proc Natl Acad Sci USA **94:** 14156–14161

**Leonhardt N, Vavasseur A, Forestier C** (1999) ATP binding cassette modulators control abscisic acid-regulated slow anion channels in guard cells. Plant Cell **11:** 1141–1151

**Leung J, Bouvier-Durand M, Morris PC, Guerrier D, Chefdor F, Giraudat J** (1994) Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. Science **264:** 1448–1452

**Li JX, Wang XQ, Watson MB, Assmann SM** (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. Science **287:** 300–303

**Li S, Assmann SM, Albert R** (2006) Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. PLoS Biol **4:** 1732–1748

**Liang YK, Dubos C, Dodd IC, Holroyd GH, Hetherington AM, Campbell MM** (2005) AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. Curr Biol **15:** 1201–1206

**Masle J, Gilmore SR, Farquhar GD** (2005) The *ERECTA* gene regulates plant transpiration efficiency in Arabidopsis. Nature **436:** 866–870

**McKay JK, Richards JH, Mitchell-Olds T** (2003) Genetics of drought adaptation in *Arabidopsis thaliana*: I. Pleiotropy contributes to genetic correlations among ecological traits. Mol Ecol **12:** 1137–1151

**Meinhard M, Grill E** (2001) Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from Arabidopsis. FEBS Lett **508:** 443–446

**Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J** (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. Plant J **25:** 295–303

**Merlot S, Mustilli AC, Genty B, North H, Lefebvre V, Sotta B, Vavasseur A, Giraudat J** (2002) Use of infrared thermal imaging to isolate Arabidopsis mutants defective in stomatal regulation. Plant J **30:** 601–609

**Meyer K, Leube MP, Grill E** (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. Science **264:** 1452–1455

**Mishra G, Zhang WH, Deng F, Zhao J, Wang XM** (2006) A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science **312:** 264–266

**Mitchell-Olds T, Schmitt J** (2006) Genetic mechanisms and evolutionary significance of natural variation in Arabidopsis. Nature **441:** 947–952

**Murata Y, Pei ZM, Mori IC, Schroeder J** (2001) Abscisic acid activation of plasma membrane $Ca^{2+}$ channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatase 2C mutants. Plant Cell **13:** 2513–2523

**Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J** (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell **14:** 3089–3099

**Nambara E, Marion-Poll A** (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol **56:** 165–185

**Papp I, Mur LA, Dalmadi A, Dulai S, Koncz C** (2004) A mutation in the Cap Binding Protein 20 gene confers drought tolerance to Arabidopsis. Plant Mol Biol **55:** 679–686

**Pei ZM, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI** (1997) Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and *abi1* and *abi2* mutants. Plant Cell **9:** 409–423

**Pigliucci M** (2002) Ecology and evolutionary biology of Arabidopsis. *In* EM Meyerowitz, CR Somerville E, eds, The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD, pp 1–20

**Rock CD** (2000) Pathways to abscisic acid-regulated gene expression. New Phytol **148:** 357–396

**Roelfsema MRG, Hedrich R** (2005) In the light of stomatal opening: new insights into 'the Watergate'. New Phytol **167:** 665–691

**Saez A, Apostolova N, Gonzalez-Guzman M, Gonzalez-Garcia MP, Nicolas C, Lorenzo O, Rodriguez PL** (2004) Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signalling. Plant J **37:** 354–369

**Saez A, Robert N, Maktabi MH, Schroeder JI, Serrano R, Rodriguez PL** (2006) Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. Plant Physiol **141:** 1389–1399

**Sanders D, Pelloux J, Brownlee C, Harper JF** (2002) Calcium at the crossroads of signaling. Plant Cell **14:** S401–S417

**Sang Y, Zheng S, Li W, Huang B, Wang X** (2001) Regulation of plant water loss by manipulating the expression of phospholipase D$\alpha$. Plant J **28:** 135–144

**Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D** (2001) Guard cell signal transduction. Annu Rev Plant Physiol Plant Mol Biol **52:** 627–658

**Sheng L** (2003) Protein phosphatases in plants. Annu Rev Plant Biol **54:** 63–92

**Song CP, Agarwal M, Ohta M, Guo Y, Halfter U, Wang PC, Zhu JK** (2005) Role of an Arabidopsis AP2/EREBP-type transcriptional repressor in abscisic acid and drought stress responses. Plant Cell **17:** 2384–2396

**Sothern RB, Tseng TS, Orcutt SL, Olszewski NE, Koukkari WL** (2002) *GIGANTEA* and *SPINDLY* genes linked to the clock pathway that controls circadian characteristics of transpiration in Arabidopsis. Chronobiol Int **19:** 1005–1022

**Sridha S, Wu KQ** (2006) Identification of AtHD2C as a novel regulator of abscisic acid responses in Arabidopsis. Plant J **46:** 124–133

**Szyroki A, Ivashikina N, Dietrich P, Roelfsema MRG, Ache P, Reintanz B, Deeken R, Godde M, Felle H, Steinmeyer R, et al** (2001) KAT1 is not essential for stomatal opening. Proc Natl Acad Sci USA **98:** 2917–2921

**Tal M** (1966) Abnormal stomatal behavior in wilty mutants of tomato. Plant Physiol **41:** 1387–1391

**Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S** (2005) Ethylene inhibits abscisic acid-induced stomatal closure in Arabidopsis. Plant Physiol **138:** 2337–2343

**Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S** (2006) Cytokinin and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in Arabidopsis. J Exp Bot **57:** 2259–2266

**Teng N, Wang J, Chen T, Wu X, Wang Y, Lin J** (2006) Elevated $CO_2$ induces physiological, biochemical and structural changes in leaves of *Arabidopsis thaliana*. New Phytol **172:** 92–103

**Umezawa T, Okamoto M, Kushiro T, Nambara E, Oono Y, Seki M, Kobayashi M, Koshiba T, Kamiya Y, Shinozaki K** (2006) CYP707A3, a major ABA 8′-hydroxylase involved in dehydration and rehydration response in *Arabidopsis thaliana*. Plant J **46:** 171–182

**Verslues PE, Zhu JK** (2005) Before and beyond ABA: upstream sensing and internal signals that determine ABA accumulation and response under abiotic stress. Biochem Soc Trans **33:** 375–379

**Wang XQ, Ullah H, Jones AM, Assmann SM** (2001) G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science **292:** 2070–2072

**Wang Y, Ying JF, Kuzma M, Chalifoux M, Sample A, McArthur C, Uchacz T, Sarvas C, Wan JX, Dennis DT, et al** (2005) Molecular tailoring of farnesylation for plant drought tolerance and yield protection. Plant J **43:** 413–424

**Wang YB, Holroyd G, Hetherington AM, Ng CKY** (2004) Seeing 'cool' and 'hot'-infrared thermography as a tool for non-invasive, high-throughput screening of Arabidopsis guard cell signalling mutants. J Exp Bot **55:** 1187–1193

**Xie XD, Wang YB, Williamson L, Holroyd GH, Tagliavia C, Murchie E, Theobald J, Knight MR, Davies WJ, Leyser HMO, et al** (2006) The identification of genes involved in the stomatal response to reduced atmospheric relative humidity. Curr Biol **16:** 882–887

**Yoshida R, Hobo T, Ichimura K, Mizoguchi T, Takahashi F, Aronso J, Ecker JR, Shinozaki K** (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. Plant Cell Physiol **43:** 1473–1483

**Yoshida R, Umezawa T, Mizoguchi T, Takahashi S, Takahashi F, Shinozaki K** (2006) The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. J Biol Chem **281:** 5310–5318

**Young JJ, Mehta S, Israelsson M, Godoski J, Grill E, Schroeder JI** (2006) $CO_2$ signaling in guard cells: calcium sensitivity response modulation, a $Ca^{2+}$-independent phase, and $CO_2$ insensitivity of the *gca2* mutant. Proc Natl Acad Sci USA **103:** 7506–7511

**Zhang JZ, Creelman RA, Zhu JK** (2004) From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant Physiol **135:** 615–621

**Zhang W, Qin C, Zhao J, Wang X** (2004) Phospholipase D$\alpha$1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci USA **101:** 9508–9513

**Zhao J, Wang XM** (2004) Arabidopsis phospholipase D$\alpha$1 interacts with the heterotrimeric G-protein $\alpha$-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. J Biol Chem **279:** 1794–1800

**Zhu JH, Verslues PE, Zheng XW, Lee B, Zhan XQ, Manabe Y, Sokolchik I, Zhu YM, Dong CH, Zhu JK, et al** (2005) HOS10 encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants. Proc Natl Acad Sci USA **102:** 9966–9971

## Chapter 3: Heterotrimeric G protein regulation of stomatal movements


### ABSTRACT

Fine-tuned regulation of stomatal aperture size is key to the survival of land plants. This chapter discusses the roles of heterotrimeric G proteins in the regulation of stomatal movements and ion channel activities of guard cells. Evidence implicating heterotrimeric G protein function in light-induced stomatal opening, ABA-induced stomatal closure, and pathogen-induced stomatal movements is described from early pharmacological experiments to phenotypic studies of *Arabidopsis* heterotrimeric G protein mutants.


### INTRODUCTION

Among the earliest identified and most extensively studied functions of heterotrimeric G proteins in plants are their roles in the regulation of stomatal movements. Stomata, which are found on the aerial surfaces of plants, consist of two guard cells surrounding a pore or stoma. The pore allows gas exchange with the atmosphere; through the pore $CO_2$ enters the leaf for photosynthesis and water evaporates, driving the transport of water from the roots to the shoots. Land plants are faced with the challenge of balancing $CO_2$ acquisition with desiccation avoidance; guard cells, by regulating the size of the stomatal pore, are critical to maintaining this balance. This chapter will review the primary guard cell signal transduction pathways that pertain to G proteins, describe the main methods used for assaying stomatal function, and discuss the pharmacological and molecular genetic evidence which has identified heterotrimeric G protein subunits as key players in stomatal aperture regulation. A summary table of the guard-cell related phenotypes of the heterotrimeric G proteins mutants (Table 1) and an integrated model of G protein regulation of abscisic acid (ABA)-induced stomatal movements (Figure 1) are provided.

## Mechanisms of stomatal movements

Guard cells are able to finely regulate the size of stomatal apertures in response to an array of environmental signals including $CO_2$ concentration, humidity, ABA, red and blue light, pathogen infection and wounding (Assmann 1993; Schroeder et al. 2001; Melotto, Underwood et al. 2006; Shimazaki et al. 2007; Underwood et al. 2007; Zhang et al. 2008b; Zhao et al. 2008). Stomatal movements are driven by turgor changes within the guard cells. Because of the radial arrangement of cellulose microfibrils in guard cell walls, an increase in cell turgor, originating from water influx into the guard cells, results in stomatal opening while water efflux from the cell, and the subsequent loss of turgor, facilitates stomatal closure (MacRobbie 1998; Schroeder et al. 2001). The turgor changes are initiated by the influx and efflux of solutes, including $K^+$ and anions, by malate synthesis, catabolism, and export, and, under some conditions, by the production and transport of soluble sugars (MacRobbie 1998; Fan et al. 2004; Shimazaki et al. 2007).

Guard cells integrate numerous signals and have become a model cell type for plant signal transduction (Assmann 1993; Schroeder et al. 2001). Mature guard cells lack plasmodesmata, so solute movements must occur across the plasma membrane (Willmer and Sexton 1979; Pandey et al. 2007). Ion channels, including inward- and outward-rectifying $K^+$ channels, anion channels, and $Ca^{2+}$ permeable channels, are capable of mediating large ion fluxes across the cell membrane (Hille 2001) and are important components of stomatal movements and G-protein regulation (Wang et al. 2001; Coursol et al. 2003; Fan et al. 2008; Zhang et al. 2008b). Two of the best characterized pathways that involve heterotrimeric G proteins are light-induced stomatal opening and ABA promotion of stomatal closure.

### *Light-induced stomatal opening*

Guard cell exposure to light, especially blue light, activates plasma membrane $H^+$-ATPases, which results in $H^+$ efflux from the cell and hyperpolarization of the cell membrane (Shimazaki et al. 2007). This hyperpolarization opens voltage-gated inward-rectifying $K^+$ channels that mediate the influx of $K^+$ into the cell. $K^+$ influx, along with

anion influx and malate$^{2-}$ production from starch metabolism reduces the cell water potential (Shimazaki et al. 2007; Lee et al. 2008). Water moves into the guard cell to compensate for the reduction in water potential, resulting in an increase in cell turgor and stomatal opening (MacRobbie 1983; Talbott and Zeiger 1998; Schroeder et al. 2001; Pandey et al. 2007).

### *ABA promotion of closure*

In response to drought stress, ABA concentration increases in the leaves (Davies and Zhang, 1991; Wilkinson and Davies, 2002). ABA both induces closure of open stomata and inhibits light-induced opening of closed stomata. ABA stimulates stomatal closure in part by activating plasma membrane channels that are permeable to $Ca^{2+}$, causing an increase in cytosolic $Ca^{2+}$. The influx of $Ca^{2+}$ also stimulates the release of $Ca^{2+}$ from intracellular stores (Gilroy et al. 1990; Staxen et al. 1999). Elevated cytosolic $Ca^{2+}$ levels can activate both slow and rapid anion channels which facilitate anion loss from the cell and plasma membrane depolarization (Schroeder and Hagiwara, 1989; Hedrich et al. 1990), however, $Ca^{2+}$-independent activation of anion channels has also been observed (Levchenko et al. 2005; Marten et al. 2007). Depolarization of the membrane stimulates outward-rectifying $K^+$ channels and $K^+$ efflux from the cell. The net loss of $K^+$ and anions from the cell increases the water potential and drives water loss from the cell, turgor reduction, and stomatal closure. ABA-induced cytosolic $Ca^{2+}$ elevations also inhibit the plasma membrane $H^+$-ATPase and inward $K^+$ channel activation, thereby inhibiting the opening of closed stomata (Schroeder and Hagiwara 1989; McAinsh et al. 1990; Lemtiri-Chlieh and MacRobbie 1994; Kinoshita et al. 1995; Schroeder et al. 2001; Pandey et al. 2007).

## Measuring stomatal movements and ion channel activities

### *Electrophysiological studies of guard cells*

Ion channel activities can be measured in guard cell protoplasts or isolated membrane patches using the patch clamp technique. (Schroeder et al. 1987; Wu and Assmann 1994; Forestier et al. 1998; Zhang et al. 2008c). This electrophysiological method can either measure net ion fluxes in whole cells or activities of single ion channels in isolated membrane patches (Neher et al. 1978; Kornreich 2007). The type of ion channel studied is controlled by the ionic composition and concentration of the patch pipette solution and the bath solution (the solution surrounding the protoplasts). Additionally, two-electrode voltage clamp is an electrophysiological technique that can be used to measure guard cell ion channels in epidermal peels or intact plants (Blatt 1992; Roelfsema et al. 2001). Electrophysiology experiments using *Vicia faba*, *Commelina communis*, and *Arabidopsis thaliana* protoplasts (both mutant and wild type) in combination with G protein modulators and hormones have greatly contributed to our understanding of the downstream targets of G protein regulation of stomatal movements.

***Stomatal aperture assays***

Stomatal aperture assays allow the measurement of stomatal movements in response to hormonal, environmental or pharmacological treatments. Typically, excised leaves are incubated in a buffer to induce stomatal closure or opening, a treatment is applied (e.g., ABA, blue light, or a G protein modifying drug) and after a period of incubation the epidermal strips are peeled off the leaves with forceps. For some species, particularly *Commelina* and *Vicia,* for which the epidermis can be peeled easily, the isolated epidermal strips, rather than the intact leaves, can be directly subjected to the incubation solution. After treatment, the peels are examined under a microscope and stomatal apertures are measured with an ocular micrometer. Alternatively, digital photographs of peels can be taken and apertures measured on the photographs using image analysis software. By comparing apertures from treated and untreated samples, stomatal movements can be assessed. This technique is particularly effective for species in which the epidermis can be easily separated from the leaf. These studies are best performed blind to avoid inadvertent bias in measurement.

### *Whole leaf/plant measures of stomatal function*

The implication of G-protein involvement in ABA-regulated stomatal movements has led researchers to conduct water loss assays from excised leaves and rosettes of G protein mutants by weighing samples periodically following excision (Wang et al. 2001; Pandey and Assmann 2004; Zhang et al. 2008a). While the excision of plant tissue does result in wilting and water loss, the physiological validity of these assays is limited given that the leaves and/or rosette and therefore the stomata are separated from the roots, a primary source of ABA. Non-uniform humidity and temperatures can also confound results. An alternative approach to measuring whole-leaf changes in stomatal functioning is to measure stomatal conductance, an indicator of how open stomata are, using a gas exchange system. Gas exchange systems allow for rates of photosynthesis and stomatal conductance to be assessed on whole plants or intact leaves under tightly controlled environmental conditions. Recent technological advances, such as the availability of a whole-plant *Arabidopsis* gas exchange chamber, will no doubt contribute to our understanding of G protein regulation of stomatal function and whole plant water status in the future.

## G PROTEIN REGULATION OF STOMATAL MOVEMENTS

Evidence suggesting that G proteins regulate stomatal movements and ion channel activities was first obtained in the early 1990s using electrophysiological and pharmacological methods mainly applied to broad bean, *Vicia faba* (Fairley-Grenot and Assmann 1991; Wu and Assmann 1994). With the sequencing of the *Arabidopsis* genome and identification of G protein encoding genes, *Arabidopsis* quickly became the model system for the study of G protein function in plants. The development of guard cell protoplast isolation and patch clamping techniques suitable for *Arabidopsis* (*Arabidopsis* guard cells are considerably smaller than those of *Vicia faba*) (Pei et al. 1997) and the acquisition and characterization of mutants lacking functional genes for heterotrimeric G proteins allowed direct testing and confirmation of the role of heterotrimeric G proteins in the regulation of stomatal movements (Wang et al. 2001).

**Early pharmacological studies**

Early and ongoing, mammalian studies have demonstrated that non-hydrolysable forms of GTP and GDP can be used to manipulate heterotrimeric G protein function in cells. G proteins can be constitutively activated upon binding to GTPγS or inactivated upon binding to GDPβS. Additionally, two pharmacological agents which function as ADP-ribosyltransferases, cholera toxin and pertussis toxin, can lock G proteins that contain an ADP-ribosylation site in either an active or an inactive state, respectively (Gilman 1987). The first evidence of G protein regulation of ion channels was found in *Vicia faba* guard cell protoplasts, when G protein activity modulators were combined with whole-cell electrophysiological recordings of $K^+$ channels (Fairley-Grenot and Assmann 1991). Inward-rectifying $K^+$ channels were found to be activated by GDPβS and inhibited by GTPγS. Additionally, both cholera and pertussis toxins were found to inhibit inward $K^+$ channels (Fairley-Grenot and Assmann 1991). No significant effects of GTPγS, GDPβS, or bacterial toxins were found on outward $K^+$ channels of *Vicia faba*. Synthetic mastoparan toxin (mas7), which essentially mimics ligand binding to a GPCR and activates heterotrimeric G proteins in mammalian species, inhibited inward $K^+$ but not outward $K^+$ channels in *Vicia* guard cell protoplasts (Armstrong and Blatt 1995). Application of GDPβS blocked the mas7 attenuation of inward $K^+$ channels, further supporting a role for heterotrimeric G proteins in ion channel regulation in plants (Armstrong and Blatt 1995). It is important to note, however, that mastoparan can activate MAP kinase signaling independent of Gα or Gβ, suggesting that mastoparan may not always specifically modulate G protein signaling in *Arabidopsis* (Miles et al. 2004).

Regulation of inward $K^+$ channels by pharmacological modulations of G proteins was also found in single-channel recordings from isolated membrane patches, showing that a cytosolic signal transduction cascade was not required for ion channel regulation by G proteins i.e. that regulation can occur via a membrane delimited pathway (Wu and Assmann 1994). However, electrophysiological studies using both calcium chelators and G protein activity modifiers also suggested a role for cytosolic $Ca^{2+}$ in G protein regulation of inward $K^+$ channels. Thus, G-protein regulation of plant ion channels may

occur via both membrane-delimited pathways and cytosolic pathways involving secondary messengers (Fairley-Grenot and Assmann 1991; Kelly et al. 1995).

In epidermal peel experiments, microinjection of GTPγS into guard cells of *Commelina communis L* somewhat promoted light-induced stomatal opening (Lee et al. 1993), supporting a regulatory role for plant G proteins in stomatal movements, although one inconsistent with the observed effects on $K^+$ channel activity. Also, application of G protein antagonists (mas17 or GP Ant-2) to *Commelina communis* epidermal peels was shown to inhibit promotion of stomatal opening by 1 mM auxin as well as promotion of stomatal closure by 100 nM ABA (Cousson et al. 1998a,b). While these early studies strongly suggested the involvement of heterotrimeric G proteins in the regulation of ion channels and stomatal movements, it was the cloning of G protein subunit genes and functional analyses of T-DNA insertional mutants that unequivocally identified heterotrimeric G proteins as regulators of stomatal movements.

### *Arabidopsis* heterotrimeric G protein genes

The *Arabidopsis* genome contains only one canonical Gα subunit, encoded by *GPA1*, and one Gβ subunit, encoded by *AGB1* (Ma et al. 1990; Weiss et al. 1994). Two Gγ subunits encoded by *AGG1* and *AGG2* have also been identified (Mason and Botella 2000; 2002). Northern analysis showed expression of *GPA1* in a mixed vegetative tissue sample that included both leaf and root tissue (Ma et al. 1990). Promoter GUS analysis shows *GPA1* expression throughout the plant body, with stronger expression in developing rather than in mature tissues (Huang et al. 1994). *GPA1* transcript can also be amplified from cDNA obtained from guard cell protoplast RNA (Wang et al. 2001). Microarray analysis also indicates *GPA1* expression in guard cell protoplasts (Yang et al. 2008). Western blotting and immunolocalization experiments indicate that GPA1 protein is present in all plant organs but mature dry seeds (Weiss et al. 1993; Huang et al. 1994; Pandey et al. 2006) and is more highly expressed in seedling roots as opposed to seedling shoots (Chen et al. 2006a). Like *GPA1*, *AGB1* has ubiquitous expression throughout the plant (Weiss et al. 1994; Anderson and Botella 2007) and is also expressed in guard cell protoplasts (Yang et al. 2008). *AGB1* promoter::GUS transgenic lines show strong GUS

activity in the guard cells of cotyledons and seedling leaves (Anderson and Botella 2007; Trusov et al. 2007). RNA blots show *AGG1* and *AGG2* expression throughout the plant. Promoter GUS fusions show guard cell expression of *AGG2* but not *AGG1* (Mason and Botella 2000, 2001; Trusov et al. 2007), however, microarray analysis of guard cell protoplasts indicates that *AGG1* is expressed in guard cells (Yang et al. 2008)

All heterotrimeric G protein subunits have been shown to associate with the plasma membrane. Immunolocalization (Weiss et al. 1997) and transient expression assays (Adjobo-Hermans et al. 2006; Chen et al. 2006a, Wang et al. 2008) show GPA1 at the plasma membrane. While more than 60% of leaf GPA1 protein is associated with the plasma membrane (Wang et al. 2007), GPA1 also has been immunolocalized to the Golgi apparatus and the endoplasmic reticulum (Weiss et al. 1997). Transgenic *Arabidopsis* overexpressing an AGB1-GFP reporter fusion show plasma membrane and nuclear localization of AGB1 in leaf epidermal cells (Anderson and Botella 2007) and 35S::YFP-AGB1 localizes to the plasma membrane of *Arabidopsis* suspension cells (Chen et al. 2006a). In transient mesophyll protoplast expression assays AGB1 localizes to the cytoplasm, but plasma membrane localization of AGB1 can be observed when AGB1 is coexpressed with either AGG1 or AGG2 (Adjobo-Hermans et al. 2006, Wang et al. 2008). Protein fractionation experiments also show that AGB1 is associated with the plasma membrane, endoplasmic reticulum, and nuclei (Obrdlik et al. 2000; Peškan and Oelmüller 2000; Wang et al. 2007). AGG1 and AGG2 both show plasma membrane localization in leaf epidermal cells in YFP-tagged AGG1 and AGG2 *Arabidopsis* lines (Zeng et al. 2007). Additionally, AGG1 also localizes to the Golgi apparatus (Zeng et al. 2007). Transient expression experiments in protoplasts show plasma membrane association for AGG1 and AGG2 (Adjobo-Hermans et al. 2006; Wang et al. 2008). Although Adjobo-Hermans et al. (2006) found that the plasma membrane localization of AGG1 requires AGB1 coexpression, this requirement was not observed by Wang et al. (2008). FRET experiments using *Arabidopsis* (Wang et al. 2008) and cowpea (Adjobo-Hermans et al. 2006) mesophyll cell protoplasts transfected with GPA1, AGB1, and AGG1 show interaction among the subunits and suggest that the heterotrimer exists at the plasma membrane. To date, no heterotrimeric G protein subcellular localization studies have been performed in guard cells or guard cell protoplasts.

In addition to the genes encoding heterotrimeric G protein subunits in *Arabidopsis*, one regulator of G protein signaling gene, *RGS1* (Chen et al. 2003), and several putative G protein coupled receptors (GPCRs), one of which is GCR1, have been identified (Josefsson and Rask 1997; Pandey and Assmann 2004; Moriyama et al. 2006, Gookin et al. 2008). *RGS1* is expressed in root and shoot meristems (Chen et al. 2003) and in guard cell protoplasts (Fan et al. 2008). RGS1-GFP localizes to the plasma membrane of *Arabidopsis* suspension cells (Chen et al. 2003). *GCR1* transcript can be detected throughout the plant body including in guard cell protoplasts (Pandey and Assmann 2004). Both western blotting (Pandey and Assmann 2004) and GFP localization studies (Humphrey and Botella 2001) show that GCR1 associates with cell membranes. The gene expression patterns and subcellular localizations of additional candidate GPCRs, predicted using bioinformatics (Moriyama et al. 2006; Gookin et al. 2008) and biochemical interaction assays with GPA1 (Gookin et al. 2008), have not been studied.

Two novel GPCR-like G proteins, GTG1 and GTG2, have been identified in *Arabidopsis* and localize at the plasma membrane (Pandey et al. 2009). The *GTG* genes are widely expressed in the plant according to RT-PCR and promoter::GUS analysis, including expression in guard cells (Pandey et al., 2009). It should be noted that another gene, *GCR2*, has been reported to function as both an ABA receptor and a GPCR and to regulate stomatal movements in response to ABA (Liu et al. 2007). However, these conclusions have been challenged; phenotypic discrepancies and erroneous topology predictions indicate it is neither an ABA receptor nor a GPCR (Gao et al. 2007; Guo et al. 2008; Illingworth et al. 2008).

**G protein regulation of ABA-inhibition of light induced stomatal opening**

Using two null mutants of *gpa1*, Wang et al. (2001) found the first definitive evidence for heterotrimeric G protein regulation of stomatal movements. ABA inhibition of light-induced stomatal opening was diminished in the mutants compared to wild type, Ws. Consistent with the aperture data, patch clamp experiments showed that *gpa1* mutants are insensitive to ABA inhibition of inward $K^+$ channels suggesting that GPA1 is a positive regulator of ABA inhibition of stomatal opening (Wang et al. 2001). Recently

it has been found that the *Arabidopsis* gene *SPHK1* encodes a sphingosine kinase (SphK) which is involved in ABA-induced stomatal closure and inhibition of stomatal opening (Worrall et al. 2008). ABA inhibits stomatal opening in part via activation of SphK, resulting in the production of spingosine-1-phosphate (S1P) which propagates the signal via GPA1 (Coursol et al. 2003). Application of S1P or the similar molecule phytospingosine (Coursol et al. 2005) inhibited stomatal opening in wild type plants, but not in *gpa1* mutants. Also, *gpa1* mutants are insensitive to S1P inhibition of inward $K^+$ channels (Coursol et al. 2003). Since S1P elicits elevation of cytosolic $Ca^{2+}$ concentration in guard cells (Ng et al. 2001) and $Ca^{2+}$ inhibits inward $K^+$ channels (Schroeder and Hagiwara 1989), this $Ca^{2+}$-elevation may be initiated by G-protein activation (Wang et al. 2001; Coursol et al. 2003).

Phospholipase Dα1 (PLDα1) is an additional downstream component of ABA regulation of stomatal movements. ABA activation of PLDα1 activity results in phosphatidic acid (PA) production which inhibits stomatal opening (Jacob et al. 1999; Zhang et al. 2004). Application of PA to *gpa1* and wild type epidermal peels inhibited stomatal opening in wild type, but not in *gpa1*, suggesting that GPA1 is a downstream component of PA-induced inhibition of opening (Mishra et al. 2006). Interestingly, PLDα1 has been shown to interact with GPA1 and stimulate GTPase activity of GPA1 and GPA1-GDP can bind PLDα1 and attenuate its activity suggesting a possible negative feedback mechanism in ABA signaling (Zhao and Wang 2004, Mishra et al. 2006).

In mammalian studies of Gα regulation of inward $K^+$ channels, Gα-GDP inhibits GIRK (G protein coupled inwardly rectifying $K^+$) channels and upon activation of the G protein the free Gβγ dimer activates GIRK channels (Riven et al. 2006). If plant heterotrimeric G proteins regulate ion channels in a similar manner it would be expected that *gpa1* and *agb1* mutants would show different phenotypes. In *Arabidopsis*, however, electrophysiology and stomatal aperture assays of *gpa1*, *agb1*, and *gpa1agb1* mutants all show statistically identical phenotypes: in the absence of ABA there is no alteration in basal $K^+$ currents in any of these mutants, while in the presence of ABA, inhibition of inward $K^+$ currents and light-induced stomatal opening is attenuated similarly in all of these mutants (Fan et al. 2008). Taken together, the mutant data suggest that plant heterotrimeric G proteins regulate $K^+$ channels differently than animal heterotrimeric G

proteins. This idea is also supported by electrophysiological analyses of Gγ mutants. According to the mammalian G protein paradigm, Gβγ always functions as a dimer, however, mutants of the two identified Gγ genes in *Arabidopsis*, *agg1* and *agg2*, show wild type ABA-induced stomatal closure, ABA inhibition of stomatal opening, and ABA inhibition of inward $K^+$ channels (Trusov et al. 2008). Given the known lack of extensive sequence similarity among mammalian Gγs, it is possible that additional *Arabidopsis* Gγ(s) which would function with AGB1 in the regulation of stomatal movements, exist but have not yet been identified (Mason and Botella 2001; Trusov et al. 2008).

Of the putative GPCRs that have been identified in *Arabidopsis* only *gcr1*, *gtg1*, and *gtg2* mutants have been phenotyped for stomatal aperture regulation. *gcr1* mutants, unlike *gpa1* or *agb1* mutants, are hypersensitive to ABA and S1P inhibition of stomatal opening (Pandey and Assmann 2004). GPA1 and GCR1 have been shown to interact in yeast and in co-immunoprecipitation from plant tissue leading to the hypothesis that GCR1 is a negative regulator of GPA1 (Pandey and Assmann 2004). GTG1 and GTG2 are membrane localized proteins, have both GPCR-like topologies and intrinsic GTPase activity, bind ABA, and interact with GPA1. While it is tempting to postulate that these ABA receptors activate heterotrimeric G proteins which then propagate the ABA signal, resulting in inhibition of $K^+$ channels, analysis of the *gtg1gtg2* mutants show wild type ABA inhibition of opening (Pandey et al. 2009). Characterization of GTG regulation of ion channels and analysis of *gtggpa1* double mutants will no doubt help to elucidate this novel component of G protein signaling in guard cells. *rgs1* mutants have also been assessed for altered ion channel regulation. The mutants did not show alterations in the magnitude of ABA inhibition of inward $K^+$ currents, however the voltage-activation kinetics of inward $K^+$ channels were accelerated, suggesting a function for RGS in the regulation of channel response to signals (Fan et al. 2008).

**G protein regulation of ABA promotion of stomatal closure**

Analysis of *Arabidopsis* heterotrimeric G protein mutants has also suggested a role for G proteins in the regulation of ABA promotion of stomatal closure. The above-mentioned *gtg1gtg2* double mutants show hyposensitivity in ABA promotion of stomatal

closure (and in all other, non-stomatal ABA responses that were assayed; Pandey et al., 2009). ABA activation of slow anion channels during stomatal closure can occur via at least two pathways, one of which is dependent on the G protein heterotrimer, while the other involves cytosolic pH. Thus, *gpa1* mutants show reduced ABA activation of slow anion channels when cytosolic pH is strongly buffered. However, under weak pH buffering, ABA activation of anion efflux channels is identical in wild type and *gpa1* mutants (Wang et al. 2001). The functional redundancy of these two pathways for ABA activation of anion channels likely explains the lack of an ABA promotion of closure phenotype in the *gpa1* mutants, unless cytosolic pH is similarly clamped (Wang et al. 2001). These identical phenomena are seen in assays of *agb1* guard cells (Fan et al. 2008).

Like ABA, S1P promotes stomatal closure (Ng et al. 2001). *gcr1* mutants show hypersensitivity toward both ABA and S1P in promotion of stomatal closure, consistent with the idea that GCR1 functions as a negative regulator of GPA1-mediated ABA and S1P signaling (Pandey and Assmann, 2004). Ion channel activity in *gcr1* mutants has not yet been assessed. However, it is known that *gpa1* mutants are insensitive to both S1P promotion of stomatal closure and S1P activation of slow anion channels (Coursol et al. 2003). The effect of S1P on anion channels, unlike that of ABA, is obligately dependent on GPA1 (Coursol et al., 2003).

S1P induces an elevation in cytosolic $Ca^{2+}$ concentration (Ng et al. 2001), and elevated cytosolic $Ca^{2+}$ is sufficient to activate slow anion channels (Schroeder and Hagiwara 1989), therefore G proteins may mediate S1P response via cytosolic $Ca^{2+}$ signals (Coursol et al. 2003). Both ABA and S1P induce cytosolic $Ca^{2+}$ transients (Hetherington 1990; Schroeder and Hagiwara 1990; McAnish et al. 1992; Allen et al. 2001; Ng et al. 2001). Experiments in which cytosolic $Ca^{2+}$ transients are stimulated in guard cells have found evidence for two distinct mechanisms for $Ca^{2+}$ dependent stomatal closure, a rapid "$Ca^{2+}$ reactive" response which contributes to the closure response itself, and a sustained "$Ca^{2+}$ programmed" response which contributes to the maintenance of closed stomata after cessation of cytosolic $Ca^{2+}$ elevation (Allen et al. 2001). S1P application to stomata induces $Ca^{2+}$ transients and rapid and temporary stomatal closure (Ng et al. 2001), presumably invoking the "$Ca^{2+}$ reactive" response and not the "$Ca^{2+}$

programmed response (Allen et al. 2001). Since G proteins function downstream of S1P they are similarly implicated in the "$Ca^{2+}$ reactive" response.

One mechanism by which ABA promotes an increase in cytosolic $Ca^{2+}$ is through the production of $H_2O_2$ which activates the plasma membrane $Ca^{2+}$-permeable channels that mediate $Ca^{2+}$ influx (Pei et al. 2000). Extracellular calmodulin can also promote stomatal closure and triggers $H_2O_2$ production and cytosolic $Ca^{2+}$ increases (Chen et al. 2004). $H_2O_2$ generation in response to extracellular calmodulin is attenuated in the *gpa1* mutants (Chen et al. 2004), consistent with the observations that stomatal closure induced by extracellular calmodulin is impaired in *gpa1* mutants and heightened in plants overexpressing GPA1 (Chen et al. 2004).

**G protein regulation of pathogen induced stomatal movements**

Stomata play critical roles in regulating plant water status and photosynthetic carbon assimilation; however their pores also can serve as convenient entry points for plant bacterial pathogens (Melotto et al. 2006; Underwood et al. 2007; Melotto et al. 2008). It has been shown that guard cells function in plant innate immunity by closing stomata and inhibiting stomatal opening in response to bacterial pathogens or their elicitors, such as the flg22 peptide derived from flagellin, a pathogen-associate molecular pattern (PAMP) (Melotto et al. 2006; Zhang et al. 2008b). The *gpa1* mutant shows insensitivity in flg22-induced inhibition of stomatal opening and inhibition of inward $K^+$ channels, implicating G proteins in PAMP signaling (Zhang et al. 2008b). Interestingly, G proteins are not the only shared signaling components between PAMP and ABA signaling pathways: nitric oxide, $H_2O_2$ and the kinase OST1 all function in both ABA- and PAMP-induced stomatal closure (Melotto et al. 2006). In addition, $Ca^{2+}$-permeable channels (CNGC2/DND1) facilitate $Ca^{2+}$ influx and act as an upstream signal component for NO production during plant hypersensitive responses (Ali et al. 2007), suggesting that cytosolic $Ca^{2+}$ signals and NO are also shared components in ABA and pathogen signaling.

## G protein regulation of whole-leaf water status and drought response

Despite the numerous studies outlined above which show that G proteins are involved in the regulation of stomatal movements, very little is known concerning G protein regulation of water status at the whole leaf or plant level. Excised leaf water loss assays (which should be interpreted with caution for reasons discussed above) show that *gpa1* mutants in the Ws background have increased water loss, however, water loss assays of cotyledons of *gpa1* mutants in the Col background show reduced water loss while *agb1* mutant cotyledons show increased water loss (Wang et al. 2001; Mishra et al. 2006; Zhang et al. 2008a). In terms of drought tolerance, it has been reported that young *gpa1* seedlings (Col background) growing on agar media exposed to dry air exhibit drought tolerance while *agb1* mutants are drought sensitive (Zhang et al. 2008a). *gcr1* mutants show reduced water loss in excised leaf water loss assays and *gcr1* plants grown in soil exhibit improved survival following drought release (Pandey and Assmann 2004). Similar results were observed for transgenic plants overexpressing RGS1 (Chen et al. 2006b). The recent identification of GPA1 and AGB1 as regulators (positive and negative, respectively) of stomatal density in cotyledons (Zhang et al. 2008a) confounds any simple predictions of how G protein regulation of stomatal movements may contribute to whole leaf and whole plant water status and indicates the need for further experimentation.

## CONCLUSIONS AND UNASWERED QUESTIONS

Pharmacological and molecular genetic approaches have identified heterotrimeric G proteins as regulators of ABA inhibition of stomatal opening, ABA promotion of stomatal closure and pathogen-induced stomatal movements (summarized in Figure 1 and Table 1). Electrophysiology experiments clearly indicate that ion channels are a target of G protein regulation. However, the mechanisms by which heterotrimeric G proteins regulate ion channels have yet to be elucidated and may involve both secondary messenger cascades and plasma membrane-based regulation. Further experimentation is needed to identify additional downstream effectors of G-protein signaling as well as G

protein coupled-receptor(s) that act directly upstream of the heterotrimeric G protein in the regulation of stomatal movements. Additional physiological studies of G protein mutants with the goal of examining stomatal conductance, stomatal density and ultimately, plant fitness, under well-watered and tightly controlled drought stress conditions are warranted in order to obtain an integrated understanding of how heterotrimeric G proteins contribute to whole-plant water status and therefore of their potential utility as biotechnological targets for crop improvement.

## LITERATURE CITED


**Adjobo-Hermans MJ, Goedhart J, Gadella TW** (2006). Plant G protein heterotrimers require dual lipidation motifs of Gα and Gγ and do not dissociate upon activation. J Cell Sci 119: 5087-5097.

**Ali R, Ma W, Lemtiri-Chlieh F, Tsaltas D, Leng Q, von Bodman S, Berkowitz GA** (2007) Death don't have no mercy and neither does calcium: *Arabidopsis* CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. Plant Cell 19: 1081-1095.

**Allen GJ, Chu SP, Harrington CL, Schumacher K, Hoffmann T, et al.** (2001) A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature 411: 1053–1057.

**Anderson DJ, Botella JR** (2007) Expression analysis and subcellular localization of the *Arabidopsis* thaliana G-protein beta-subunit AGB1. Plant Cell Rep 26: 1469-80.

**Armstrong F, Blatt MR** (1995) Evidence for $K^+$ channel control in *Vicia* guard cells coupled by G-proteins to a 7TMS receptor mimetic. Plant J 8: 187-198.

**Assmann SM** (1993) Signal transduction in guard cells. Annu Rev Cell Biol 9: 345-375.

**Assmann SM** (2002) Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. Plant Cell 14: S355-373.

**Blatt MR** (1992) $K^+$ channels of stomatal guard cells. Characteristics of the inward rectifier and its control by pH. J Gen Physiol 99: 615-644.

**Chen JG**, Willard FS, Huang J, Liang J, Chasse SA, Jones AM, Siderovski DP (2003) A seven-transmembrane RGS protein that modulates plant cell proliferation. Science 301: 1728-31.

**Chen JG, Gao Y, Jones AM** (2006a) Differential roles of Arabidopsis heterotrimeric G-protein subunits in modulating cell division in roots. Plant Physiol 141: 887-897.

**Chen Y, Ji F, Xie H, Liang J** (2006b) Overexpression of the regulator of G-protein signalling protein enhances ABA-mediated inhibition of root elongation and drought tolerance in *Arabidopsis*. J Exp Bot 57: 2101-2110

**Chen YL, Huang R, Xiao YM, Lu P, Chen J, Wang XC** (2004). Extracellular calmodulin-induced stomatal closure is mediated by heterotrimeric G protein and $H_2O_2$. Plant Physiol 136: 4096-103.

**Coursol S, Fan LM, Le Stunff H, Spiegel S, Gilroy S, Assmann SM** (2003) Sphingolipid signalling in *Arabidopsis* guard cells involves heterotrimeric G proteins. Nature 423: 651-654.

**Coursol S, Le Stunff H, Lynch DV, Gilroy S, Assmann SM, Spiegel S** (2005) *Arabidopsis* sphingosine kinase and the effects of phytosphingosine-1-phosphate on stomatal aperture. Plant Physiol 137: 724-737.

**Cousson A, Vavasseur A** (1998a) Putative involvement of cytosolic $Ca^{2+}$ and GTP-binding proteins in cyclic-GMP-mediated induction of stomatal opening by auxin in *Commelina communis* L. Planta 206: 308-314.

**Cousson A, Vavasseur A** (1998b) Two potential $Ca^{2+}$-dependent transduction pathways in stomatal closing in response to abscisic acid. Plant Physiol Biochem 36: 257-262.

**Davies WJ, Zhang JH** (1991). Root signals and the regulation of growth and development of plants in drying soil. Annu Rev Plant Physiol Plant Mol Biol 42: 55-76.

**Fairley-Grenot K, Assmann SM** (1991) Evidence for G-protein regulation of inward $K^+$ channel current in guard cells of fava bean. Plant Cell 3: 1037-1044.

**Fan LM, Zhang W, Chen JG, Taylor JP, Jones AM, Assmann SM** (2008) Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ- and RGS-deficient *Arabidopsis* lines. Proc Natl Acad Sci USA 105: 8476-8481.

**Fan LM, Zhao Z, Assmann SM** (2004) Guard cells: a dynamic signaling model. Curr Opin Plant Biol 7: 537-546.

**Forestier C, Bouteau F, Leonhardt N, Vavasseur A** (1998) Pharmacological properties of slow anion currents in intact guard cells of *Arabidopsis* . Application of the discontinuous single-electrode voltage-clamp to different species. Pflugers Arch 436: 920-7.

**Gao Y, Zeng Q, Guo J, Cheng J, Ellis BE, Chen JG** (2007) Genetic characterization reveals no role for the reported ABA receptor, GCR2, in ABA control of seed germination and early seedling development in *Arabidopsis*. Plant J 52: 1001-1013.

**Gilman AG** (1987) G Proteins: Transducers of receptor-generated signals. Annu Rev Biochem 56: 615-649.

**Gilroy S, Read ND, Trewavas AJ** (1990) Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. Nature 346: 769-771.

**Gookin TE, Kim J, Assmann SM** (2008) Whole proteome identification of plant candidate G-protein coupled receptors in *Arabidopsis*, rice, and poplar: computational prediction and in-vivo protein coupling. Genome Biol 9: R120.

**Guo J, Zeng Q, Emami M, Ellis BE, Chen J-G** (2008) The GCR2 gene family is not required for ABA control of seed germination and early seedling development in *Arabidopsis*. PLoS ONE 3: e2982.

**Hedrich R, Busch H, Raschke K** (1990) $Ca^{2+}$ and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. EMBO J 9: 3889-3892

**Hetherington AM** (1990) Abscisic acid-induced elevation of guard cell cytosolic $Ca^{2+}$ precedes stomatal closure. Nature 343: 186-188.

**Hille B** (2001) Ion channels of excitable membranes. Sinauer Associates, Inc. Sunderland, Msssachusetts,

**Huang H, Weiss CA, Ma H** (1994) Regulated expression of the *Arabidopsis* G protein α subunit gene GPA1. Int J Plant Sci 155: 3-14.

**Humphrey TV, Botella JR** (2001) Re-evaluation of the cytokinin receptor role of the *Arabidopsis* gene GCR1. J Plant Physiol 158: 645-653.

**Illingworth CJR, Parkes KE, Snell CR, Mullineaux PM, Reynolds CA** (2008). Criteria for confirming sequence periodicity identified by Fourier transform analysis: application to GCR2, a candidate plant GPCR? Biophys Chem 133: 28-35.

**Jacob T, Ritchie S, Assmann SM, Gilroy S** (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. Proc Natl Acad Sci USA 96: 12192-12197.

**Josefsson L-G, Rask L** (1997) Cloning of a putative G-protein-coupled receptor from *Arabidopsis thaliana.* Eur J Biochem 249: 415-420.

**Kelly WB, Esser JE, Schroeder JI** (1995). Effects of cytosolic calcium and limited, possible dual, effects of G protein modulators on guard cell inward potassium channels. Plant J 8: 479-489.

**Kinoshita T, Nishimura M, Shimazaki K** (1995). Cytosolic concentration of $Ca^{2+}$ regulates the plasma membrane $H^+$-ATPase in guard cells of fava bean. Plant Cell **7**: 1333-1342.

**Kornreich BG** (2007) The patch clamp technique: Principles and technical considerations. J Vet Cardiol 9: 25-37.

**Lee M, Choi Y, Burla B, Kim Y-Y, Jeon B, Maeshima M, Yoo J-Y, Martinoia E, Lee Y** (2008) The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to $CO_2$. Nat Cell Biol 10: 1217-1223.

**Lee HJ, Tucker EB, Crain RC, Lee Y** (1993) Stomatal opening is induced in epidermal peels of *Commelina communis* L. by GTP analogs or pertussis toxin. Plant Physiol 102: 95-100.

**Lemtiri-Chlieh F, MacRobbie EAC** (1994) Role of calcium in the modulation of *Vicia* guard cell potassium channels by abscisic acid: a patch-clamp study. J Membr Biol 137: 99-107.

**Levchenko V, Konrad KR, Dietrich P, Roelfsema MRG, Hedrich R** (2005) Cytosolic abscisic acid activates guard cell anion channels without preceding $Ca^{2+}$ signals. Proc Natl Acad Sci USA 102: 4203–4208.

**Liu X, Yue Y, Li B, Nie Y, Li W, Wu W-H, Ma L** (2007) A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science 315: 1712-1716.

**Ma H, Yanofsky MF, Meyerowitz EM** (1990) Molecular cloning and characterization of GPA1, a G protein alpha subunit gene from *Arabidopsis thaliana*. Proc Natl Acad Sci USA 87: 3821-3825.

**MacRobbie EAC** (1983) Effects of light/dark on cation fluxes in guard cells of *Commelina communis* L. J Exp Bot 34: 1695-1710.

**MacRobbie EAC** (1998) Signal transduction and ion channels in guard cells. Philos Trans R Soc Lond B Biol Sci 353: 1475-1488.

**Marten H, Konrad KR, Dietrich P, Roelfsema MRG, Hedrich R** (2007) $Ca^{2+}$-dependent and -independent abscisic acid activation of plasma membrane anion channels in guard cells of *Nicotiana tabacum*. Plant Physiol. 143: 28-37.

**Mason MG, Botella JR** (2000) Completing the heterotrimer: isolation and characterization of an *Arabidopsis thaliana* G protein γ-subunit cDNA. Proc Natl Acad Sci USA 97: 14784-1478.

**Mason MG, Botella JR** (2001) Isolation of a novel G-protein γ-subunit from *Arabidopsis thaliana* and its interaction with Gβ. Biochim Biophys Acta 1520: 147-153.

**McAinsh MR, Brownlee C, Hetherington AM** (1990) Abscisic acid-induced elevation of guard cell cytosolic $Ca^{2+}$ precedes stomatal closure. Nature 343: 186-188.

**McAinsh MR, Brownlee C, Hetherington AM** (1992) Visualizing changes in cytosolic-free $Ca^{2+}$ during the response of stomatal guard cells to abscisic acid. Plant Cell 7: 1113–1122.

**Melotto M, Underwood W, He SY** (2008) Role of stomata in plant innate immunity and foliar bacterial diseases. Annu Rev Phytopathol 46: 101-122.

**Melotto M, Underwood W, Koczan J, Nomura K, He SY** (2006) Plant stomata function in innate immunity against bacterial invasion. Cell 126: 969-980.

**Miles GP, Samuel MA, Jones AM, Ellis BE** (2004) Mastoparan rapidly activates plant MAP kinase signaling independent of heterotrimeric G proteins. Plant Physiol 134: 1332-1336.

**Mishra G, Zhang W, Deng F, Zhao J, Wang X** (2006) A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in *Arabidopsis*. Science 312: 264-266.

**Moriyama E, Strope P, Opiyo S, Chen Z, Jones A** (2006) Mining the *Arabidopsis thaliana* genome for highly-divergent seven transmembrane receptors. Genome Biol **7**: R96.

**Neher E, Sakmann B, Steinbach JH** (1978) The extracellular patch clamp: A method for resolving currents through individual open channels in biological membranes. Pflugers Arch 375: 219-228.

**Ng CK, Carr K, McAnish MR, Powell B, Hetherington AM** (2001) Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. Nature 410: 596-599.

**Obrdlik P, Neuhaus G, Merkle T** (2000). Plant heterotrimeric G protein beta subunit is associated with membranes via protein interactions involving coiled-coil formation. FEBS Lett 476: 208-212.

**Pandey S, Assmann SM** (2004) The *Arabidopsis* putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signaling. Plant Cell 16: 1616-1632.

**Pandey S, Chen JG, Jones AM, Assmann SM** (2006) G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. Plant Physiol 141: 243-256.

**Pandey S, Nelson DC, Assmann SM** (2009) Two novel GPCR-type G proteins are abscisic acid receptors in *Arabidopsis*. Cell 136: 136-148.

**Pandey S, Zhang W, Assmann SM** (2007) Roles of ion channels and transporters in guard cell signal transduction. FEBS Lett 581: 2325-2336.

**Pei ZM, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI** (1997) Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and *abi1* and *abi2* mutants. Plant Cell 9: 409-423.

**Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI** (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406: 731-734.

**Peškan T, Oelmüller R** (2000) Heterotrimeric G-protein β-subunit is localized in the plasma membrane and nuclei of tobacco leaves. Plant MolBiol 42: 915-922.

**Riven I, Iwanir S, Reuveny E** (2006) GIRK channel activation involves a local rearrangement of a preformed G protein channel complex. Neuron 51: 561-573.

**Roelfsema MR, Steinmeyer R, Staal M, Hedrich R** (2001) Single guard cell recordings in intact plants: light-induced hyperpolarization of the plasma membrane. Plant J 26: 1-13.

**Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D** (2001) Guard cell signal transduction. Annu Rev Plant Physiol Plant Mol Biol 52: 627-658.

**Schroeder JI, Hagiwara S** (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. Nature 338: 427-430.

**Schroeder JI, Hagiwara S** (1990) Repetitive increases in cytosolic $Ca^{2+}$ of guard cells by abscisic acid activation of nonselective $Ca^{2+}$ permeable channels. Proc Natl Acad Sci USA 87: 9305–9309.

**Schroeder JI, Raschke K, Neher E** (1987) Voltage dependence of K channels in guard-cell protoplasts. Proc Natl Acad Sci USA 84: 4108-4112.

**Shimazaki K, Doi M, Assmann SM, Kinoshita T** (2007) Light regulation of stomatal movement. Annu Rev Plant Biol 58: 219-247.

**Staxen I, Pical C, Montgomery LT, Gray JE, Hetherington AM, McAinsh MR** (1999) Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. Proc Natl Acad Sci USA 96: 1779-1784.

**Talbott L, Zeiger E** (1998) The role of sucrose in guard cell osmoregulation. J Exp Bot 49: 329-337.

**Trusov Y, Rookes JE, Tilbrook K, Chakravorty D, Mason MG, Anderson D, Chen J-G, Jones AM, Botella JR** (2007) Heterotrimeric G protein γ subunits provide functional selectivity in Gβγ dimer signaling in *Arabidopsis*. Plant Cell 19: 1235-1250.

**Trusov Y, Zhang W, Assmann SM, Botella JR** (2008) Gγ1 + Gγ2 not equal to Gβ: heterotrimeric G protein Gγ-deficient mutants do not recapitulate all phenotypes of Gβ-deficient mutants. Plant Physiol 147: 636-649.

**Underwood W, Melotto M, He SY** (2007) Role of plant stomata in bacterial invasion. Cell Microbiol 9: 1621-1629.

**Wang XQ, Ullah H, Jones AM, Assmann SM** (2001) G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. Science 292: 2070-2072.

**Wang SY, Narendra S, Fedoroff N** (2007) Heterotrimeric G protein signaling in the Arabidopsis unfolded protein response. Proc Natl Acad Sci USA 104: 3817-3822.

**Wang SY, Assmann SM, Fedoroff NV** (2008). Characterization of the Arabidopsis heterotrimeric G protein. J Biol Chem 283: 13913-13922.

**Weiss CA, Garnaat CW, Mukai K, Hu Y, Ma H** (1994) Isolation of cDNAs encoding guanine nucleotide-binding protein beta-subunit homologues from maize (ZGB1) and *Arabidopsis* (AGB1). Proc Natl Acad Sci USA 91: 9554-9558.

**Weiss CA, Huang H, Ma H** (1993) Immunolocalization of the G protein α subunit encoded by the GPA1 gene in *Arabidopsis*. Plant Cell 5: 1513-1528.

**Weiss CA, White E, Huang H, Ma H** (1997) The G protein α subunit (GPα1) is associated with the ER and the plasma membrane in meristematic cells of *Arabidopsis* and cauliflower. FEBS Lett 407: 361-367.

**Wilkinson S, Davies WJ** (2002) ABA-based chemical signalling: the co-ordination of responses to stress in plants. Plant Cell Enviro 25: 195-210.

**Willmer CM, Sexton R** (1979) Stomata and plasmodesmata. Protoplasma 100: 113-124.

**Worrall D, Liang YK, Alvarez S, Holroyd GH, Spiegel S, Panagopulos M, Gray JE, Hetherington AM** (2008) Involvement of sphingosine kinase in plant cell signalling. Plant J 56: 64-72.

**Wu WH, Assmann SM** (1994) A membrane-delimited pathway of G-protein regulation of the guard-cell inward $K^+$ channel. Proc Natl Acad Sci USA 91: 6310-6314.

**Yang Y, Costa A, Leonhardt N, Siegel RS, Schroeder JI** (2008) Isolation of a strong guard cell promoter and its potential as a research tool. Plant Methods 4:6.

**Zeng Q, Wang X, Running MP**. (2007) Dual lipid modification of Arabidopsis Gγ-subunits is required for efficient plasma membrane targeting. Plant Physiol 143: 1119-1131.

**Zhang L, Hu G, Cheng Y, Huang J** (2008a) Heterotrimeric G protein α and β subunits antagonistically modulate stomatal density in *Arabidopsis thaliana*. Dev Biol 324: 68-75.

**Zhang W, He SY, Assmann SM** (2008b) The plant innate immunity response in stomatal guard cells invokes G-protein-dependent ion channel regulation. Plant J 56: 984-996.

**Zhang W, Nilson SE, Assmann SM** (2008c) Isolation and whole-cell patch clamping of *Arabidopsis* guard cell protoplasts. CSH Protocol 2008: pdb.prot5014.

**Zhang W, Qin C, Zhao J, Wang X** (2004) Phospholipase Dα1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci USA 101: 9508-9513.

**Zhao J, Wang X** (2004) *Arabidopsis* phospholipase Dα1 interacts with the heterotrimeric G-protein α-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. J Biol Chem 279: 1794-1800.

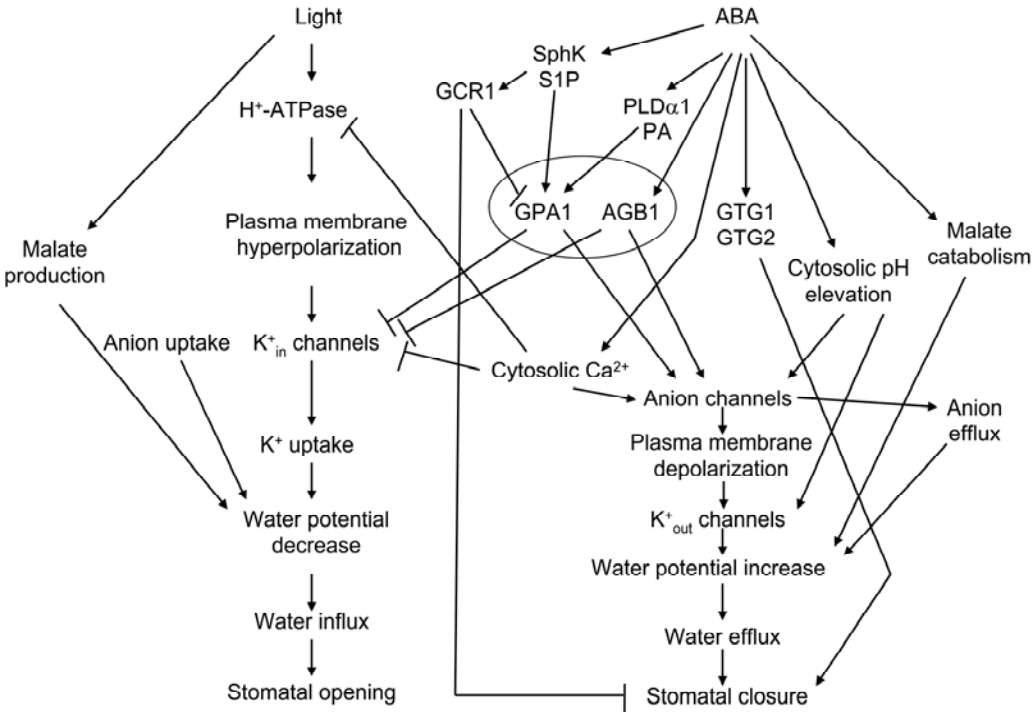
**Zhao Z, Zhang W, Stanley BA, Assmann SM** (2008) Functional proteomics of *Arabidopsis thaliana* guard cells uncovers new stomatal signaling pathways. Plant Cell 20:3210-3226 .

**Table 1**. Stomatal-regulation and density-related phenotypes of *Arabidopsis* heterotrimeric G protein mutants

| Allele | Ecotype | Ion channel activities | Stomatal movements | Excised leaf water loss | Drought tolerance | Stomatal density |
|---|---|---|---|---|---|---|
| *gpa1-1*<br>*gpa1-2* | Ws<br>Ws | Insensitive to ABA inhibition of inward $K^+$ channels and pH independent ABA activation of slow anion channels (Wang *et al.* 2001)<br><br>Insensitive to S1P inhibition of inward $K^+$ channels and S1P promotion of slow anion channels (Coursol *et al.* 2003) | Insensitive to ABA inhibition of opening (Wang *et al.* 2001)<br><br>Wild type ABA promotion of closure (Wang *et al.* 2001)<br><br>Insensitive to S1P promotion of closure and inhibition of opening (Coursol *et al.* 2003)<br><br>Reduced extracellular calmodulin-induced closure (Chen *et al.* 2004) | Increased water loss from excised leaves (Wang *et al.* 2001) | Not phenotyped | Not phenotyped |
| *gpa1-3*<br>*gpa1-4* | Col<br>Col | *gpa1-4*: Hyposensitive to ABA inhibition of inward $K^+$ channels (Fan *et al.* 2008)<br><br>Insensitive towards flg22 induced inhibition of inward $K^+$ channels (Zhang *et al.* 2008b) | *gpa1-4*: Hyposensitive to ABA inhibition of opening (Fan *et al.* 2008)<br><br>*gpa1-4*: Wild type ABA induced closure<br><br>*gpa1-3*: Insensitive to PA inhibition of opening (Mishra *et al.* 2006)<br>Insensitive towards flg22 induced inhibition of opening (Zhang *et al.* 2008b) | Allele not specified: Reduced water loss from detached cotyledons (Zhang *et al.* 2008a)<br><br>*gpa1-3*: Increased water loss from detached leaves (Mishra *et al.* 2006) | Allele not specified: Reduced seedling dessication under low humidity conditions (Zhang *et al.* 2008a) | Allele not specified: Reduced cotyledon stomatal density (Zhang *et al.* 2008a) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *agb1-1*<br>*agb1-2* | Col<br>Col | Hyposensitive to ABA inhibition of inward $K^+$ channels and pH independent activation of outward anion channels (Fan *et al.* 2008) | Hyposensitive to ABA inhibition of opening (Fan *et al.*, 2008)<br><br>Wild type ABA-promotion of closure (Fan *et al.* 2008) | Allele not specified: Increased water loss from cotyledons (Zhang *et al*. 2008a) | Allele not specified: Increased seedling dessication under low humidity conditions (Zhang *et al.* 2008a) | Allele not specified: Increased cotyledon stomatal density (Zhang *et al.* 2008a) |
| *agg1-1c* | Ws allele introgressed into Col | Wild type ABA-inhibition of inward $K^+$ channels (Trusov *et al.* 2008) | Wild type ABA inhibition of opening and promotion of closure (Trusov *et al.* 2008) | Not phenotyped | Not phenotyped | Not phenotyped |
| *agg2-1*<br>*agg2-2* | Col | Wild type ABA-inhibition of inward $K^+$ channels (Trusov *et al.* 2008) | Wild type ABA inhibition of opening and promotion of closure (Trusov *et al.* 2008) | Not phenotyped | Not phenotyped | Not phenotyped |
| *gcr1-3*<br>*gcr1-4* | Ws<br>Col | Not phenotyped | Hypersensitive to ABA and S1P inhibition of stomatal opening and ABA and S1P promotion of closure (Pandey and Assmann 2004) | Reduced water loss from excised leaves (Pandey and Assmann, 2004) | Plants exhibit drought tolerance (Pandey and Assmann 2004) | Not phenotyped |
| *rgs1-1*<br>*rgs1-2* | Col<br>Col | Wild type ABA inhibition of inward $K^+$ channels for current amplitude but channels show accelerated voltage activation kinetics (Fan *et al.* 2008) | Not phenotyped | Not phenotyped | Not phenotyped | *rgs1-2*: Increased stomatal density in cotyledons (Zhang *et al.* 2008a) |
| *gtg1gtg2* (double mutant) | Ws | Not phenotyped | Wild type ABA inhibition of stomatal opening, hyposensitive to ABA promotion of closure (Pandey *et al.* 2009) | Not phenotyped | Not phenotyped | Not phenotyped |

**Figure 1**. Integrated model of G protein regulation of light induced stomatal opening and ABA induced stomatal closure. (→) indicates positive regulation while (⊣) indicates negative regulation.



## Chapter 4: The α subunit of the *Arabidopsis* heterotrimeric G protein, GPA1, is a regulator of transpiration efficiency


### ABSTRACT

Land plants must balance $CO_2$ assimilation with transpiration in order to minimize drought stress and to maximize their reproductive success. The ratio of assimilation to transpiration is called transpiration efficiency (TE). TE is under genetic control, although only one gene, *ERECTA*, has been shown to regulate TE. We have found that the α subunit of the heterotrimeric G protein in *Arabidopsis thaliana*, GPA1, is a regulator of TE. *gpa1* mutants, despite having guard cells which are hyposensitive to ABA-induced inhibition of stomatal opening, have increased TE under ample water and drought stress conditions and when treated with exogenous ABA. Leaf level gas exchange analysis shows that *gpa1* mutants have wild type assimilation vs. internal $CO_2$ concentration responses but exhibit reduced stomatal conductance compared to Col at ambient and below-ambient internal $CO_2$ concentrations. The increased TE and reduced stomatal conductance of *gpa1* can be primarily attributed to stomatal density which is reduced in *gpa1* mutants. GPA1 regulates stomatal density via control of epidermal cells size and stomata formation. *GPA1 promoter::GUS* lines indicate that the *GPA1* promoter is active in the stomatal cell lineage further supporting a function for GPA1 in stomatal development in true leaves.


### INTRODUCTION

Land plants, in particular plants which utilize C3 photosynthesis, must balance $CO_2$ acquisition with water loss in order to maintain turgor and maximize fitness. The water loss cost per unit of biomass acquired can be expressed as transpiration efficiency (TE, also referred to as water-use efficiency), the ratio of $CO_2$ assimilation (A) to transpiration. TE strongly correlates with the $\delta^{13}C$ of plant tissue, the ratio of $^{13}C$ to $^{12}C$

relative to a standard (Farquhar et al., 1982; Farquhar et al., 1989; Dawson et al., 2002). The physiological basis of this correlation is that in plants there is diffusional and biochemical discrimination against $^{13}C$, the heavier and less abundant stable isotope of C. Discrimination against $^{13}C$ decreases with decreasing internal $CO_2$ concentration ($C_i$), which can result from either increased A or reduced stomatal conductance ($g_s$) (Farquhar et al., 1982). While it is known that $g_s$ (a main factor controlling transpiration) correlates with A (Wong et al., 1979), genetic variation for TE and/or $\delta^{13}C$ has been documented in a number of species (Virgona et al., 1990; Ehleringer et al., 1991; Comstock and Ehleringer, 1992; Hammer et al., 1997; Lambrides et al., 2004). In *Arabidopsis thaliana*, multiple QTL associated with TE have been identified indicating that TE is under genetic control (Juenger et al., 2005; Masle et al., 2005; McKay et al., 2008). However, only one gene, *ERECTA*, has been specifically identified as a regulator of TE (Masle et al., 2005). *ERECTA* encodes a leucine-rich repeat receptor-like kinase (Torii et al., 1996) and regulates TE via control of stomatal density, $g_s$, mesophyll cell proliferation and photosynthetic capacity (Masle et al., 2005).

Heterotrimeric G proteins are GTP-binding proteins that function in the transduction of extracellular signals into intracellular responses. In its inactive state, the G protein classically exists as a trimer consisting of an α subunit (Gα) bound to GDP, a β subunit (Gβ), and a γ subunit (Gγ). When a ligand binds to a G protein coupled receptor (GPCR), a confirmational change occurs in the G protein resulting in the exchange of GDP for GTP and the dissociation of Gα-GTP from the Gβγ dimer. The G protein subunits remain active until the intrinsic GTPase activity of Gα results in the hydrolysis of GTP to GDP and the reassociation of the inactive trimer. The Arabidopsis genome contains canonical Gα and Gβ genes, *GPA1* and *AGB1*, and two genes known to encode Gγs, *AGG1* and *AGG2* (Assmann, 2002). One likely GPCR, GCR1 has been functionally characterized (Pandey and Assmann, 2004) and additional GPCRs have been predicted using bioinformatics (Moriyama et al., 2006; Gookin et al., 2008) and interaction with GPA1 in yeast-based protein-protein interaction assays (Gookin et al., 2008). Recently a new class of G proteins, GPCR-type G proteins (GTG1 and GTG2) have been identified in Arabidopsis which also serve as ABA receptors (Pandey et al., 2009).

Despite the paucity of heterotrimeric G protein subunit genes in the Arabidopsis genome as compared to mammalian systems, functional studies of heterotrimeric G protein mutants suggest that G protein function is diverse in Arabidopsis. G proteins have been shown to function in developmental processes and hormonal and environmental signaling, including stomatal aperture regulation (Perfus-Barbeoch et al., 2004; Joo et al., 2005; Chen et al., 2006; Pandey et al., 2006; Trusov et al., 2006; Warpeha et al., 2007; Fan et al., 2008; Zhang et al., 2008; Zhang et al., 2008). In response to drought stress, ABA concentration increases in the leaves (Davies and Zhang, 1991; Davies et al., 2005) where it promotes stomatal closure and inhibits stomatal opening (Schroeder et al., 2001). *gpa1* and *agb1* mutants are hyposensitive to ABA inhibition of stomatal opening while displaying wild type ABA promotion of stomatal closure (Wang et al., 2001; Fan et al., 2008). ABA inhibits stomatal opening in part by inhibiting inward-rectifying $K^+$ channels, reducing $K^+$ influx and therefore water entry into the cell (Schroeder et al., 2001). ABA inhibition of inward $K^+$ channel activity is reduced in both *gpa1* and *agb1* mutants (Wang et al., 2001; Fan et al., 2008). *agg1* and *agg2* mutants show no altered regulation of ABA-induced stomatal movements or ion channel activities suggesting that the genome contains additional unknown Gγ(s) or that heterotrimeric G protein signaling in plants does not operate according to the mammalian paradigm (Trusov et al., 2008). *gcr1* mutants are hypersensitive to both ABA-inhibition of opening and ABA promotion of stomatal closure (Pandey et al., 2006). *gtg1gtg2* double mutants show wild type response for ABA inhibition of stomatal opening and are hyposensitive in ABA promotion of stomatal closure (Pandey et al., 2009).

While the altered stomatal sensitivities of the G protein mutants to ABA suggest that heterotrimeric G proteins may function in the regulation of whole-plant water status, few experiments have been performed at the whole leaf or whole plant level. *gpa1* mutants in the Ws background display increased water loss from excised leaves (Wang et al., 2001), however there are no published reports of experiments assessing whole-plant water status in *gpa1* or *agb1* mutants. *gcr1* mutants show reduced water loss from excised leaves, drought tolerance, and improved recovery following the cessation of drought stress (Pandey and Assmann, 2004). In addition to their altered guard cell sensitivities to ABA, *gpa1*, *agb1*, and *gcr1* mutants are hypersensitive to ABA inhibition

of root and seedling development (Pandey et al., 2006) which could have impacts on whole-plant water status. Finally, it has been recently reported that *gpa1* and *agb1* mutants have reduced and increased stomatal densities, respectively, in cotyledons (Zhang et al., 2008). While stomatal density of leaves can be an important component of whole-plant water status, the Zhang et al. study was performed on cotyledons only, whose developmental programs are often independent from those of true leaves (Chandler, 2008). Therefore, it is difficult to infer how this cotyledon phenotype will affect water relations at the whole-plant level. Taken together, the stomatal aperture, electrophysiology, and tissue-specific ABA phenotypes of the G protein mutants, in addition to the possibility for altered stomatal density in the G protein mutant leaves, makes it difficult to predict how G proteins contribute to the regulation of whole-plant water status. It is important to note that previous attempts to address the contributions of G proteins to plant fitness (and water relations) using excised leaf/rosette assays to measure TE are not adequate, because both transpiration and A must be taken into account. Therefore, we investigated the role of *GPA1* in regulating TE under ample water and drought stress conditions and in the presence of ABA. We have identified *GPA1* as a negative regulator of TE in Arabidopsis via control of $g_s$ and stomatal proliferation.

## RESULTS

### *gpa1* mutants have increased TE under ample and low soil water conditions

Given the involvement of GPA1 in the regulation of stomatal movements and ABA signaling (Wang et al., 2001; Pandey et al., 2006), TE was measured on *gpa1-3*, *gpa1-4*, and Col under soil water conditions equivalent to 90% (ample water) or 30% (drought stress) of the soil water carrying capacity. ANOVA found significant effects for both genotype and water level and no significant genotype by treatment interaction (Supplemental Table 1). As would be expected, drought stress significantly increased TE for all genotypes (Figure 1A). Interestingly, *gpa1* mutants displayed increased TE under both ample water and drought stress conditions (Figure 1A). Under ample water

conditions *gpa1* mutants had approximately a 12% increase in TE compared to Col ($p=0.0107$ for *gpa1-3*, $p<.0001$ for *gpa1-4*). Under drought stress, *gpa1* mutants had a 14% increase in TE compared to Col ($p < .0001$ for both *gpa1* alleles).

In order to corroborate the whole-plant TE phenotypes of the *gpa1* mutants, stable carbon isotope analysis was performed on a subset of plants from the TE experiment (Figure 1B). Consistent with the whole-plant TE data, the drought stress treatment resulted in an increase in the ratio of $^{13}C$ to $^{12}C$ (i.e., drought stress resulted in reduced discrimination against $^{13}C$) in the rosette tissue of all genotypes and *gpa1* mutants had increased ratios of $^{13}C$ to $^{12}C$ (reduced discrimination against $^{13}C$) compared to Col under both drought stress and ample soil water conditions ($p < .0001$ for all *gpa1* vs. Col comparisons). Rosette percent carbon and percent nitrogen were consistent between the genotypes within a water treatment but both percent carbon and percent nitrogen were affected by water treatment ($p < .0001$ for both percent carbon and percent nitrogen). Drought stress treatment resulted in a 2% decrease in percent carbon and a 2% increase in percent nitrogen compared to the ample water treatment for all genotypes.

### *gpa1* mutants have reduced carbon isotope discrimination when treated with ABA

To directly assess the role of ABA in regulation of TE by GPA1, the rosettes of *gpa1* and Col were exogenously treated with ABA and carbon isotope analysis was performed. *gpa1* and Col plants were grown under ample soil water conditions (90% of the soil water carrying capacity) in a similar fashion as in the TE experiments. Carbon isotope analysis was performed on rosettes following 4 weeks of exogenous 25 μM ABA treatment (Figure 2). Because the plants were sprayed with ABA dissolved in water, whole-plant TE could not be reliably calculated. ABA treatment significantly and dramatically increased the ratio of $^{13}C$ to $^{12}C$ (reduced discrimination against $^{13}C$) in the rosette tissue; ABA treatment (Fig. 2) had a much stronger effect on carbon isotope ratio than drought stress for all genotypes (Fig. 1B). Interestingly, despite the altered stomatal physiology of *gpa1* mutants in response to ABA, *gpa1* mutants had reduced discrimination against $^{13}C$ in rosette tissue compared to Col even in this experiment

where 25 μM ABA was applied directly to the leaves ($p < .001$ for *gpa1-3* and $p=0.004$ for *gpa1-4*).

### *gpa1* mutants show a wild type A-$C_i$ response but a reduced $g_s$-$C_i$ response

Gas exchange analysis was performed to determine whether the increased TE of the *gpa1* mutants was due to an effect of the mutation that enhanced A, reduced $g_s$, or both of these processes. A and $g_s$ were measured under a range of external $CO_2$ concentrations under non-light limiting conditions in intact leaves. A-$C_i$ curves indicate that the *gpa1* mutants resemble wild type in their A responses (Fig. 3A). $V_{cmax}$ and $J_{max}$ were calculated for each individual plant curve and no significant differences were found between the *gpa1* mutants and Col for either parameter (Supplemental Fig. 1).

Stomatal conductance data taken simultaneously with the photosynthetic data in Fig. 3A indicate that *gpa1* mutants have altered $g_s$ response to $C_i$ compared to wild type (Fig. 3B). At high $C_i$s (over 600 ppm) $g_s$ is at a baseline minimum value (stomata are closed) for all genotypes (approximately 0.075 mol $H_2O$ $m^{-2}s^{-1}$). As $C_i$ is reduced, $g_s$ increases for all genotypes, however, in *gpa1* mutants this increase is attenuated. At $C_i$s corresponding to external $CO_2$ concentrations at or below ambient $CO_2$ level, *gpa1* mutants have significantly reduced $g_s$ compared to Col. At minimum $C_i$s (approximately 40 ppm), *gpa1-3* and *gpa1-4* show respective 35% ($p < .001$) and 25% ($p = 0.004$) reductions in $g_s$ compared to Col.

### *gpa1* mutants are hypersensitive to low $CO_2$-induced stomatal opening

One hypothesis for the altered $g_s$ responses to $C_i$ observed in the *gpa1* mutants is that the mutants may be hyposensitive to low $CO_2$-induced stomatal opening. To investigate this hypothesis, we performed stomatal aperture measurements under reduced $CO_2$ and ambient $CO_2$ conditions. Incubation of leaves in buffer which had been bubbled with $CO_2$-free air resulted in increased stomatal opening in all genotypes compared to

leaves incubated in buffer equilibrated to ambient $CO_2$ levels (Fig. 4). *gpa1* mutants, however, had slightly enhanced stomatal opening compared to Col (Fig. 4; $p = 0.06$ for *gpa1-3*, $p = 0.008$ for *gpa1-4*). Control experiments using Col leaves incubated in buffer bubbled with ambient $CO_2$ air or with no bubbling showed no significant differences in stomatal aperture, therefore bubbling alone did not induce stomatal opening. The result suggests that *gpa1* mutant guard cells are actually hypersensitive to low $CO_2$-induced stomatal opening and that altered $CO_2$ sensing or response by the guard cells is not limiting whole-leaf $g_s$ in *gpa1* mutants at $C_i$s below 250 ppm.

### *gpa1* mutants have reduced stomatal density and index in mature leaves

Another hypothesis to explain the reduced $g_s$ of *gpa1* mutants is that stomatal density is reduced in mature leaves of *gpa1* plants. In fact, we found that *gpa1* mutants do have approximately 50% fewer stomata on fully expanded leaves compared to Col (Fig.s 5A and 5D; $p = 0.0001$ for *gpa1-3*, $p < .0001$ for *gpa1-4*). The reduction in stomatal density can be attributed to both increased cell size of epidermal cells and reduced formation of stomata. Epidermal cell density was reduced in *gpa1* mutants by 20% compared to Col ($p= 0.0203$ for *gpa1-3*, $p= 0.0027$ for *gpa1-4*) indicating the mutants have larger leaf epidermal cells than wild type (Fig. 5B). Stomatal index, the number of stomata relative to total cell number in the epidermis, was also significantly reduced in *gpa1* mutants ($p < .0001$ for both *gpa1* alleles) suggesting a role for GPA1 in stomatal development and/or proliferation in true leaves (Fig. 5C).

### *GPA1prom::GUS* activity is observed in stomatal precursor cells and immature guard cells

The stomatal density results supported a role for GPA1 in stomatal development. Therefore, we created *GPA1prom::GUS* reporter lines to examine *GPA1* promoter activity in stomata and stomatal precursor cells. Analysis of multiple independent

transgenic lines expressing the *GPA1prom::GUS* construct shows GUS activity in meristemoids (Fig. 6A), guard mother cells (Fig. 6B) and immature stomata (Fig. 6C) of developing true leaves. GUS activity decreases as stomata develop and is absent or faint in the majority of mature stomata (Fig. 6D). The epidermal cells directly adjacent to the immature stomata also show reporter gene activity albeit weaker than that of the developing stomatal complex (Fig. 6E). The results indicate that *GPA1* promoter activity is associated with developing stomatal complexes.

### *gpa1* mutants show reduced biomass allocation to the inflorescence

We have reported that *gpa1* mutants have reduced fitness (defined as total seed production) under both well-watered and drought stress conditions (Chapter 6). In order to potentially reconcile the increased TE phenotype with the reduced fitness observation we performed an additional TE experiment examining TE in both vegetative and reproductive phases of Arabidopsis. Interestingly, consistent with our previous TE experiments, *gpa1* mutants showed increased TE compared to wild type when harvested prior to bolting (Fig. 7A; $p < .0001$ for both *gpa1* alleles) and also showed increased TE when plants were harvested 5 weeks after bolting when all plants were flowering and setting seed ($p = 0.004$ for *gpa1-3*, $p < .001$ for *gpa1-4*). However, calculation of TE for the inflorescence only (inflorescence dry weight/ total water transpired) showed that *gpa1* mutants have reduced inflorescence TE compared to wild type (Fig. 7B; $p = 0.0407$ for *gpa1-3* and $p= 0.0116$ for *gpa1-4*). Additionally, analysis of dry biomass partitioning between the rosette and the inflorescence show that *gpa1* mutants allocate less biomass to the inflorescence compared to wild type plants (Fig. 7C, $p = 0.0049$ for *gpa1-3*, $p=0.0004$ for *gpa1-4*). The data suggest that despite the increased TE of *gpa1*, reduced biomass partitioning to the inflorescence has negative fitness consequences for *gpa1* mutants.

## DISCUSSION

### GPA1 regulation of TE

Increasing global populations necessitate the development of crop species that can thrive in inhospitable environments such as drought-prone areas. Breeding programs focusing on different plant physiological traits, including high TE/low $^{13}C$ discrimination, have been successful at developing high yielding drought tolerant crop cultivars (Condon et al., 2002; Rebetzke et al., 2002; Richards, 2006). Understanding the genetic basis of TE and other plant traits which contribute to plant survival and productivity under drought stress will benefit both traditional breeding programs and biotechnological crop enhancement. QTL that affect TE have been identified in Arabidopsis (Juenger et al., 2005; Masle et al., 2005) but only one specific gene, *ERECTA*, has been shown to regulate TE (Masle et al., 2005). Here, we identify a second gene, *GPA1*, as a regulator of TE in Arabidopsis. Despite the hyposensitivity of *gpa1* guard cells to ABA-induced inhibition of stomatal opening (Wang et al., 2001; Fan et al., 2008), *gpa1* mutants have increased vegetative TE and reduced carbon isotope discrimination compared to Col under both ample water and drought conditions. *gpa1* mutants also have reduced carbon isotope discrimination when ABA is directly applied to leaves. Since the increased TE of *gpa1* mutants could be due to reduced transpiration, enhanced photosynthesis or both of these processes, we examined a number of physiological (A, $g_s$, stomatal aperture size) and developmental (stomatal index and density, epidermal cell size) traits of *gpa1* mutants in order to gain insight into the mechanism by which GPA1 regulates TE.

### GPA1 regulation of leaf stomatal density

A number of leaf morphological traits can affect the level of water loss from the leaf including cuticle thickness, leaf thickness and anatomy, and the distribution and density of stomata on leaf surfaces. Therefore, we examined *gpa1* for altered leaf

developmental traits that could contribute to the enhanced TE and reduced $g_s$ observed for *gpa1*. Histological analysis of cross-sections of *gpa1* and Col leaves found no significant differences in leaf thickness or anatomy (Supplemental Fig. 2). Recently it has been shown that *gpa1* mutants have reduced stomatal density in cotyledons (Zhang et al., 2008). However, because cotyledons and leaves have at least partially independent developmental programs (Chandler, 2008) it could not be concluded from Zhang et al. (2008) that mature *gpa1* leaves would also have reduced stomatal densities. We did find though that *gpa1* mutants have approximately 50% fewer stomata on leaves compared to Col, which is similar to the reduction in density observed in cotyledons (Zhang et al., 2008). Taking our data as a whole, reduced stomatal density in *gpa1* is likely the major contributing factor to *gpa1* having reducing $g_s$ and increased TE compared to Col.

It has been reported that *gpa1* mutants have reduced cell division in shoots; *gpa1* mutants have fewer and larger hypocotyls cells and leaf epidermal cells (Ullah et al., 2001). Additionally, inducible overexpression of GPA1 has been shown to cause ectopic cell division in seedlings (Ullah et al., 2001). The epidermal cell density of *gpa1* was significantly reduced in mature leaves indicating that *gpa1* mutants have larger epidermal cells in mature leaves, therefore increased cell size and reduced cell division both contribute to the reduced stomatal density observed in *gpa1*. Stomatal index was also attenuated in mature leaves of *gpa1* indicating there is also reduced stomata formation. However, the reduction in stomatal index (≈ 15%) in mature leaves, while significant, was less than the reduction in stomatal index (28%) reported for *gpa1* cotyledons (Zhang et al., 2008). While Zhang et al. (2008) report that the reduced stomatal density in cotyledons could be explained in entirety by reduced stomata formation, we found a more complex developmental role for GPA1 in regulating stomatal density in leaves: GPA1 modulates both stomatal formation and epidermal cell division/size.

*GPA1* has been shown to be expressed in roots, shoots and reproductive organs and guard cell protoplasts (Ma et al., 1990; Weiss et al., 1993; Huang et al., 1994; Wang et al., 2001; Chen et al., 2006; Fan et al., 2008) however, *GPA1* expression in developing stomata has never been assessed. Using *GPA1*prom::GUS reporter lines we found significant GUS activity in stomatal precursor cells including meristemoids, guard mother cells and immature stomata. Reporter activity is strongest in immature stomata

and is severely reduced in mature guard cells. The finding of *GPA1* promoter activity in developing stomata, in addition to the reduced stomatal densities of *gpa1* mutants in mature leaves, suggest that GPA1 is a positive regulator of stomatal density in leaves.

It is interesting that the one other identified genetic regulator of TE in Arabidopsis, ERECTA, also functions in stomatal development, albeit as a negative regulator (Masle et al., 2005; Shpak et al., 2005). Heterotrimeric G proteins have additional overlapping functions with ERECTA including in flower, fruit, and leaf development, cell division and pathogen responses (Lease et al., 2001; Shpak et al., 2004; Llorente et al., 2005) and double mutant analysis of *erecta agb1* mutants show that ERECTA and AGB1 likely function in the same pathway in regulating fruit shape (Lease et al., 2001). Additionally, both GPA1 and ERECTA are membrane localized proteins with putative functions in signal transduction (Torii et al., 1996; Weiss et al., 1997; Adjobo-Hermans et al., 2006; Chen et al., 2006; Wang et al., 2008). Unfortunately, we were unable to assess whether or not ERECTA and GPA1 function in the same pathway to regulate TE and/or stomatal density because tight linkage between the two loci (1.7 cM genetic distance and 7 kb physical distance) to date has prevented the isolation of F2 recombinants that are heterozygous for both *erecta* and *gpa1*. Yeast-based biochemical interaction assays between ERECTA and GPA1 were inconclusive and there was no identifiable phosphorylation of GPA1 or ABG1 by the kinase domain of ERECTA in *in vitro* phosphorylation assays (Chapter 5). Further investigation is needed in order to ascertain if GPA1 and ERECTA function in the same pathway to regulate TE and stomatal development.

**Leaf level gas exchange, stomatal aperture regulation and stomatal density**

The increased TE exhibited by *gpa1* mutants could have been a consequence of enhanced A, reduced gs, or both these processes. Gas exchange analysis revealed that *gpa1* mutants had wildtype A vs. $C_i$ responses but had reduced $g_s$ compared to Col, under ambient and below-ambient $CO_2$ levels. This indicated that reduced $g_s$ is the primary means by which *gpa1* mutants have increased TE. We observed that stomatal density

contributes to the reduced $g_s$ of *gpa1*, but we also needed to evaluate the possibility that *gpa1* stomata failed to open in response to low $CO_2$ concentrations, a phenomenon that would also contribute to a reduced $g_s$. However, we found that *gpa1* mutants did not exhibit this phenotype and in fact had the opposite phenotype: *gpa1* mutants have larger stomatal apertures compared to wild type when leaves with closed stomata are exposed to low $CO_2$ buffer. In other words, low $CO_2$ sensing is altered in *gpa1*, but *gpa1* mutants are hypersensitive, not hyposensitive, for low $CO_2$ induced opening. Therefore, the reduced $g_s$ and increased TE observed in *gpa1* is most likely a consequence of *gpa1* mutants having reduced stomatal density. At approximately ambient $C_a$, $C_i$s were slightly lower for the *gpa1* mutants compared to wild type (Fig. 3). As would be expected, A is reduced at ambient $C_a$ in the *gpa1* mutants, however, $g_s$ is also reduced in *gpa1* compared to wild type. Therefore, we hypothesize that the water savings experienced by *gpa1* mutants at ambient $C_a$ due to reduced $g_s$, outweighs the reductions in A, resulting in *gpa1* mutants having an increased TE. While there is no consistent relationship reported in the literature between stomatal density, $g_s$ and A, gas exchange analysis of the Arabidopsis *sdd1-1* mutant which has an elevated stomatal density showed that $g_s$ was affected more so by the *sdd1-1* mutation than A, although the extent of this difference depended on the light intensity (Schlüter et al. 2003). This report combined with our data on the *gpa1* mutants, suggest that at least in Arabidopsis, transpiration may be more sensitive to changes in stomatal density than A. However, we can not rule out the possibility that a *gpa1* mutation does enhance A by some mechanism that we did not detect in our assays, resulting in wild type A vs $C_i$ responses in the *gpa1* mutants.

*gpa1* mutants also have stomata which are opened more widely than wild type, as measured in stomatal aperture assays, when closed stomata are treated with ABA during light-induced stomatal opening (Wang et al., 2001; Fan et al., 2008). Therefore, GPA1 functions in both ABA and $CO_2$ signaling. Growing evidence suggests that $CO_2$ signaling and ABA signaling may operate in part via shared signaling components (reviewed in Vavasseur and Raghavendra, 2005). In particular, the 2C Ser-Thr protein phosphatases ABI1 and ABI2 appear to function in both ABA and $CO_2$ signaling (Webb and Hetherington, 1997; Leymarie et al., 1998; Leymarie et al., 1998). *abi1-1* and *abi2-1* mutants which are ABA insensitive are also insensitive to high $CO_2$-induced and

extracellular-$Ca^{2+}$-induced stomatal closure suggesting that $CO_2$, ABA, and extracellular $Ca^{2+}$ signaling converge at these signaling nodes (Webb and Hetherington, 1997). Interestingly, $CO_2$ and ABA may also converge in mediating stomatal density. Recently it has been proposed by Lake and Woodward (2008) that changes in stomatal density due to altered $CO_2$ concentration and humidity may be signaled via ABA regulation of stomatal aperture and transpiration rate.

Another possible explanation for *gpa1* plants' altered $CO_2$ and ABA stomatal aperture responses is that the *gpa1* mutants compensate for their reduced stomatal density in order to more closely achieve a wild type level of leaf water status. This phenomenon of stomatal aperture compensation for changes in stomatal density has been reported for the stomatal development mutant *sdd1*, which has increased stomatal density, and for SDD1 overexpressing lines which have reduced stomatal densities (Berger and Altmann, 2000; Bussis et al., 2006). While *gpa1* may be able to partially compensate for the reduced density under certain environmental conditions by increasing stomatal aperture size, it cannot fully compensate for the reduced stomatal density; at low $C_i$s *gpa1* mutants have markedly reduced $g_s$.

Recently, Zhao et al. (submitted) found using quantitative proteomic techniques that 17 out of 18 proteins enriched in the guard cells of *gpa1* mutants are members of the Arabidopsis chloroplast proteome: 12 proteins function in photosynthesis and five proteins are associated with the Calvin cycle (Zhao et al. submitted). Chloroplast abundance in guard cells varies by species although guard cell chloroplasts are typically smaller and fewer than mesophyll chloroplasts (Lawson, 2009). The functions of guard cell chloroplasts are still debated (Lawson, 2009) although four main functions have been proposed: electron transport in guard cell results in an energy source for osmoregulation, chlorophyll of guard cell chloroplasts may function as a receptor for light-induced stomatal movements, guard cell chloroplasts are storage reservoirs for starch from which malate or sucrose can be produced, and the Calvin cycle activity within guard cells produces sugars which function in osmoregulation and stomatal opening (Lawson, 2009). GPA1 has also been shown to interact with a THF1, a plastid membrane protein which has a putative function in glucose signaling, in yeast and root epidermal cells (Huang et al., 2006). Zhao et al. (submitted) speculate that, in wild type plants, GPA1 may

negatively regulate guard cell photosynthesis in response to glucose levels by suppressing photosynthesis-related proteins. An alternative hypothesis, given the reduced stomatal densities of *gpa1* mutants and the possibility of stomatal aperture compensation for density under certain environmental conditions (low $CO_2$, ABA), is that the increase in photosynthesis-related proteins is utilized in *gpa1* to produce the energy and solutes required to drive more extreme stomatal movements, such is the case for low-$CO_2$ induced stomatal opening (Fig. 4). Again, heightened stomatal movements may be a way by which *gpa1* can compensate for reduced stomatal density and allow leaf water status to approach more wild type levels. Determining whether GPA1 directly regulates guard cell photosynthesis as proposed by Zhao et al. (submitted) or whether the increased photosynthetic machinery in *gpa1* guard cells is a compensatory response to the reduction in stomatal density in *gpa1* requires further study.

**GPA1, TE and plant fitness**

We have found that *gpa1* mutants have increased vegetative TE under well-watered and drought stress conditions and when ABA is applied directly to the leaves. However, *gpa1* mutants do not appear to have an increase in fitness (quantified as seed production) when grown under well-watered, moderate drought or severe drought conditions (Chapter 6). We have also found that total above-ground biomass TE is increased in *gpa1* even when the plants are harvested after five weeks of flowering. However, inflorescence TE is reduced in *gpa1* mutants compared to Col. One possible explanation for the lack of a fitness benefit is altered biomass partitioning in *gpa1* mutants. Indeed, as reported here, despite the increased TE of *gpa1* plants, *gpa1* mutants allocate a smaller proportion of their total biomass to the inflorescence compared to Col which may be due in part to reduced lateral branch number in *gpa1* mutants (Nilson and Assmann submitted, Chapter 7). Finally, analysis of *gpa1* mutants indicates that *GPA1* functions in a number of different stress responses (pathogens, ozone, ROS), leaf and flower development, and hormonal signaling (Perfus-Barbeoch et al., 2004; Joo et al., 2005; Llorente et al., 2005; Pandey et al., 2006; Trusov et al., 2006; Zhang et al., 2008).

Therefore, pleiotropic effects on fitness caused by the *gpa1* mutation may counteract any fitness benefit achieved from enhanced TE. In future experiments, it will be interesting to explore the mechanistic basis of the stomatal proliferation phenotype which underlies the TE effect, and to assess whether other G protein subunits also contribute to the regulation of TE.

## CONCLUSION

Using whole-plant approaches we found that GPA1 is a negative regulator of TE under well-watered and limited water growth conditions and when ABA is exogenously applied to rosettes. GPA1 regulates TE via promotion of stomata formation, stomatal density and $g_s$ in rosette leaves. This finding is opposite to what would be predicted based solely on the altered stomatal aperture responses of *gpa1* mutants to the plant stress hormone ABA. Despite enhanced TE, *gpa1* mutants do not show increased fitness when grown under a range of water levels (Nilson and Assmann submitted, Chapter 6). The reduced fitness of *gpa1* may be due in part to reduced biomass allocation to reproductive structures and/or additional pleiotropic effects of the *gpa1* mutation on other environmental stress and developmental responses e.g., *gpa1* hypersensitivity in non-guard cell ABA responses (Pandey and Assmann, 2004). More experimentation is needed to determine if other G protein subunits also contribute to the regulation of TE and also to investigate the explicit mechanism by which G proteins regulate stomatal density.

## MATERIALS AND METHODS

### Plant material and growth conditions

All *Arabidopsis thaliana* seed used in these experiments were collected from co-grown Col, *gpa1-3*, and *gpa1-4* parent plants whose genotypes were confirmed via PCR of genomic DNA. Seed was stratified on wet filter paper at 4° C in the dark for 48 hours prior to planting to synchronize germination. Plants were grown in 8 $cm^2$ pots in soil mix (Miracle-Gro Potting Mix, Scotts, Marysville, OH) augmented with perlite, unless otherwise specified. Plants were grown in walk-in growth chambers (Controlled Environments Limited, Winnipeg, Manitoba) under extended short-day light conditions (12 hour light, 21°C /12 hour dark, 19°C), at a light intensity of 110-120 $\mu molm^{-2}s^{-1}$, and 65 percent relative humidity.

### Whole plant TE experiments

The protocol was modified from Juenger et al. (2005). Plants were grown in a 16:8:1 volume mixture of potting mix, fritted clay (Turface Greens Grade, Profile Products, Buffalo Grove, IL) and perlite. The carrying capacity of the soil mix was determined following a 24 hour gravimetric drain of saturated soil. A known dry weight of soil mixture was placed in 250 mL plastic containers with four holes punched in the bottom. A circle of landscape fabric was placed at the bottom of each pot to prevent soil loss. Water was added to the pots to either 90% (ample water) or 30% (drought stress) of the soil water carrying capacity and sealed with a layer of parafilm and a tight fitting lid with a small central hole. One stratified seed was placed on the soil surface, centered under the hole of the lid. Pots were weighed every 2 to 3 days and water was added to the pots with a syringe to return soil to the appropriate water level. Blank pots containing no plants indicated that evaporative losses from the soil under these conditions were minimal. Eight-week old plants were harvested prior to bolting for vegetative TE

measurements. For the phase change TE experiment the plants were harvested at 13 weeks; all plants were flowering and setting seed at harvest. The rosette and inflorescence (where applicable) were removed and dried at 70°C until a constant weight was achieved for dry weight determination. TE was calculated by dividing the dry weight of the rosette, inflorescence or entire above ground biomass where applicable (mg) by the total volume of water transpired (ml). For the ample water/drought stress TE experiment, three replicate genotype by water level replicates were planted in each of 7 blocks. Plant mortality resulted in final genotype by water level replicates ranging from 13-21 plants for a total n = 120 plants. For the phase change TE experiment 8 blocks were planted, each block containing 6 replicate genotypes. 4 blocks were harvested prior to bolting and 4 blocks after flowering. Plant mortality resulted in final sample sizes ranging from 20-24 plants per genotype per harvest time resulting in n = 135 plants.

**Exogenous ABA treatment**

Plants were grown as described for the TE experiments at 90% of the soil water carrying capacity in 5 blocks containing 6 genotype x ABA concentration replicates (total n = 174 plants after plant mortality). After 4 weeks of growth, plants were sprayed twice weekly with either ABA solution (25 µM ABA, 0.05% ethanol, 0.02% Silwett L-77) or control solution (0.05% ethanol, 0.02% Silwett L-77). After four weeks of ABA applications, rosettes were harvested for carbon isotope analysis.

**Carbon isotope analysis**

Dried rosette were ground to a fine powder using a mortar and pestle and sent to the Cornell Stable Isotope Laboratory (http://www.cobsil.com) where the carbon isotope ratios of the samples (Rs) were determined. The ratios given are relative to the V-PDB standard (RPDB) where $\delta^{13}C$ (‰) = $(R_s/R_{PDB}-1) \times 1000$. Carbon isotope ratio values rather than carbon isotope discrimination are shown because the carbon signature of the

growth chamber air is unknown. Sample size was 12-15 genotype by water level replicates for the ample water/drought stress experiment and 11-12 genotype x ABA concentration replicates for the ABA treatment experiment.

**Stomatal density and index measurements**

The abaxial epidermes were peeled from fully-expanded leaves of 7 week old plants, wet-mounted, and photographed at 400X power using a digital camera mounted to a Nikon Diaphot 300. Stomatal density and epidermal cell density were determined for each image using Image J. The pixel scale was determined by photographing a slide micrometer. Two or three leaves were sampled per plant and approximately 24 images were analyzed for cell densities per plant. Stomatal index (100 x stomatal density/(stomatal density + epidermal cell density)) was calculated for each image. For each plant, the density and index values were averaged and the mean value was used for statistical analysis. Six replicate plants were measured for each genotype.

***GPA1pro::GUS* lines and GUS staining**

The 1500 base pair region directly upstream of the *GPA1* translational start site was amplified using Accuprime Pfx Supermix (Invitrogen) with the forward primer: CTCGAGTTTAAGTGGTTAGGGAAGCTATGTATT and the reverse primer: GCGGCCGCGATTGTTTCTATATCCCCACAG. The fragment was blunt cloned into the TOPO PCR Blunt II vector (Invitrogen), sequenced, and subcloned into the binary GUS reporter vector pORE R1 at the Xho1 and Not1 sites (Coutu et al., 2007). pORE R1 *GPA1*pro::GUS was transformed into Agrobacteria cells (C58C1) and a modified floral dip method was used to infect the floral buds with the transformed Agrobacteria (Clough and Bent, 1998). Seeds were plated on 0.5x MS, 50 μg/ml kanamycin, 0.8% agar to select for transformants. All transformants were confirmed via PCR genotyping.

GUS staining was performed on multiple independent T1 lines. The abaxial epidermes of developing leaves were removed with forceps and immediately placed in acetone on ice. Samples were vacuum-infiltrated with acetone for 10 minutes and fixed at room temperature for 30 minutes. The acetone was removed and samples were vacuum infiltrated on ice with GUS staining buffer (50 mM NaPO4 pH 7.2, 0.2% Triton X, 2mM potassium ferrocyanide, 2mM potassium ferricyanide) for 10 minutes. The staining buffer was removed and the samples were infiltrated under vacuum with GUS staining buffer containing 2mM X-gluc in dimethylformamide for 20 minutes on ice. Samples were incubated overnight at 37°C in the dark. Samples were dehydrated in an ethanol series (20%, 35%, 50%) for 30 minutes at room temperature for each concentration. The samples were fixed with FAA (50% ethanol, 3.7% formaldehyde, 5% acetic acid) for 30 minutes at room temperature and then placed in 70% ethanol. Samples were mounted in 70% ethanol 30% glycerol and examined under a light microscope.

**Gas exchange analysis**

Gas exchange analysis was performed on intact, fully expanded leaves of 7 week old plants using a Licor-6400 photosynthesis system (Licor Biosciences, Lincoln, NE) equipped with the Licor-6400-40 leaf chamber fluorometer. Light curves performed on wild type plants prior to A-$C_i$ analyses showed that a light level of 1000 μmol $m^{-2}s^{-1}$ was appropriate for non-light limiting and non-photoinhibitory conditions (data not shown). The following variables were held constant during gas exchange analyses: vapor pressure difference (1.2 kPa), leaf temperature (23°C), and light intensity (1000 μmol $m^{-2}s^{-1}$, 10% blue light). Assimilation and $g_s$ were measured at the following external $CO_2$ values in the order shown: 2000, 1500, 1000, 800, 400, 200, 100, 50, and 0 μL $L^{-1}$. The IRGAs were matched after each change in $CO_2$ concentration and data were logged for 2 minutes at each $CO_2$ level. Estimates of $V_{cmax}$ and $J_{max}$ were calculated for each A- $C_i$ curve using a web-based curve fitting utility (Sharkey et al., 2007). For each genotype, gas exchange measurements were performed on 10-12 replicate plants. Data shown are averaged curves where average $g_s$ or A is plotted against average $C_i$.

**Stomatal aperture assay**

Leaves from 6 week-old plants were excised prior to the beginning of the day's light cycle and incubated in buffer (10 mM KCl, 7.5 mM iminodiacetic acid, 10 mM MES, pH 6.15 with KOH) in the dark for 2 hours to ensure stomatal closure (Leymarie et al., 1998). A Licor-6400 was used to scrub $CO_2$ and pump $CO_2$ -free air into a 600 mL glass beaker containing 150 mL of buffer (flow rate 500 μmol $s^{-1}$). The buffer was equilibrated with $CO_2$ free air overnight. The leaves were placed in the low $CO_2$ buffer or ambient $CO_2$ buffer, abaxial side down, in the dark. For the low $CO_2$ treatment, low $CO_2$ air was continuously pumped into the buffer during the leaf incubation. After one hour of incubation, the epidermes of the leaves were removed and wet-mounted onto slides. 8 genotype by treatment replicates were assayed over a 4 day period. Photographs were obtained using a digital camera mounted to a Nikon Diaphot 300 microscope. Stomatal aperture measurements were performed blind using Image J; a slide micrometer was photographed at the same resolution for scale.

**Statistical analyses**

All statistical analyses were carried out using Minitab 14 Statistical Software. GLM ANOVA was used for all TE and $\delta^{13}C$ analyses with Dunnett or Tukey-corrected multiple comparisons. For all ANOVAs, data and residuals were examined to confirm that all ANOVA assumptions were met. ANOVA tables can be found in the Supplemental Tables 1-7. Student's t-test was used to test all other means comparisons for statistical significance.

## LITERATURE CITED


**Adjobo-Hermans MJ, Goedhart J, Gadella TW, Jr.** (2006) Plant G protein heterotrimers require dual lipidation motifs of Gα and Gγ and do not dissociate upon activation. J Cell Sci **119:** 5087-5097

**Assmann SM** (2002) Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. Plant Cell **14:** S355-373

**Berger D, Altmann T** (2000) A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. Genes Dev **14:** 1119-1131

**Bussis D, von Groll U, Fisahn J, Altmann T** (2006) Stomatal aperture can compensate altered stomatal density in *Arabidopsis thaliana* at growth light conditions. Func Plant Biol **33:** 1037-1043

**Chandler JW** (2008) Cotyledon organogenesis. J Exp Bot **59:** 2917-2931

**Chen JG, Gao Y, Jones AM** (2006) Differential roles of *Arabidopsis* heterotrimeric G-protein subunits in modulating cell division in roots. Plant Physiol **141:** 887-897

**Clough SJ, Bent AF** (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735-743

**Comstock JP, Ehleringer JR** (1992) Correlating genetic-variation in carbon isotopic composition with complex climatic gradients. Proc Natl Acad Sci USA **89:** 7747-7751

**Condon AG, Richards RA, Rebetzke GJ, Farquhar GD** (2002) Improving intrinsic water-use efficiency and crop yield. Crop Sci **42:** 122-131

**Coutu C, Brandle J, Brown D, Brown K, Miki B, Simmonds J, Hegedus DD** (2007) pORE: a modular binary vector series suited for both monocot and dicot plant transformation. Transgenic Res **16:** 771-781

**Davies WJ, Kudoyarova G, Hartung W** (2005) Long-distance ABA signaling and its relation to other signaling pathways in the detection of soil drying and the mediation of the plant's response to drought. J Plant Growth Regul **24:** 285-295

**Davies WJ, Zhang JH** (1991) Root signals and the regulation of growth and development of plants in drying soil. Annu Rev of Plant Physiol Plant Mol Biol **42:** 55-76

**Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP** (2002) Stable isotopes in plant ecology. Annu Rev Ecol Syst **33:** 507-559

**Ehleringer JR, Klassen S, Clayton C, Sherrill D, Fullerholbrook M, Fu QN, Cooper TA** (1991) Carbon isotope discrimination and transpiration Efficiency in common bean. Crop Sci **31:** 1611-1615

**Fan LM, Zhang W, Chen JG, Taylor JP, Jones AM, Assmann SM** (2008) Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ- and RGS-deficient *Arabidopsis* lines. Proc Natl Acad Sci USA **105:** 8476-8481

**Farquhar GD, Ehleringer JR, Hubick KT** (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **40:** 503-537

**Farquhar GD, Oleary MH, Berry JA** (1982) On the relationship between carbon isotope discrimination and the inter-cellular carbon-dioxide concentration in leaves. Aust J Plant Physiol **9:** 121-137

**Gookin TE, Kim J, Assmann SM** (2008) Whole proteome identification of plant candidate G-protein coupled receptors in *Arabidopsis*, rice, and poplar: computational prediction and in-vivo protein coupling. Genome Biol **9:** R120

**Hammer GL, Farquhar GD, Broad IJ** (1997) On the extent of genetic variation for transpiration efficiency in sorghum. Aust J of Agric Res **48:** 649-655

**Huang H, Weiss CA, Ma H** (1994) Regulated expression of the *Arabidopsis* G Protein α subunit gene *GPA1*. Int J Plant Sci **155:** 3

**Huang J, Taylor JP, Chen JG, Uhrig JF, Schnell DJ, Nakagawa T, Korth KL, Jones AM** (2006) The plastid protein THYLAKOID FORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar-signaling mechanism in *Arabidopsis*. Plant Cell **18:** 1226-1238

**Joo JH, Wang S, Chen JG, Jones AM, Fedoroff NV** (2005) Different signaling and cell death roles of heterotrimeric G protein α and β subunits in the *Arabidopsis* oxidative stress response to ozone. Plant Cell **17:** 957-970

**Juenger TE, Mckay JK, Hausmann N, Keurentjes JJB, Sen S, Stowe KA, Dawson TE, Simms EL, Richards JH** (2005) Identification and characterization of QTL underlying whole-plant physiology in *Arabidopsis thaliana*: $\delta^{13}C$, stomatal conductance and transpiration efficiency. Plant Cell and Environ **28:** 697-708

**Lake JA, Woodward FI** (2008) Response of stomatal numbers to $CO_2$ and humidity: control by transpiration rate and abscisic acid. New Phytol **179:** 397-404

**Lambrides CJ, Chapman SC, Shorter R** (2004) Genetic variation for carbon isotope discrimination in sunflower: association with transpiration efficiency and evidence for cytoplasmic inheritance. Crop Sci **44:** 1642-1653

**Lawson T** (2009) Guard cell photosynthesis and stomatal function. New Phytol **181:** 13-34

**Lease KA, Wen J, Li J, Doke JT, Liscum E, Walker JC** (2001) A mutant *Arabidopsis* heterotrimeric G-protein β subunit affects leaf, flower, and fruit development. Plant Cell **13:** 2631-2641

**Leymarie J, Lasceve G, Vavasseur A** (1998) Interaction of stomatal responses to ABA and $CO_2$ in *Arabidopsis thaliana*. Aust J Plant Physiol **25:** 785-791

**Leymarie J, Vavasseur A, Lasceve G** (1998) $CO_2$ sensing in stomata of *abi1-1* and *abi2-1* mutants of *Arabidopsis thaliana*. Plant Physiol Biochem **36:** 539-543

**Llorente F, Alonso-Blanco C, Sanchez-Rodriguez C, Jorda L, Molina A** (2005) ERECTA receptor-like kinase and heterotrimeric G protein from *Arabidopsis* are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Plant J **43:** 165-180

**Ma H, Yanofsky MF, Meyerowitz EM** (1990) Molecular cloning and characterization of *GPA1*, a G protein α subunit gene from *Arabidopsis thaliana*. Proc Natl Acad Sci USA **87:** 3821-3825

**Masle J, Gilmore SR, Farquhar GD** (2005) The ERECTA gene regulates plant transpiration efficiency in Arabidopsis. Nature **436:** 866-870

**McKay JK, Richards JH, Nemali KS, Sen S, Mitchell-Olds T, Boles S, Stahl EA, Wayne T, Juenger TE** (2008) Genetics of drought adaptation in *Arabidopsis thaliana* II. QTL analysis of a new mapping population, Kas-1 X Tsu-1. Evolution **62:** 3014-3026

**Moriyama E, Strope P, Opiyo S, Chen Z, Jones A** (2006) Mining the *Arabidopsis thaliana* genome for highly-divergent seven transmembrane receptors. Genome Biol **7:** R96

**Pandey S, Assmann SM** (2004) The *Arabidopsis* putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signaling. Plant Cell **16:** 1616-1632

**Pandey S, Chen JG, Jones AM, Assmann SM** (2006) G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. Plant Physiol **141:** 243-256

**Pandey S, Nelson DC, Assmann SM** (2009) Two Novel GPCR-Type G Proteins are abscisic acid receptors in *Arabidopsis*. Cell **136:** 136-148

**Perfus-Barbeoch L, Jones AM, Assmann SM** (2004) Plant heterotrimeric G protein function: insights from *Arabidopsis* and rice mutants. Curr Opin Plant Biol **7:** 719-731

**Rebetzke GJ, Condon AG, Richards RA, Farquhar GD** (2002) Selection for reduced carbon isotope discrimination increases aerial biomass and grain yield of rainfed bread wheat. Crop Sci **42:** 739-745

**Richards RA** (2006) Physiological traits used in the breeding of new cultivars for water-scarce environments. Agric Water Manage **80:** 197-211

**Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D** (2001) Guard cell signal transduction. Annu Rev Plant Physiol Plant Mol Biol **52:** 627-658

**Schlüter U, Muschak M, Berger D, Altmann T** (2003) Photosynthetic performance of an *Arabidopsis* mutant with elevated stomatal density (*sdd1-1*) under different light regimes. J Exp Bot **54:** 867-874.

**Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL** (2007) Fitting photosynthetic carbon dioxide response curves for C-3 leaves. Plant Cell Environ **30:** 1035-1040

**Shpak ED, Berthiaume CT, Hill EJ, Torii KU** (2004) Synergistic interaction of three ERECTA-family receptor-like kinases controls *Arabidopsis* organ growth and flower development by promoting cell proliferation. Development **131:** 1491-1501

**Shpak ED, McAbee JM, Pillitteri LJ, Torii KU** (2005) Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science **309:** 290-293

**Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, Komeda Y** (1996) The Arabidopsis *ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell **8:** 735-746

**Trusov Y, Rookes JE, Chakravorty D, Armour D, Schenk PM, Botella JR** (2006) Heterotrimeric G proteins facilitate Arabidopsis resistance to necrotrophic pathogens and are involved in jasmonate signaling. Plant Physiol **140:** 210-220

**Trusov Y, Rookes JE, Chakravorty D, Armour D, Schenk PM, Botella JR** (2006) Heterotrimeric G proteins facilitate *Arabidopsis* resistance to necrotrophic pathogens and are involved in jasmonate signaling. Plant Physiol. **140:** 210-220

**Trusov Y, Zhang W, Assmann SM, Botella JR** (2008) Gγ1 + Gγ2 not equal to Gβ: heterotrimeric G protein Gγ-deficient mutants do not recapitulate all phenotypes of Gβ-deficient mutants. Plant Physiol **147:** 636-649

**Ullah H, Chen JG, Young JC, Im KH, Sussman MR, Jones AM** (2001) Modulation of cell proliferation by heterotrimeric G protein in *Arabidopsis*. Science **292:** 2066-2069

**Vavasseur A, Raghavendra AS** (2005) Guard cell metabolism and $CO_2$ sensing. New Phytol **165:** 665-682

**Virgona JM, Hubick KT, Rawson HM, Farquhar GD, Downes RW** (1990) Genotypic variation in transpiration efficiency, carbon-isotope discrimination and carbon allocation during early growth in sunflower. Aust J Plant Physiol **17:** 207-214

**Wang SY, Assmann SM, Fedoroff NV** (2008) Characterization of the *Arabidopsis* heterotrimeric G protein. J Biol Chem **283:** 13913-13922

**Wang XQ, Ullah H, Jones AM, Assmann SM** (2001) G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. Science **292:** 2070-2072

**Warpeha KM, Upadhyay S, Yeh J, Adamiak J, Hawkins SI, Lapik YR, Anderson MB, Kaufman LS** (2007) The GCR1, GPA1, PRN1, NF-Y signal chain mediates both blue light and abscisic acid responses in *Arabidopsis*. Plant Physiol **143:** 1590-1600

**Webb AA, Hetherington AM** (1997) Convergence of the abscisic acid, $CO_2$, and extracellular calcium signal transduction pathways in stomatal guard cells. Plant Physiol **114:** 1557-1560

**Weiss CA, Huang H, Ma H** (1993) Immunolocalization of the G protein α subunit encoded by the *GPA1* gene in *Arabidopsis*. Plant Cell **5:** 1513-1528

**Weiss CA, White E, Huang H, Ma H** (1997) The G protein α subunit (GPA1) is associated with the ER and the plasma membrane in meristematic cells of *Arabidopsis* and cauliflower. FEBS Lett **407:** 361-367

**Wong SC, Cowan IR, Farquhar GD** (1979) Stomatal conductance correlates with photosynthetic capacity. Nature **282:** 424-426

**Zhang L, Hu G, Cheng Y, Huang J** (2008) Heterotrimeric G protein α and β subunits antagonistically modulate stomatal density in *Arabidopsis thaliana*. Dev Biol **324:** 68-75

**Zhang W, He SY, Assmann SM** (2008) The plant innate immunity response in stomatal guard cells invokes G-protein-dependent ion channel regulation. Plant J **56:** 984-996

**Zhao Z, Stanley B, Zhang W, Assmann SM** (submitted) ABA-regulated G protein signaling in Arabidopsis guard cells: a proteomic perspective. Submitted to J Proteome Res

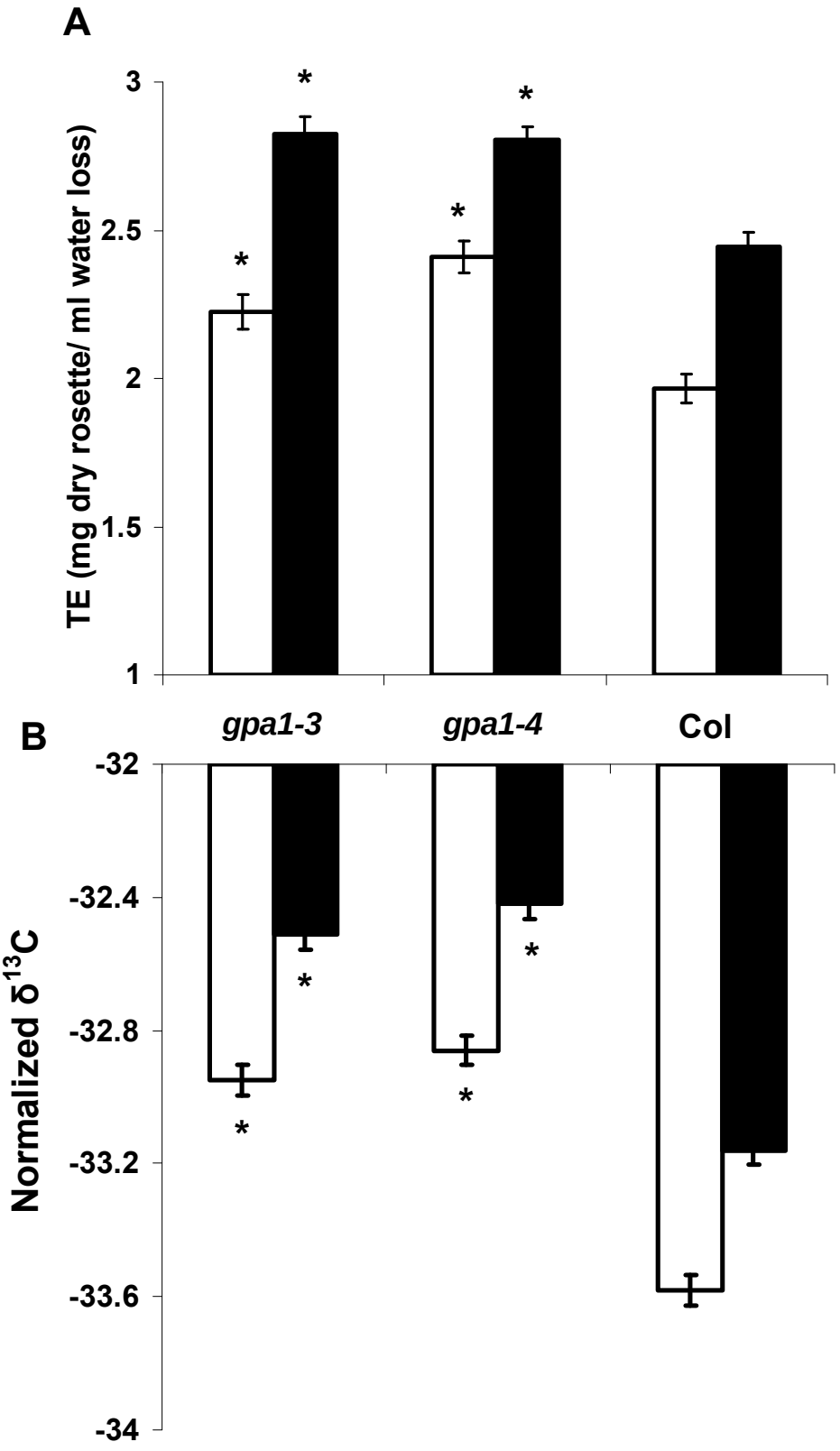



**Figure 1**. *gpa1* mutants have increased TE and increased $\delta^{13}C$ (reduced discrimination) compared to Col. Mean TE (A) and mean normalized $\delta^{13}C$ values of rosette tissue (B) of *gpa1* and Col under ample water (open bars) and drought stress (black bars) conditions. Error bars represent standard error. Asterisks indicate mean differs significantly from the mean of Col within the treatment ($p < 0.05$).

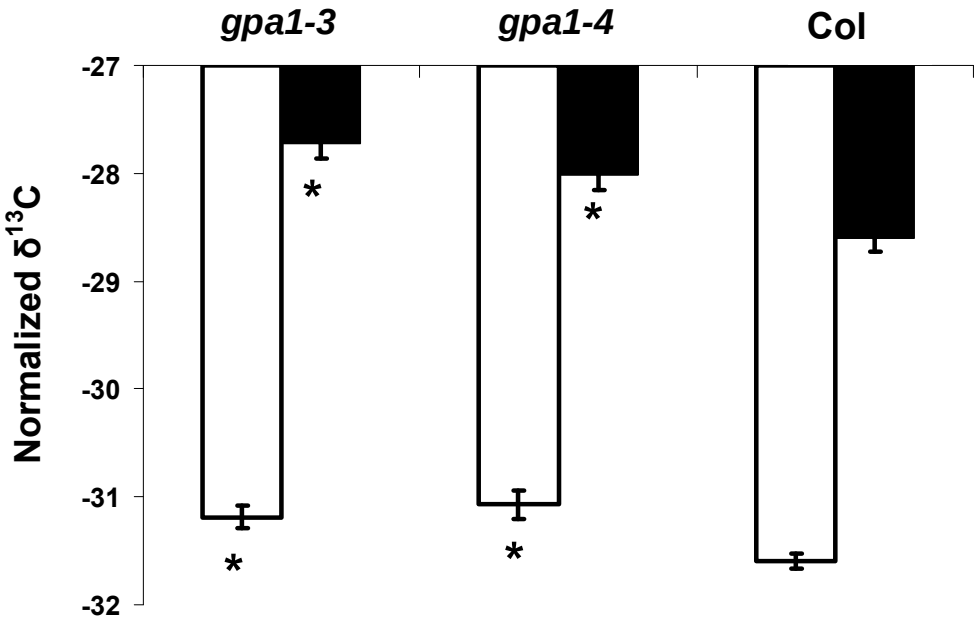
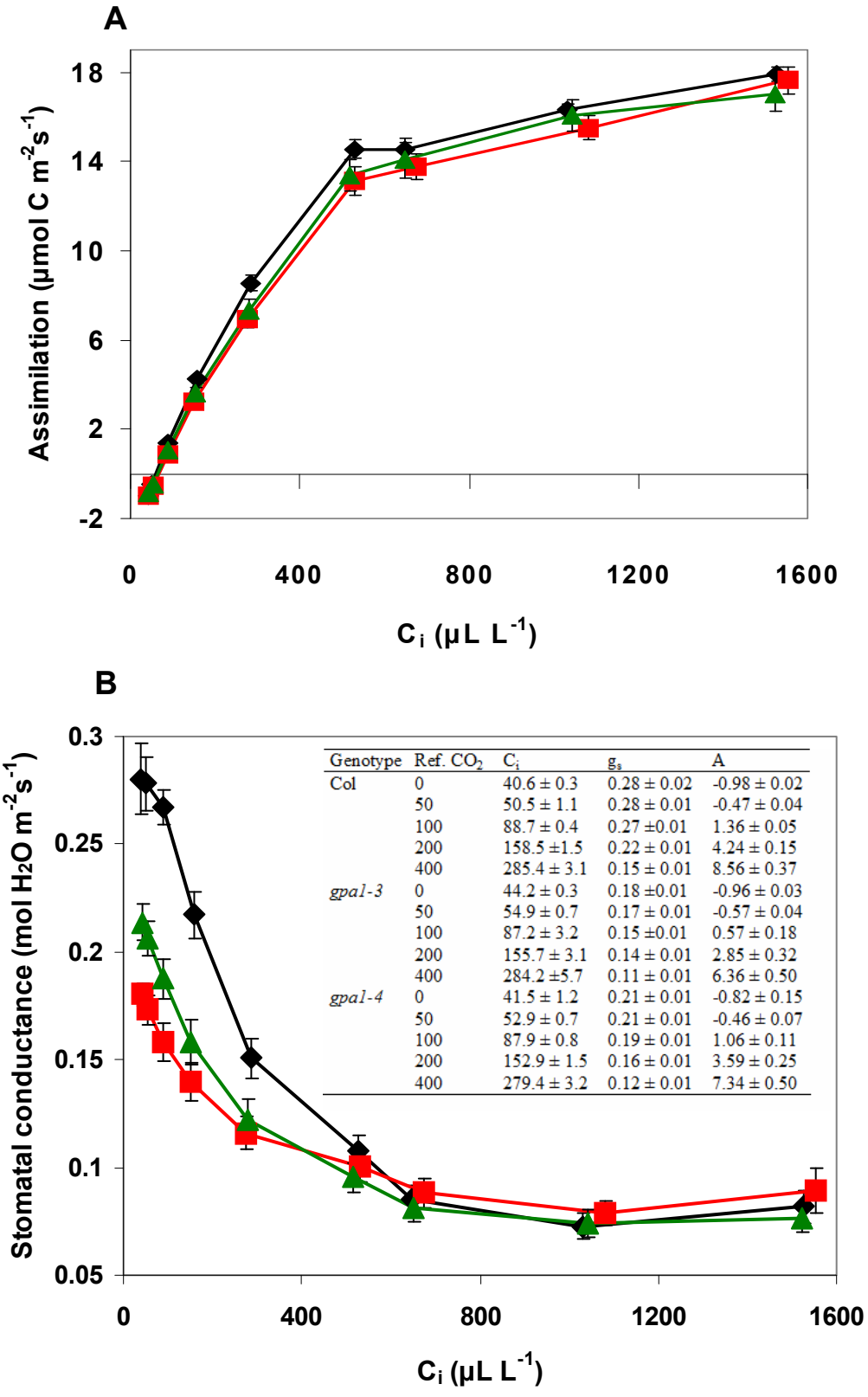



**Figure 2**. *gpa1* mutants have increased $\delta^{13}C$ (reduced discrimination) in the absence and presence of ABA as compared to Col. Mean normalized $\delta^{13}C$ values of rosette tissue from *gpa1* and Col plants treated with no ABA (open bars) or 25 μM ABA (black bars). Error bars represent standard error. Asterisks indicate means differ significantly from mean of Col ($p < 0.05$).




| Genotype | Ref. $CO_2$ | $C_i$ | $g_s$ | A |
|---|---|---|---|---|
| Col | 0 | 40.6 ± 0.3 | 0.28 ± 0.02 | -0.98 ± 0.02 |
| | 50 | 50.5 ± 1.1 | 0.28 ± 0.01 | -0.47 ± 0.04 |
| | 100 | 88.7 ± 0.4 | 0.27 ±0.01 | 1.36 ± 0.05 |
| | 200 | 158.5 ±1.5 | 0.22 ± 0.01 | 4.24 ± 0.15 |
| | 400 | 285.4 ± 3.1 | 0.15 ± 0.01 | 8.56 ± 0.37 |
| *gpa1-3* | 0 | 44.2 ± 0.3 | 0.18 ±0.01 | -0.96 ± 0.03 |
| | 50 | 54.9 ± 0.7 | 0.17 ± 0.01 | -0.57 ± 0.04 |
| | 100 | 87.2 ± 3.2 | 0.15 ±0.01 | 0.57 ± 0.18 |
| | 200 | 155.7 ± 3.1 | 0.14 ± 0.01 | 2.85 ± 0.32 |
| | 400 | 284.2 ±5.7 | 0.11 ± 0.01 | 6.36 ± 0.50 |
| *gpa1-4* | 0 | 41.5 ± 1.2 | 0.21 ± 0.01 | -0.82 ± 0.15 |
| | 50 | 52.9 ± 0.7 | 0.21 ± 0.01 | -0.46 ± 0.07 |
| | 100 | 87.9 ± 0.8 | 0.19 ± 0.01 | 1.06 ± 0.11 |
| | 200 | 152.9 ± 1.5 | 0.16 ± 0.01 | 3.59 ± 0.25 |
| | 400 | 279.4 ± 3.2 | 0.12 ± 0.01 | 7.34 ± 0.50 |

**Figure 3**. *gpa1* mutants show essentially wild type A-$C_i$ but altered $g_s$-$C_i$ responses. Photosynthesis (A) and stomatal conductance (B) of *gpa1-3* (squares), *gpa1-4* (triangles) and Col (diamonds) at different internal $CO_2$ concentrations (inset $C_i$, $g_s$, and A means and standard errors for the lowest 5 external $CO_2$ concentrations for all genotypes); significant differences are observed for $g_s$ of *gpa1* vs. Col for $g_s$ at the 5 lowest $C_i$s ($p < 0.05$).

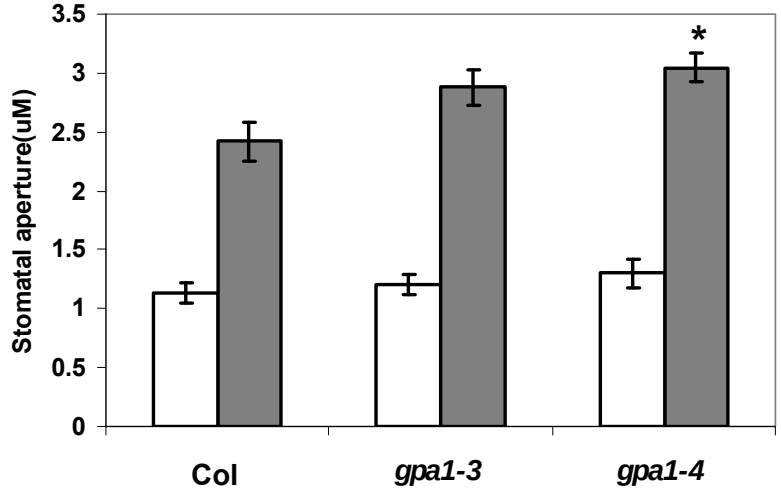



**Figure 4.** *gpa1* mutants are hypersensitive to low $CO_2$-induced stomatal opening. Mean stomatal apertures from epidermal peels after incubation of leaves in low $CO_2$ buffer (gray bars) or ambient $CO_2$ buffer (open bars). Errors bars represent standard errors and asterisk indicates a significant difference from Col. $p = 0.06$ for *gpa1-3* and $p = 0.008$ for *gpa1-4* for *gpa1* low $CO_2$ aperture size vs. Col low $CO_2$ aperture size.

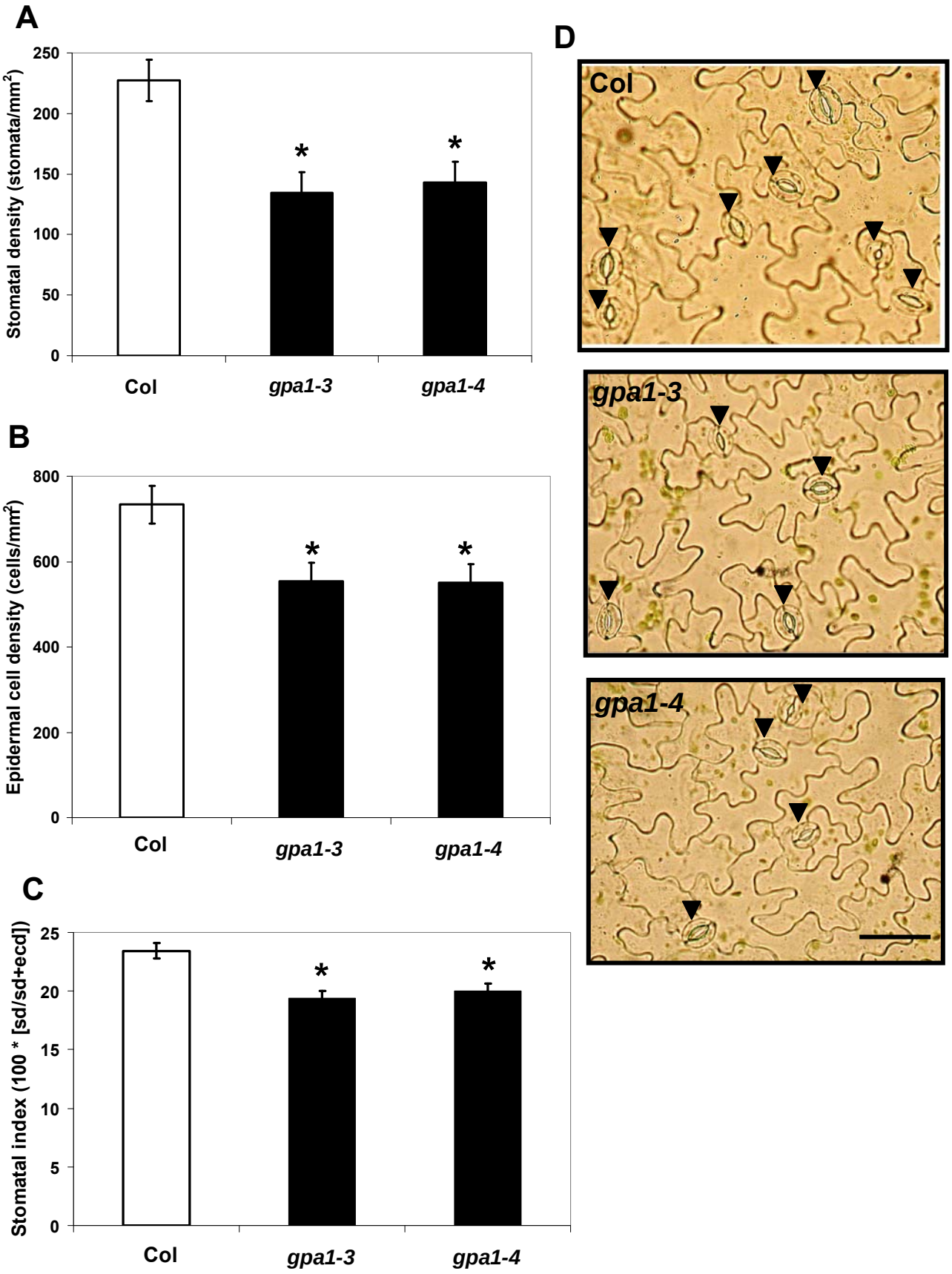



**Figure 5.** *gpa1* mutants have reduced stomatal density (A), epidermal cell density (B) and stomatal index (C). Shown are mean values with error bars indicating standard error. Asterisks indicate the mean differs significantly from the Col mean ($p < 0.05$). Photographs of representative epidermal peels (D) of Col and *gpa1* mutants; arrowheads indicate stomata, scale bar = 50 μm.

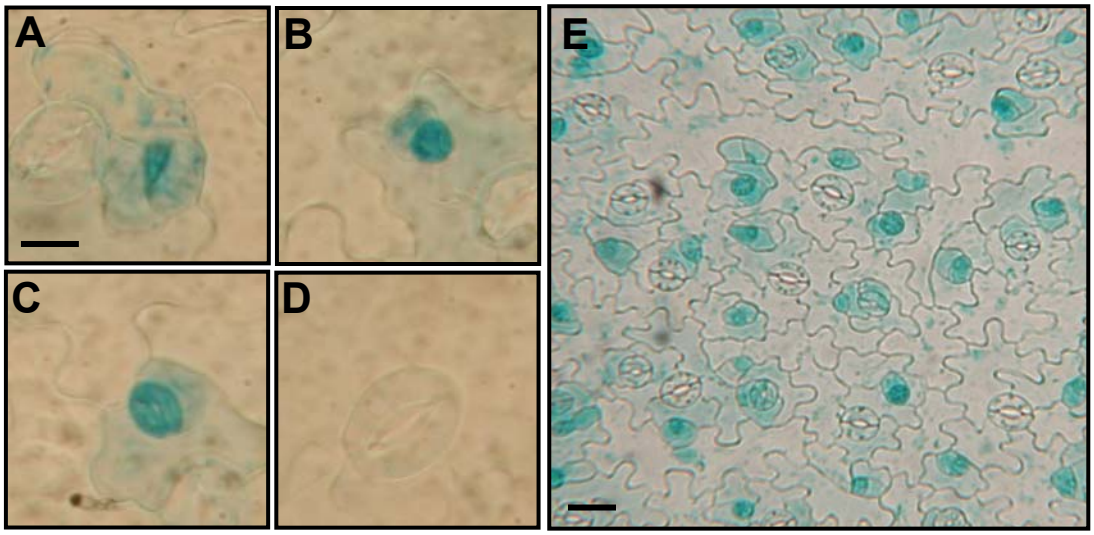



**Figure 6.** *GPA1* prom :: GUS activity is observed in stomatal precursor cells and immature stomata. Images are from an epidermal peel of a true leaf of a 15 day old soil grown seedling. (A) meristemoid; (B) guard mother cell; (C) immature stomate; (D) mature stomate, scale bar = 10 μm; (E) GUS activity in developing stomatal complexes and neighboring epidermal cells, scale bar = 20 μm.

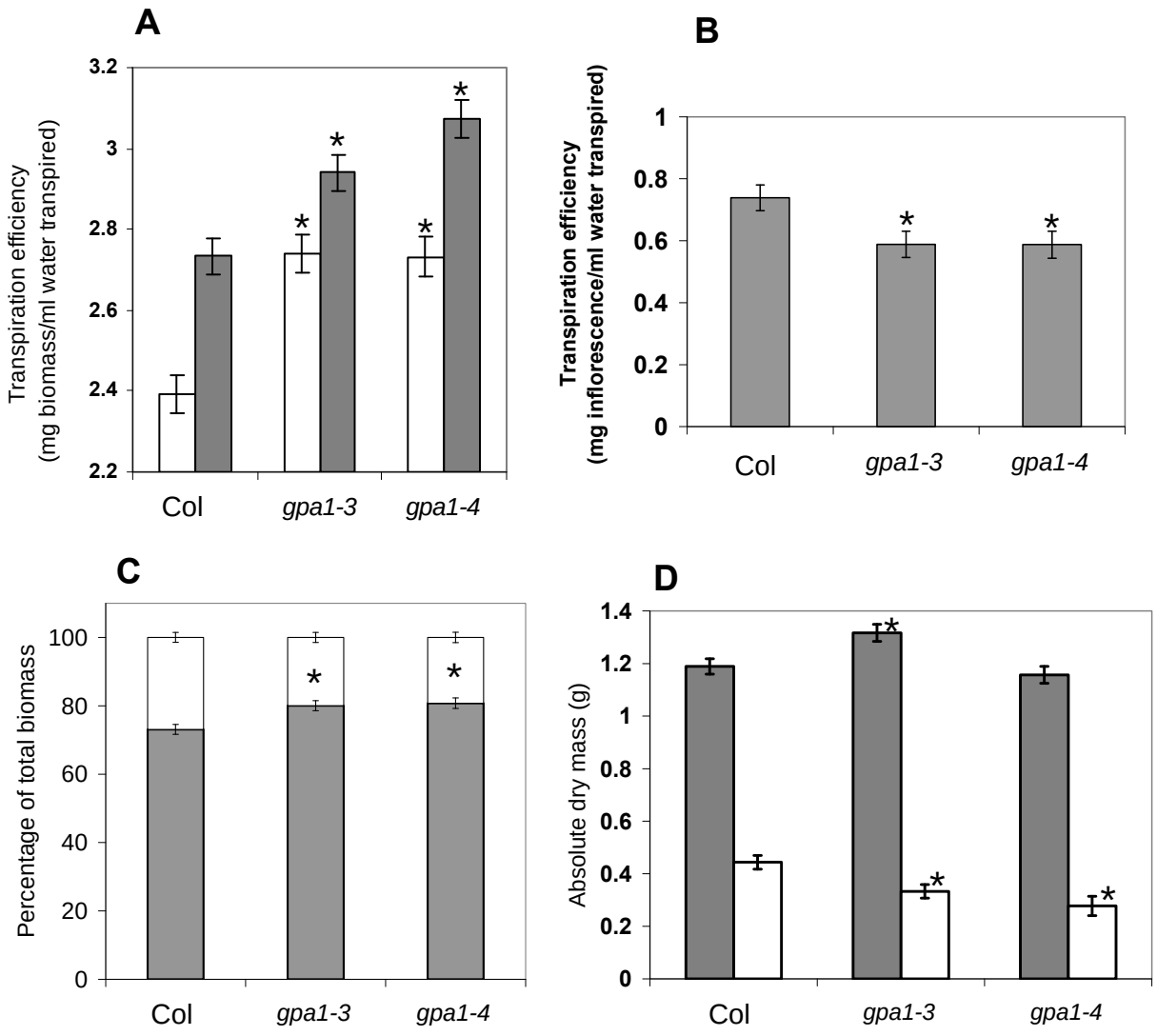
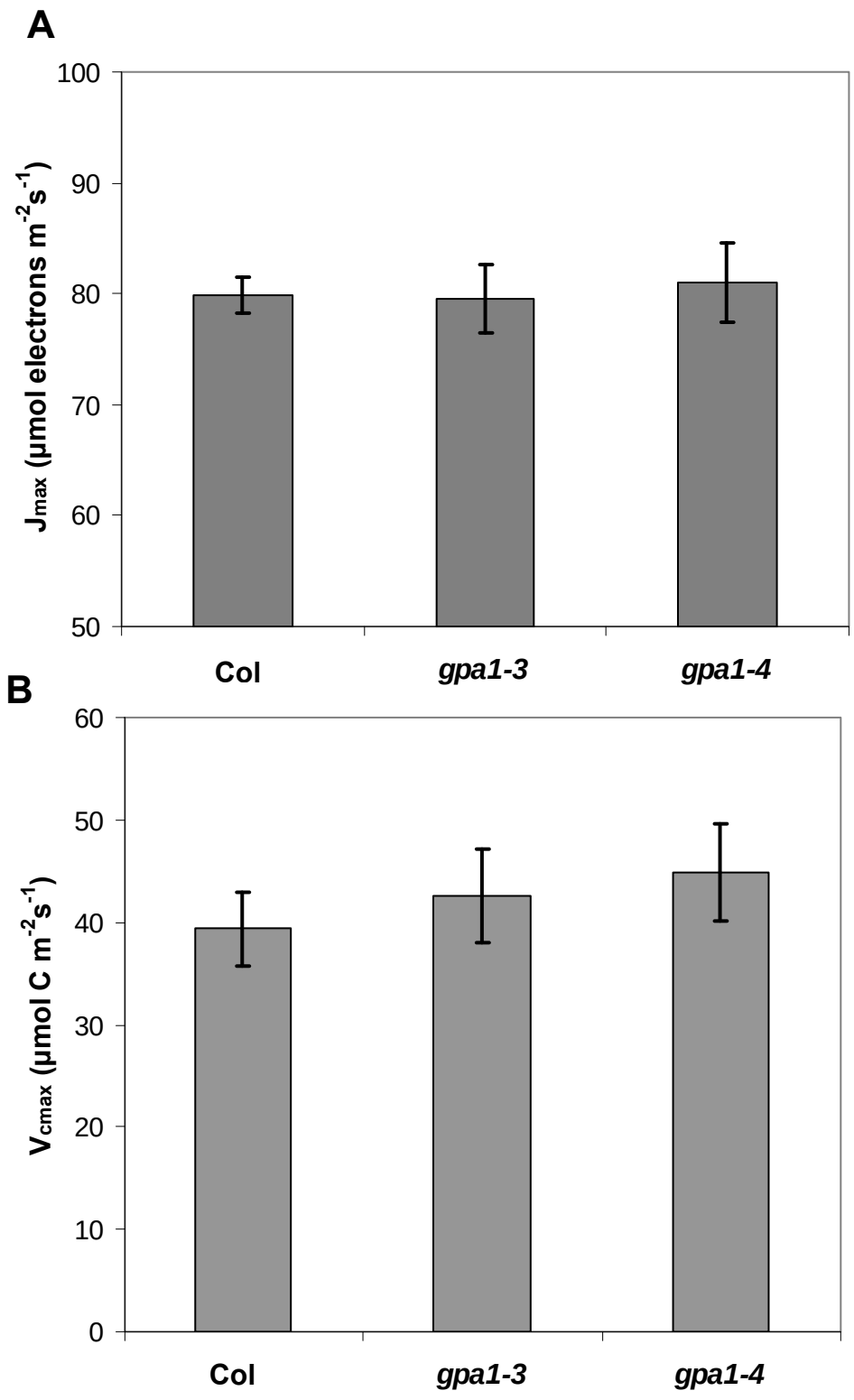
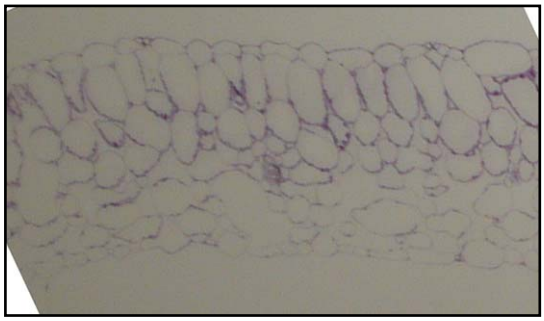



**Figure 7**. *gpa1* mutants have increased TE before and after flowering but reduced inflorescence TE and reduced biomass allocation to the inflorescence compared to Col. TE (A) of Col and *gpa1* mutants before flowering (open bars) and after flowering (gray bars), inflorescence TE (B) of Col and *gpa1* mutants, and biomass partioning (C) and absolute biomass (D) for Col and *gpa1* mutants for rosette (gray bars) and inflorescence tissues (open bars). Errors bars represent standard error and asterisks indicate significant mean differences from Col.

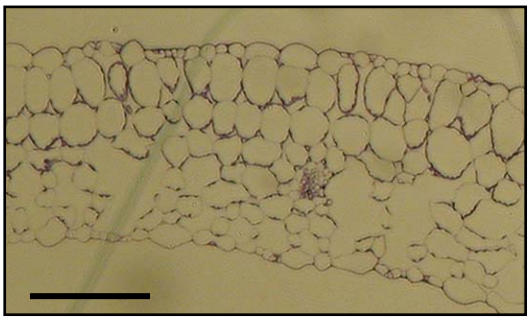



**Supplemental Figure 1**. *gpa1* mutants do not significantly differ from Col for $J_{max}$ (A) or $V_{cmax}$ (B). Error bars represent standard error.

Col



*gpa1-3*



**Supplemental Figure 2**. There are no obvious differences in leaf thickness or anatomy between Col and *gpa1-3*. Scale bar = 100 μm.

**Supplemental Table 1**. ANOVA table for ample water/ drought TE experiment

| Source | d.o.f. | Mean Square | F-statistic | p-value |
|---|---|---|---|---|
| Genotype | 2 | 1.650 | 38.81 | <.001 |
| Water level | 1 | 5.873 | 138.16 | <.001 |
| Block | 6 | 0.103 | 2.41 | 0.033 |
| Genotype x water level | 2 | 0.081 | 1.90 | 0.156 |
| Error | 91 | 0.0425 | | |

**Supplemental Table 2**. ANOVA table for ample water/ drought $\delta^{13}C$ analysis

| Source | d.o.f. | Mean Square | F-statistic | p-value |
|---|---|---|---|---|
| Genotype | 2 | 4.4590 | 158.76 | <.001 |
| Water level | 1 | 3.8378 | 136.64 | <.001 |
| Block | 6 | 0.4253 | 15.14 | <.001 |
| Genotype x water level | 2 | 0.0016 | 0.06 | 0.946 |
| Error | 82 | 0.0281 | | |

**Supplemental Table 3**. ANOVA table for ABA treatment $\delta^{13}C$ analysis

| Source | d.o.f. | Mean Square | F-statistic | p-value |
|---|---|---|---|---|
| Genotype | 2 | 2.895 | 15.50 | <.001 |
| ABA treatment | 1 | 175.298 | 938.82 | <.001 |
| Block | 1 | 0.001 | 0.00 | 0.955 |
| Error | 65 | 0.187 | | |

**Supplemental Table 4**. ANOVA table for TE before flowering

| Source | d.o.f. | Mean Square | F-statistic | p-value |
|---|---|---|---|---|
| Genotype | 2 | 0.864 | 17.30 | <.001 |
| Block | 3 | 0.089 | 1.78 | 0.160 |
| Error | 60 | 0.050 | | |

**Supplemental Table 5**. ANOVA table for TE after flowering

| Source | d.o.f. | Mean Square | F-statistic | p-value |
|---|---|---|---|---|
| Genotype | 2 | 0.666 | 14.34 | <.001 |
| Block | 3 | 0.363 | 7.81 | <.001 |
| Error | 63 | 0.046 | | |

**Supplemental Table 6.** ANOVA table for inflorescence TE

| Source | d.o.f. | Mean Square | F-statistic | p-value |
|---|---|---|---|---|
| Genotype | 2 | 0.178 | 4.29 | 0.018 |
| Block | 3 | 0.185 | 4.46 | 0.007 |
| Error | 63 | 0.041 | | |

**Supplemental Table 7**. ANOVA table for percent of total biomass allocated to inflorescence

| Source | d.o.f. | Mean Square | F-statistic | p-value |
|---|---|---|---|---|
| Genotype | 2 | 417.77 | 8.27 | <.001 |
| Block | 3 | 142.75 | 2.83 | 0.046 |
| Error | 63 | 50.49 | | |

# Chapter 5:
# Testing for GPA1 interaction with ERECTA and other Arabidopsis regulators of stomatal development


## ABSTRACT

GPA1 has been recently identified as a regulator of transpiration efficiency and stomatal density in Arabidopsis (Chapter 4). To better understand the mechanism by which GPA1 regulates transpiration efficiency yeast-based and in vitro biochemical assays were performed to test if GPA1 physically interacted with the only other known regulator of transpiration efficiency, ERECTA, as well as with other regulators of stomatal development. While some yeast-based assays supported an interaction between GPA1 and ERECTA this interaction could not be confirmed using additional biochemical or genetic approaches. Additionally, no significant interactions were observed between GPA1 and the other known regulators of stomatal development. Further investigation is warranted in order to understand the explicit mechanism by which GPA1 regulates stomatal development.


## INTRODUCTION

Stomata are small pores found on the aerial surfaces of plants where gas exchange occurs between the plant and the atmosphere. One of first identified and most extensively studied functions of heterotrimeric G proteins in plants is in the regulation of stomatal aperture size (reviewed in Chapter 3). Additionally, heterotrimeric G proteins have been recently implicated as regulators of stomatal density and development (Chapter 4, Zhang et al., 2008), however the mechanism of this regulation is unknown.

Stomata form from stomatal cell lineages which are found in developing tissues. The lineage begins when a meristematic epidermal cell, a meristemoid mother cell, undergoes an asymmetric division called an entry division which produces a larger daughter cell and a smaller, triangular-shaped meristemoid cell. Meristemoids eventually convert into oval-shaped guard mother cells which each undergo one symmetric division to form the guard cells of a stomate. Prior to this conversion, meristemoids can undergo additional asymmetric divisions (two or three usually) called amplifying divisions which

form additional daughter cells while at the same time regenerating a meristemoid. Amplifying divisions result in an increase in the number of epidermal cells since daughter cells either become epidermal cells or undergo spacing divisions. Spacing divisions occur when cells adjacent to meristemoids, guard mother cells, or guard cells become meristemoid mother cells and asymmetrically divide so that the new meristemoid forms away from the existing guard cell or guard cell precursor. Spacing divisions are responsible for maintaining the single cell spacing rule found in wild type Arabidopsis namely that there is always at least one epidermal cell in between two stomata (Bergmann and Sack, 2007; Nadeau, 2009).

A number of genes have been identified that regulate stomatal density and patterning in Arabidopsis (Bergmann and Sack, 2007; Nadeau, 2009). Putative functions and localizations of these gene products combined with genetic interaction studies have led to the formation of a stomatal development model for Arabidopsis. A signal, possibly the small secreted peptide EPF, may be modified by the putative protease, SDD1, and serves as a mobile positional signal from a stomate or a stomatal precursor cell (Berger and Altmann, 2000; Von Groll et al., 2002; Hara et al., 2007). EPF could hypothetically activate a receptor complex at the cell membrane which includes TMM (a putative receptor-like protein), ERECTA, (LRR-RLK), ERL1 (LRR-RLK), ERL2 (LRR-RLK), but note that formation of a complex between these proteins has never been biochemically investigated (Yang and Sack, 1995; Nadeau and Sack, 2002; Masle et al., 2005; Shpak et al., 2005). Downstream of the proposed receptor complex activation is a MAP kinase signaling cascade consisting of YODA (MAP kinase) and MKK4/5 (MAP kinase kinase), and MPK3/6 (MAP kinase kinase kinase) (Bergmann et al., 2004; Wang et al., 2007). Recently it has been found that MPK3/6 can directly phosphorylate the bHLH transcription factor SPEECHLESS thereby negatively regulating its activity (Lampard et al., 2008). SPEECHLESS is a positive regulator of stomatal development and is required for the initial asymmetric cell division of the protodermal cell (MacAlister et al., 2007; Pillitteri et al., 2007). MUTE, a transcription factor related to SPEECHLESS, is required for the transition of a meristemoid into a guard mother cell (MacAlister et al., 2007; Pillitteri et al., 2007). The transcription factors FAMA, FLP,

and MYB88 are required to end symmetric division of the guard mother cell (Lai et al., 2005; Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007).

We have previously shown that *GPA1* is expressed in developing guard cells and functions as a positive regulator of stomatal density by modulating cell expansion and stomata formation in rosette leaves (Chapter 4). Additionally, it has been recently reported that GPA1 and AGB1 antagonistically regulate stomata formation in cotyledons, with GPA1 serving as a positive regulator and AGB1 as a negative regulator of stomatal development (Zhang et al., 2008). *gpa1* mutant cotyledons show a lower proportion of higher order stomatal complexes suggesting that amplifying and spacing divisions are attenuated in *gpa1* (Zhang et al., 2008). Conversely, cotyledons of *agb1* mutants showed increased stomatal density and increased proportion of higher order stomatal complexes. Cotyledons of the double mutant *gpa1agb1* showed an intermediate phenotype for stomatal density in cotyledons (Zhang et al., 2008). Zhang et al. (2008) also claim that *MUTE* and *SPEECHLESS* are down regulated in *gpa1* and up regulated in *agb1*. However, it is difficult to conclude this from the gel image of the RT-PCR analysis provided (Zhang et al. 2008, Figure 4). Given the low resolution and non-quantitative nature of gel-based RT-PCR, I believe this conclusion is premature.

Because ERECTA and heterotrimeric G proteins are signaling proteins that have either putative or confirmed membrane localizations and share a number of mutant phenotypes, ERECTA and G protein subunits may function in the same pathway(s) and could potentially interact with one another (Torii et al., 1996; Weiss et al., 1997; Obrdlik et al., 2000; Lease et al., 2001; Llorente et al., 2005; Masle et al., 2005; Shpak et al., 2005). Our identification of GPA1 as a regulator of transpiration efficiency and stomatal development (Chapter 4) further supported a potential interaction, thus, we explored this possibility using biochemical and genetic approaches.

The split-ubiquitin system (SUS) is a yeast-based system that can be used to detect interactions between membrane-associated proteins (Johnsson and Varshavsky, 1994; Obrdlik et al., 2004; Thaminy et al., 2004). Briefly, protein 1 is fused to the C-terminal half of ubiquitin and a transcription factor (XCub-PLV, where X is the protein, Cub is C-terminal ubiquitin, and PLV is the transcription factor). Protein 2 is fused to the N-terminal half of ubiquitin (XNub or NubX, where X is the protein and Nub is N-

terminal ubiquitin). When protein 1 and protein 2 are expressed together in yeast the two halves of ubiquitin spontaneously reassemble and are recognized by ubiquitin-specific proteases (USPs). USPs then cleave the transcription factor from the fusion protein allowing the transcription factor to relocate to the nucleus and activate reporter genes (*Ade2, His3, lacZ)* which can be detected using growth and X-gal assays. When a mutated form of N-terminal ubiquitin (XNubG or NubGX) is coexpressed with an XCub-PLV the two ubiquitin halves can reassemble only when in close proximity with each other (e.g., when proteins 1 and 2 interact). Methionine (Met) is a repressor of the promoter driving the expression of XCub-PLV so the strength of the interaction can be assayed by growing the diploid yeast on media containing different levels of Met.

The SUS assay was used to examine putative interactions of GPA1 and AGB1 with other membrane localized stomatal development regulators, specifically, ERECTA, ERL1, ERL2, TMM, and SDD1. Very little biochemistry has been performed on these stomatal development regulators so potential interactions among the known stomatal development regulators were also examined using SUS. While SUS experiments identified a positive interaction between GPA1 and ERECTA, this interaction could not be confirmed by co-immunoprecipitation (co-IP) or kinase assays, or by SUS assays using different vectors. Attempts to examine the genetic interaction of *GPA1* and *ERECTA* were also unsuccessful; linkage between the two loci prevented the isolation of *gpa1er* recombinants. Finally, assays examining interactions among additional stomatal development regulators found evidence for heterodimerization only among the ERECTA family of receptor-like kinases.

## MATERIALS AND METHODS

### Split ubiquitin assays

#### *Generation of ERECTA Cub and Nub fusions*

The entire open reading frame of *ERECTA* was PCR amplified using Pfx Supermix (Invitrogen) with split-ubiquitin system linker adapted primers (Table 1 for sequences) that included the translation start codon with no translation stop codon. The PCR product was A-tailed using ExTaq (Takara) and was cloned into the TOPO pCR2.1 (Invitrogen) vector and sequenced. SUS clones for *GPA1*, *AGB1*, and *GCR1* were

provided by Tim Gookin, Dr. Zhixin Zhao, and Anne Gibson. The linker-adapted ORFs were excised from the vectors via restriction digest and gel-purified. The Cub and Nub fusions were generated via homologous recombination with linearized Cub and Nub split ubiquitin vectors. 100 ng of insert and 100 ng of linearized vector were co-transformed into chemically competent haploid AP4 or AP5 yeast using the Yeast Maker II (Clonetech) yeast transformation protocol. Transformants were selected on the appropriate media. AP4 strains containing Cub fusions were mated with AP5 strains containing Nub fusions for 6 to 8 hours on YPD media. Diploid yeast were selected on drop out media and then replica-plated on SD containing 0, 200 μM, or 1 mM of Met. Yeast growth was assayed after 5 days of growth at 28°C.

***Generation of ERECTA, ERL1, ERL2, TMM1, and SDD1 Gateway Cub and Nub fusions***

The open reading frames of *ERECTA*, *ERL1*, *ERL2*, *TMM1*, and *SDD1* excluding a translational stop codon (primer sequences in Table 2) were amplified using Pfx supermix (Invitrogen), A-tailed with ExTaq (Takara), cloned into the Gateway entry vector pCR4 TOPO (Invitrogen) and sequenced. Clonase II LR (Invitrogen) reactions were performed using 150 ng of each entry clone with 150 ng of each Gateway compatible split-ubiquitin destination vector to generate the Cub and Nub fusions. *GPA1* and *AGB1* entry vectors were provided by Dr. Biswa Archaya. The constructs were transformed into yeast and the split-ubiquitin assay was performed as described above except the Met concentrations for the growth assays were 0 μM, 50 μM, and 150 μM as recommended by Dr. Archaya.

***lacZ activity assay***

*lacZ* reporter gene activity was assayed using an X-gal filter assay. Sterile Whatman filters were placed on SC -Leu/-Trp media and diploid yeast was streaked on the filter. After 2 days of yeast growth the cells were lysed in liquid nitrogen twice for 10 seconds. The filters were incubated in X-gal buffer (100 mM sodium phosphate buffer pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 0.27% β-mercaptoethanol, 1.4 mg/ml X-gal in DMF) for 1 hour in the dark.

## Co-IP experiments

### *In-vitro protein expression*

The cDNA encoding the kinase domain of *ERECTA* (ERECTAkd), corresponding to amino acids 610-976, was amplified from seedling cDNA using Platinum High Fidelity PCR Supermix (Invitrogen) with primers containing EcoR1 (F) and SalI (R) restriction site extensions (primer sequences in Table 3). The PCR product was gel purified, blunt cloned into TOPO Blunt II vector, and sequenced. The insert was recovered by restriction digest with EcoR1 and SalI, gel purified, and cloned into the bacterial N-terminal His expression vector pET28a. The expression vector was sequenced and transformed into BL21-A1 cells. His-ERECTAkd protein expression was induced with 1 mM IPTG and 0.2% L-arabinose for 4 hours. GPA1 in the N-terminal FLAG tagged vector pT7 was provided by Dr. Zhixin Zhao. pT7-GPA1 was transformed into BL21 DE3 cells. FLAG-GPA1 expression was induced with 1 mM IPTG for 2 hours. Induced cells were spun down and stored at -20C. Protein expression was confirmed via Western blotting with the appropriate anti-His or anti-FLAG antibodies.

His-ERECTAkd was batch purified using Ni-NTA resin (Novagen) for the co-IP experiment using leaf microsomal protein. The induced bacterial cell pellet was thawed and resuspended in 5 mL of BugBuster protein extraction reagent (Novagen). Five units of rLysozyme (Novagen) was added and the mixture was incubated on a rotator for 20 minutes at room temperature. The mixture was spun down for 20 minutes at 19,000g and the cleared lysate was applied to 1 ml of Ni-NTA slurry prepared with binding buffer (300 mM NaCl, 50mM sodium phosphate buffer, 10 mM imidazole, pH 8.0) according to the manufacturer's instructions and incubated at 4°C with shaking for 1 hour. The slurry was transferred to a resin column and washed twice with Ni-NTA wash buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0) and eluted four times with Ni-NTA elution buffer (300 mM NaCl, 250 mM imidazole, 50 mM sodium phosphate buffer, pH 8.0). The elution fractions were aliquoted and stored at -80°C.

### *Microsomal protein isolation from Col and gpa1 leaves*

30 leaves (for *gpa1-3*) and 60 leaves (for Col) were harvested from the rosettes of 5 week old plants grown under short day light conditions (120 $\mu molm^{-2}s^{-1}$). The leaves were ground in a cold mortar and pestle in buffer (250 mM Tris pH 8.0, 300 mM sucrose,

10 mM EDTA, 1 mM DTT, 0.5% insoluble PVP, 1 mM PMSF, with 1 X protease inhibitor cocktail). The mixture was filtered through cheesecloth and spun at 15,000g to isolate total protein. Total protein was spun at 100,000g for 45 minutes in an ultracentrifuge to pellet membranes. The soluble fraction was removed and stored at -80°C. The membrane pellet was homogenized in 200 µl (*gpa1-3*) or 400 µl (Col) of buffer ( 50mM Tris pH 7.5, 300 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5% PVP, and 1X protease inhibitor cocktail).

***Bacterial lysate co-IP***

Frozen induced bacterial pellets were thawed and resuspended in 5 mL of lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100 ). The mixture was strongly vortexed and incubated at 4°C for 30 minutes with shaking to promote cell lysis. The protein mixture was spun at 21,000g and the soluble protein was removed. To remove proteins that bound nonspecifically to the Protein A Sepharose resin (GE Healthcare), 600 µl of each protein extract was incubated with 40 µl of washed resin for 1 hour. The resin was pelleted and supernatants were transferred to a fresh tube. Anti-His or Anti-FLAG antibody was added to a final concentration of 5 µg/ml. The protein antibody mixtures were incubated overnight at 4°C on a rotator. The protein mixtures were applied to 40 µl of washed Protein A Sepharose resin and incubated at 4°C for 2 hours. The resin was washed six times with wash buffer (50 mM $NaH_2PO_4$, pH 8.0, 300 mM NaCl). Next, 20 µl of 2X SDS loading buffer was added to the resin and boiled for 5 minutes. The samples were run on a 12% SDS-PAGE gel and until the dye front migrated off the gel.

Protein was transferred to a nitrocellulose membrane and blocked overnight in blocking buffer containing 3% nonfat dry milk in TBS (10 mM Tris-Cl, pH 7.5, 150 mM NaCl). The membrane was incubated with anti-FLAG or anti-His (1:1000) antibody in blocking buffer for 1 hour, washed 3 times for 30 minutes each with TBSTT (20 mM Tris-Cl, pH 7.5, 500 mM NaCl, 0.05% Tween 20, 0.2% Triton x-100) and incubated with anti-mouse IgG-HRP (1:5000) for 30 minutes in TBSTT. The blot was washed with twice with TBSTT and twice with TBS (30 minutes each wash). The membrane was incubated in Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific)

according to the manufacturer's directions and X-ray film (Kodak) was exposed to the membrane. Blots were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific), reblocked, and probed with the antibody used for the pull-down to confirm that the appropriate protein was pulled down.

***Co-IP with leaf microsomal proteins***

125 μg of leaf microsomal protein was incubated with 5 μg of purified His-ERECTAkd protein and Protein A Sepharose Resin for 1 hour to remove proteins which non-specifically bind to the resin. The unbound protein was transferred to a fresh tube and 200 μg/ml of anti-penta His antibody (Qiagen) was added to precipitate His-ERECTAkd. The remaining steps were performed as described for the bacterial lysate Co-IP experiments. Western analysis was performed using rabbit anti-GPA1 antibody (gift from Alan Jones) and anti-Rabbit IgG-HRP was used for detection.

**Kinase assays**

***Protein expression and purification***

The cDNA corresponding to the kinase domain of ERECTA and the full length ORFs of GPA1 and AGB1 were cloned into a number of different bacterial expression vectors using standard molecular biology techniques (primer sequences in Table 3, vector information in Table 4). All constructs were transformed into competent BL21 cells (either DE3 or A1) and induced with the concentrations of IPTG and/or arabinose suggested by the manufacturer (Invitrogen). All proteins were batch purified using the appropriate affinity resin: Ni-NTA (Novagen) for His, glutathione for GST (GE Healthcare), and maltose (New England BioLabs) for maltose binding protein (MBP). Buffers and conditions used for purification of His-tagged proteins are as described above. For GST-ERECTAkd, induced cells were resuspended in GST binding buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.4% Triton X-100, 1 mM DTT, and 1X protease inhibitor cocktail). MBP-ERECTAkd induced cells were resuspended in MBP column buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA). The proteins were stored at -20°C to aid in cell lysis. Thawed cells were briefly probe-sonicated and spun down. The supernatant was applied to 400 μl of prepared resin slurry and incubated

with rotation for 1 hour at 4°C. Resin was washed with MBP column buffer or GST wash buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 10 mM EDTA, 0.4% Triton X-100, 1mM DTT, and 1X protease inhibitor cocktail). Resin was transferred to a microcolumn and proteins were eluted three times with GST elution buffer (50 mM Tris, pH 8.0, 10 mM reduced glutathione) or MBP elution buffer (10 mM maltose in MBP column buffer). Proteins were stored at -80°C.

***Kinase assays***

Equivalent amounts of protein were incubated in a 100 µl kinase reaction containing 10 µCi γATP in kinase buffer (50 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT) for 1 hour on a rotator at room temperature. 10 µl of StrataClean protein binding resin (Stratagene) was added to the kinase reaction and the mixture was incubated for 20 minutes at room temperature on a rotator. The resin was spun down to remove excess γATP from the mixture. Proteins were eluted with 1X SDS-PAGE loading buffer by heating at 100°C for 5 minutes. Proteins were loaded and run on a 10% SDS-PAGE for 4-6 hours. The gel was stained with Coomassie blue, destained, dried and placed on X-ray film at -80°C for 8 hours or overnight.

## RESULTS

### Split-ubiquitin results for ERECTA interaction with GPA1, AGB1, and GCR1

Split-ubiquitin assays showed no interaction between ERECTA and AGB1 or GCR1. Growth was observed for $Nub_G$ ERECTA and AGB1 Cub and $Nub_G$ ERECTA and GCR1 Cub at 0 µM Met but was abolished with the addition of Met indicating a lack of interaction (Table 5). Similar results were observed for the $Nub_G$ AGB1 and ERECTA Cub and $Nub_G$ GCR1 and ERECTA Cub interactions; no growth was observed upon addition of Met (Table 6). The corresponding $Nub_{wt}$ positive controls for these four interactions were positive (Tables 5 and 6). For the interaction of GPA1 and ERECTA, moderate growth was observed at 1 mM Met when $Nub_G$ ERECTA with GPA1 Cub were coexpressed in yeast suggesting a possible interaction of GPA1 and ERECTA only when ERECTA has a free C-terminus which is the location of the kinase domain (Table 5

and Figure 1). *lacZ* activity also showed putative interaction between GPA1 Cub and $Nub_G$ ERECTA (Figure 2). The interaction assays were repeated three times for ERECTA and ABG1 and ERECTA and GCR1 and five times for ERECTA and GPA1 with independent homologous recombination events to generate the Cub and Nub fusions. Similar results were observed in each repeat.

**GPA1-ERECTA co-IP experiments**

Like the yeast-2-hybrid assay, the SUS assay can generate false positives so all putative interactions must be confirmed using another method such as co-IP. anti-His pulldown (Figure 3) of a mixture of the induced bacterial lysates GPA1-FLAG and His-ERECTAkd failed to demonstrate a GPA1-FLAG/His-ERECTAkd interaction. There were no bands present in the co-IP lane (lane 3) except those corresponding to the heavy chain (~55 kD) and light chain (~25 kD) IgGs of the mouse anti-His antibody used for the co-IP which the anti-mouse secondary antibody detected. These antibody protein bands are present in all samples which contained anti-His antibody (lanes 3-5). The Western positive control GPA1-FLAG on the blot indicated that there were no problems with the Western blot (lane 2). Stripping of the membrane and detection with anti-His antibody showed that the anti-His antibody was pulling down the His-ERECTAkd protein (lower panel lanes 3 and 5; note that the membrane was not fully stripped and there is residual binding of anti-FLAG antibody in lane 2). The size of the His-ERECTAkd protein pulled down is consistent with the size of the His-ERECTAkd protein (lane 1). Again, the heavy chain IgG of the mouse Anti-His antibody used for the pulldown are being detected with the anti-mouse secondary antibody in lanes 3-5 and should be ignored.

Attempts to pull down GPA1 from a mixture of leaf microsomal proteins and purified His-ERECTAkd using anti-His antibody for the pulldown and anti-GPA1 for immunoblot detection (gift from Alan Jones) also failed to demonstrate an interaction of His-ERECTAkd with Arabidopsis GPA1 (Figure 4). GPA1 was detected in both Western positive control samples (lanes 1 and 2); lane 1 contained only recombinant GPA1-TRX and lane 2 contained only total microsomal protein isolated from Col. As would be expected no GPA1 was detected in the microsomal protein isolated from *gpa1-*

*3* (lane 3). These positive and negative Western controls indicate that the Western detection was successful. No GPA1 was detected in the co-IP lane (lane 4), which contained total microsomal protein from Col, recombinant His-ERECTAkd and anti-His antibody. However, there is a non-specific band present in lanes 4-6 of similar molecular mass to GPA1, which could be obscuring GPA1. If this was the case, I would expect the band to be darker in lane 4 compared to lanes 5 and 6 (negative control lanes for the co-IP), since GPA1 would be enriched in lane 4, but this is not observed. Therefore, these co-IP experiments did not support an interaction between GPA1 and ERECTAkd.

**Kinase assays**

In order to determine whether the recombinant His-ERECTAkd protein was functional, kinase activity was assayed using an in vitro kinase assay. The assay showed no autophosphorylation for His-ERECTAkd, however, the soluble kinase, OST1, (protein from Dr. Archaya) showed very high autophosphorylation activity indicating that the kinase reaction conditions were good (data not shown). Taken together, these results suggest that the His-ERECTAkd protein lacked kinase activity. The lack of kinase assay could be a consequence of the His tag or the protein purification procedure. This assay was repeated three times with similar results.

Because no autophosphorylation activity was observed in His-ERECTAkd, GST-ERECTAkd and MBP-ERECTAkd were expressed in bacteria, purified and tested for autophosphorylation activity. The purification of GST-ERECTAkd is shown in Figure 5. A GST-ERECTAkd induction band is not observed in the soluble protein (lane 1) but can be clearly seen in the insoluble protein (lane 2). Lanes 3 and 4 are wash fractions and lanes 5-7 are the elution lanes. GST-ERECTAkd elution 2 was used for the kinase assays. The purification of MBP-ERECTAkd is shown in Figure 6. The induction band MBP-ERECTAkd is visible in the insoluble protein (lane 2) but not in the soluble protein (lane 1). Lane 3 is unbound protein and lanes 4 and 5 are washes. MBP-ERECTAkd elutions are found in lanes 6-8. MBP-ERECTAkd elution 2 was used for the kinase assays.

The autophosphorylation assay is shown in Figure 7B. GST-ERECTAkd showed no autophosphorylation activity (lane 1). The phosphorylation band present in lane 1 is

much larger than GST-ERECTAkd (68 kD) and is likely a co-purifiying bacterial kinase. MBP-ERECTAkd, however, showed a strong autophosphorylation band at 83 kD corresponding to MBP-ERECTAkd on the Coomassie stained gel (Figure 7A, lane 2). Lane 3 contains MBP-ERECTAkd(mutant) protein expressed from a clone I received from the laboratory of John Walker, which contained two mutations resulting in one amino acid change in the protein. Nucleotide 37 of the kinase domain had a thymine to cytosine mutation causing amino acid 13 to change from serine to proline. However, the mutations do not appear to affect the autophosphorylation activity, because a strong phosphorylation band is also present in lane 3 (Figure 7B) which corresponds to the MBP-ERECTAkd(mutant) protein on the gel (Figure 7A, lane 3). The smear and the smaller phosphorylation bands in lanes 2 and 3 (Figure 7B) are likely protein degradation products that have retained some kinase activity. To ensure that the phosphorylation signal detected was due to MBP-ERECTAkd and not a copurifying bacterial kinase of the same size as the MBP-ERECTAkd, proteins purified using the MBP procedure from induced untransformed cells were assayed for phosphorylation. No signal was observed (data not shown) indicating that MBP-ERECTAkd was the source of autophosphorylation activity.

TRX-GPA1 was then expressed and purified in order to see if MBP-ERECTAkd could phosphorylate TRX-GPA1. The purifications of TRX and TRX-GPA1 are shown in Figure 8. Lane 1 shows insoluble protein from TRX induction and the TRX elution fractions are in lanes 2-4. TRX elution 3 was used for the kinase assays. Lane 5 contains the insoluble protein from the TRX-GPA1 purification and the TRX-GPA1 elution fractions are in lanes 6-8. TRX-GPA1 elution 2 was used for the kinase assays.

In the kinase assay, MBP-ERECTAkd again showed strong autophosphorylation activity (Figure 9, lane 1). When TRX-GPA1 was incubated with MBP-ERECTAkd, no additional phosphorylation band was present at the molecular mass of TRX-GPA1 (65 kD) indicating that MBP-ERECTAkd was not phosphorylating TRX-GPA1 (lane 2). While there is strong background signal from the MBP-ERECTAkd phosphorylation, if TRX-GPA1 was being phosphorylated a 65 kD band would be observed in lane 2 and not in lane 1 and this not the case. However, if the phosophorylation of GPA1 was weak it is possible that the non-specific background could be obscuring the signal. Lanes 3- 6 are

all negative control lanes. Lane 3 contains TRX-GPA1 only and the 75 kD band present is a co-purifying bacterial kinase which can also be observed in lanes 2 and 6 which both contain TRX-GPA1. Lane 4 contains another TRX-tagged protein, TRX-XLG1 (118 kD) and MBP-ERECTAkd. The negative control in lane 5 contains MBP-ERECTAkd and TRX and it appears that TRX protein is phosphorylated by MBP-ERECTAkd given the signal detected at 18 kD. This band is not likely to be a degradation product of MBP-ERECTAkd because it is not present in the other lanes which contain MBP-ERECTAkd sample (lanes 1, 2, and 4). Therefore it is not recommended that TRX-tagged proteins be used in kinase assays.

The kinase assays were repeated once more with His-GPA1 as well as with His-AGB1. Figures 10 and 11 show the purification of His-GPA1 and His-AGB1, respectively. Elution fractions are found in lanes 3-5 in both figures. The first elution fraction was used for both His-GPA1 and His-AGB1 in the kinase assay. MBP-ERECTAkd was freshly purified but had reduced autophosphorylation activity compared to previous kinase assays as evidenced by the weaker signal observed in lane 1 of Figure 12 than previous kinase assays (Fig.7, lane 2 and Fig. 9, lane 1). The smaller phosphorylation bands in lane 1 are likely MBP-ERECTAkd degradation products. GST-ERECTAkd was also freshly purified but once again showed no autophosphorylation activity (lane 2). Lane 3 contains His-GPA1 and the phosphorylation band present at 80 kD is a copurifying bacterial kinase that is present in all lanes containing His-GPA1 (lanes 3-5). There are no phosphorylation bands corresponding to His-GPA1 (52 kD) in lanes 4 or 5, which indicates that His-GPA1 is not phosphorylated. Similarly, there are no phosphorylation bands corresponding to His-AGB1 (47 kD) in lanes 7 or 8 which suggests that His-AGB1 is also not phosphorylated.

**Genetic interaction of GPA1 and ERECTA**

To determine if GPA1 and ERECTA may be in the same pathway regulating stomatal development and/or transpiration efficiency, crosses between *gpa1* and *er105* mutants were performed. *er105, gpa1-3* and *gpa1-4* mutants were backcrossed for 3 generations before *er105* was crossed to each *gpa1* allele. Heterozygous F1 plants were confirmed by PCR and F2 seed was collected. Over 300 F2 plants were genotyped for

*er105* and *gpa1* and no recombinants were isolated. All plants were either double heterozygote or homozygote/wild type for the alleles. *GPA1* and *ERECTA* are tightly linked on chromosome 2, separated by only 7 kb of sequence (Figure 13). Genetic mapping shows a genetic distance between *GPA1* and *ERECTA* markers of approximately 1.7 cM (Figure 13). While this distance should correspond to a recombination frequency of 1.7% the genotyping results suggest it may in fact be much lower. *er105 agb1* double mutants were generated but not analyzed because I was not able to observe significant stomatal density and TE phenotypes in both *agb1-1* and *agb1-2* alleles. However, though not studied extensively, *agb1-1* mutants may have increased stomatal density and reduced TE (Chapter 8). The *er105 agb1* double mutants are available for future use by the Assmann lab.

**Interaction of GPA1 with other regulators of stomatal development**

SUS assays using Gateway compatible SUS vectors showed no evidence for interaction of GPA1 with the known regulators of stomatal development, ERL1, ERL2, SDD1, and TMM (Tables 7 and 8). It is important to note that growth of the yeast coexpressing the Cubs and NubW was highly suppressed compared to the original SUS vectors. Additionally, many of the Cub-NubW positive control interactions showed no growth. Even at very low Met levels GPA1-Cub and NubG-ERECTA showed no growth, suggesting the original SUS assays may have identified a false positive interaction between GPA1 and ERECTA . However, it is also possible that the LR reactions to generate the SUS fusions did not work properly which could affect the translation of the fusion protein. If the SUS fusion constructs are used in the future, they should be sequenced to ensure that recombination of the entry and destination vectors was correct. Among the stomatal development regulators, only the ERECTA family of receptors showed some interaction. ERL1 heterodimerized with both ERECTA and ERL2, but only when ERL1 was fused to Cub (Table 9). ERL1-Cub also showed a very low level interaction with NubG-GPA1 (Table 8) when grown on 50 μM Met.

## DISCUSSION

Heterotrimeric G proteins are membrane localized proteins that generally function in transduction of extracellular signals into intracellular responses. ERECTA is a LRR-RLK (Torii et al., 1996) and, characteristic of LRR-RLKs, has a predicted protein topology consisting of an extracellular ligand-binding domain with a number of leucine-rich repeats, a single transmembrane domain and an intracellular kinase domain (Shiu and Bleecker, 2001; Torii, 2004). LRR-RLKs function in cell signaling and genes which encode LRR-RLKs are prolific in the Arabidopsis genome and comprise the largest subgroup of the RLK family in Arabidopsis (Shiu and Bleecker, 2001). Functional analyses of mutants have suggested roles for LRR-RLKs in both development and environmental responses (Shiu and Bleecker, 2001; Torii, 2004). While some ligands have been identified for LRR-RLKs (Torii, 2004), a ligand for ERECTA has not been identified.

ERECTA, GPA1, and AGB1 have a number of overlapping functions including in pathogen responses, stomatal development, transpiration efficiency, and leaf, silique, flower, and inflorescence development (Chapter 3, Lease et al., 2001; Llorente et al., 2005; Masle et al., 2005; Shpak et al., 2005). The G protein β subunit mutant, *agb1-1*, was first identified in a screen for *erecta-like* mutations (Lease et al., 2001). Lease et al. (2001) examined the putative genetic interaction between AGB1 and ERECTA by phenotyping *agb1er* double mutants. Analysis of *agb1er* mutants found that for some traits AGB1 and ERECTA function in parallel pathways (silique length, leaf shape, inflorescence height) while for other traits (silique width, pedicel length) the two genes likely share a developmental pathway (Lease et al., 2001). However, pull-down experiments between ERECTA and AGB1 did not support a physical interaction between the two proteins (Lease et al., 2001).

Chapter 4 reports that GPA1 functions in stomatal development and transpiration efficiency. GPA1 is a positive regulator of stomatal development and a negative regulator of transpiration efficiency while ERECTA is a negative regulator of stomatal development and a positive regulator of transpiration efficiency. Therefore, we focused on identifying a possible interaction between ERECTA and GPA1. While initial split-

ubiquitin assays suggested that ERECTA and GPA1 physically interact, subsequent in-vitro co-IP and kinase assays did not support an interaction between the ERECTAkd with GPA1. However, the kinase domain only was used for these in vitro experiments because transmembrane proteins are difficult to express and purify in vitro, and it has been previously shown that the kinase domain of ERECTA has autophosphorylation activity in vitro (Lease et al., 2001). My results also supported ERECTAkd autophosphorylation but interestingly, only when fused to the MBP tag. The His and GST tagged ERECTAkd showed no autophosphorylation activity. MBP is the same tag used by Lease et al. (2001). Differences in purification procedures (buffer components, detergents) could contribute to changes in protein folding or activity. Additionally, the protein tags themselves may alter protein folding or inhibit kinase activity. It is possible that the full length protein is required for ERECTA phosophorylation of and/or interaction with GPA1. The lack of an extracellular ligand binding domain is one limitation of my in vitro co-IP and kinase assays and may explain why an interaction between full length ERECTA and GPA1 was observed in yeast but not in the co-IP or kinase assays. However, addition yeast-based assays using different SUS vectors also failed to support an interaction between GPA1 and ERECTA. This result suggests that the interaction observed in the earlier SUS assays may have been a false positive or possibly that there was a problem with the later Gateway LR recombination reactions which affected translation or folding of the fusion protein. Even though the full length ERECTA protein was expressed in yeast, if ligand binding and activation of ERECTA was required for protein interactions to occur, and the ligand was not present in yeast, than the SUS system would not be able to identify protein interactions with ERECTA. Additionally, misfolding of the Arabidopsis protein in yeast can also contribute to changes in protein topologies which can limit the utility of this assay.

Surprisingly, despite data supporting genetic epistasis between ERECTA and TMM and negative regulation of ERECTA-family receptors by TMM (Shpak et al., 2005), no interaction was observed between ERECTA and TMM in the SUS assays. While the lack of ligand binding, discussed above, could also be responsible for the failure to detect a TMM ERECTA interaction, the interaction could also be indirect via a heterodimerization partner of ERECTA. Because ERECTA regulates a number of

different functions in plants (Torii et al., 1996; Shpak et al., 2004; Llorente et al., 2005; Masle et al., 2005; Shpak et al., 2005), it has been hypothesized that the function of ERECTA in stomatal development may be specified by heterodimerization with ERL1 or ERL2 (Shpak et al., 2005) . Therefore TMM (and possibly even GPA1) may physically interact with ERL1 or ERL2 which may dimerize with ERECTA. ERL1 and ERL2 are functional paralogues of ERECTA; *erl1* and *erl2* mutants show no mutant phenotypes but the triple mutant *erecta erl1 erl2* enhances a number of the *erecta* phenotypes including stomatal density, dwarfism and flower development (Shpak et al., 2004; Shpak et al., 2005). ERECTA promoter activity drops off before epidermal cell differentiation while ERL1 and ERL2 promoter activities, similarly to GPA1 and TMM, are high in the stomatal cell lineage and decrease in mature guard cells (Shpak et al., 2005), which lends supports to the hypothesis that ERL1 and ERL2 provide ERECTA with specificity of function in stomatal development. The SUS assays did show some heterodimerization between ERL1 and ERECTA and ERL1 and ERL2. However, the possibility that ERL1/ERl2 and TMM interact was not tested. GPA1 showed some slight interaction with ERL1 using the SUS system but this putative interaction was not further investigated or confirmed.

Genetic approaches to examine the putative interaction of *GPA1* and *ERECTA* were unsuccessful because of tight linkage between the two loci. Additional screening for *er105 gpa1* recombinants or RNAi-based transgenic lines would be required to characterize any *ERECTA – GPA1* genetic interaction. Interaction between *ERL1* or *ERL2* with *GPA1* is difficult to examine genetically, because *erl1* and *erl2* mutants have no stomatal development phenotype (Shpak *et al.*, 2004) but the double mutants could shown enhanced phenotypes indicating potential interactions between *GPA1* and *ERL1* or *GPA1* and *ERL2*..

## CONCLUSION

Overlapping mutant phenotypes, subcellular localizations, expression patterns, and molecular functions of GPA1 and ERECTA suggested a possible interaction between these two proteins. However, the yeast-based and in vitro interaction assays did not

support an interaction between GPA1 and ERECTA. Additionally, assessment of the interaction of *GPA1* and *ERECTA* in regulating stomatal development and transpiration efficiency at the genetic level was not possible because of tight genetic linkage between the two genes. While G proteins have emerged as regulators of stomatal development (Chapter 4, Zhang *et al.*, 2008), determining where G proteins function in the current stomatal development model will require further inquiry.

## LITERATURE CITED


**Berger D, Altmann T** (2000) A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. Genes Dev **14:** 1119-1131

**Bergmann DC, Lukowitz W, Somerville CR** (2004) Stomatal development and pattern controlled by a MAPKK kinase. Science **304:** 1494-1497

**Bergmann DC, Sack FD** (2007) Stomatal development. Annu Rev Plant Biol **58:** 163-181

**Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T** (2007) The secretory peptide gene *EPF1* enforces the stomatal one-cell-spacing rule. Genes Dev **21:** 1720-1725

**Johnsson N, Varshavsky A** (1994) Split ubiquitin as a sensor of protein interactions *in vivo*. Proc Natl Acad Sci USA **91:** 10340-10344

**Lai LB, Nadeau JA, Lucas J, Lee EK, Nakagawa T, Zhao L, Geisler M, Sack FD** (2005) The *Arabidopsis* R2R3 MYB proteins FOUR LIPS and MYB88 restrict divisions late in the stomatal cell lineage. Plant Cell **17:** 2754-2767

**Lampard GR, Macalister CA, Bergmann DC** (2008) *Arabidopsis* stomatal initiation is controlled by MAPK-mediated regulation of the bHLH SPEECHLESS. Science **322:** 1113-1116

**Lease KA, Lau NY, Schuster RA, Torii KU, Walker JC** (2001) Receptor serine/threonine protein kinases in signalling: analysis of the ERECTA receptor-like kinase of *Arabidopsis thaliana*. New Phytologist **151:** 133-143

**Lease KA, Wen J, Li J, Doke JT, Liscum E, Walker JC** (2001) A mutant *Arabidopsis* heterotrimeric G-protein β subunit affects leaf, flower, and fruit development. Plant Cell **13:** 2631-2641

**Llorente F, Alonso-Blanco C, Sanchez-Rodriguez C, Jorda L, Molina A** (2005) ERECTA receptor-like kinase and heterotrimeric G protein from *Arabidopsis* are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Plant Journal **43:** 165-180

**MacAlister CA, Ohashi-Ito K, Bergmann DC** (2007) Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. Nature **445:** 537-540

**Masle J, Gilmore SR, Farquhar GD** (2005) The *ERECTA* gene regulates plant transpiration efficiency in *Arabidopsis*. Nature **436:** 866-870

**Nadeau JA** (2009) Stomatal development: new signals and fate determinants. Curr Opin Plant Biol **12:** 29-35

**Nadeau JA, Sack FD** (2002) Control of stomatal distribution on the *Arabidopsis* leaf surface. Science **296:** 1697-1700

**Obrdlik P, El-Bakkoury M, Hamacher T, Cappellaro C, Vilarino C, Fleischer C, Ellerbrok H, Kamuzinzi R, Ledent V, Blaudez D, Sanders D, Revuelta JL, Boles E, Andre B, Frommer WB** (2004) $K^+$ channel interactions detected by a genetic system optimized for systematic studies of membrane protein interactions. Proc Natl Acad Sci USA **101:** 12242-12247

**Obrdlik P, Neuhaus G, Merkle T** (2000) Plant heterotrimeric G protein β subunit is associated with membranes via protein interactions involving coiled-coil formation. Febs Letters **476:** 208-212

**Ohashi-Ito K, Bergmann DC** (2006) *Arabidopsis* FAMA controls the final proliferation/differentiation switch during stomatal development. Plant Cell **18:** 2493-2505

**Pillitteri LJ, Sloan DB, Bogenschutz NL, Torii KU** (2007) Termination of asymmetric cell division and differentiation of stomata. Nature **445:** 501-505

**Shiu SH, Bleecker AB** (2001) Plant receptor-like kinase gene family: diversity, function, and signaling. Sci STKE **2001:** RE22

**Shiu SH, Bleecker AB** (2001) Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA **98:** 10763-10768

**Shpak ED, Berthiaume CT, Hill EJ, Torii KU** (2004) Synergistic interaction of three ERECTA-family receptor-like kinases controls *Arabidopsis* organ growth and flower development by promoting cell proliferation. Development **131:** 1491-1501

**Shpak ED, McAbee JM, Pillitteri LJ, Torii KU** (2005) Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science **309:** 290-293

**Thaminy S, Miller J, Stagljar I** (2004) The split-ubiquitin membrane-based yeast two-hybrid system. Methods Mol Biol **261:** 297-312

**The Arabidopsis Information Resource (TAIR)**, [http://www.arabidopsis.org/servlets/mapper?value=GPA1&action=search], on www.arabidopsis.org, [November 2008].

**Torii KU** (2004) Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways. Int Rev Cytol **234:** 1-46

**Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, Komeda Y** (1996) The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell **8:** 735-746

**Von Groll U, Berger D, Altmann T** (2002) The subtilisin-like serine protease SDD1 mediates cell-to-cell signaling during *Arabidopsis* stomatal development. Plant Cell **14:** 1527-1539

**Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S** (2007) Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in *Arabidopsis*. Plant Cell **19:** 63-73

**Weiss CA, White E, Huang H, Ma H** (1997) The G protein α subunit (GPA1) is associated with the ER and the plasma membrane in meristematic cells of *Arabidopsis* and cauliflower. Febs Letters **407:** 361-367

**Yang M, Sack FD** (1995) The *too many mouths* and *four lips* mutations affect stomatal production in *Arabidopsis*. Plant Cell **7:** 2227-2239

**Zhang L, Hu G, Cheng Y, Huang J** (2008) Heterotrimeric G protein α and β subunits antagonistically modulate stomatal density in Arabidopsis thaliana. Dev Biol **324:** 68-75

**Table 1**. Linker-adapted primer sequences for ERECTA SUS cloning

| Primer ID | |
|---|---|
| ERECTA Sub Forward primer | ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATGGCTCTGTTTAGAGATATT |
| ERECTA Sub Reverse primer | TCCGCCACCACCAACCACTTTGTACAAGAAAGCTGGGTACTCACTGTTCTGAGAAATAAC |

**Table 2**. Primer sequences for Gateway SUS cloning

| ORF | Primer ID | Forward Primer Sequence | Primer ID | Reverse Primer Sequence |
|---|---|---|---|---|
| ERECTA | ER F SUS | ATGGCTCTGTTTAGAGATATTGTTC | ER R SUS | CTCACTGTTCTGAGAAATAACTTGTC |
| ERL1 | ERL1 F SUS | ATGAAGGAGAAGATGCAGCGA | ERL1 R SUS | TATGCTACTTTTGGAGATGACTTCACGG |
| ERL2 | ERL2 F SUS | ATGAGAAGGATAGAGACCATG | ERL2 R SUS | TAAGCTACTTTTGGAGATATCTTC |
| TMM | TMM F SUS | ATGGCACGATATGAATTCTTC | TMM R SUS | ACTAGATATTAGCATAAAAATGAAATTAGG |
| SDD1 | SDD1 F SUS | ATGGAACCCAAACCTTTCTTT | SDD1 R SUS | GTTAGTCTTCAAGGTTACAGA |

**Table 3**. Primer sequences for generating protein expression clones

| ORF | Primer ID | Forward Primer Sequence | Primer ID | Reverse Primer Sequence |
|---|---|---|---|---|
| GPA1 | EcoRI GPA1 F | GAATTCGGCTTACTCTGCAGT | XhoI GPA1 R | CTCGAGTCATAAAAGGCCAGCCTC |
| AGB1 | EcoRI AGB1 F | GAATTCTCTGTCTCCGAGCTC | HindIII AGB1 R | AAGCTTTCAAATCACTCTCCTGTG |
| ERECTAkd | EcoRI ERECTA kd forward | GAATTCTTCCTTGATGGATCACTT | SalI ERECTA kd reverse | GTCGACCTACTCACTGTTCTGAGA |

**Table 4.** Fusion proteins used for co-IP and kinase assays

| Vector | N-terminal tag | C-terminal tag | ORF | Protein size (kD) |
|---|---|---|---|---|
| pET28a | HIS/T7 | - | $ERECTA_{kd}$ | 47 |
| pGEX5x-1 | GST | - | $ERECTA_{kd}$ | 68 |
| pTRX | TRX | - | $ERECTA_{kd}$ | 60 |
| pMal | MBP | - | $ERECTA_{kd}$ | 83 |
| pT7* | - | FLAG | GPA1 | 48 |
| pET28a | HIS/T7 | - | GPA1 | 52 |
| pTRX# | TRX | | GPA1 | 65 |
| pET28a | HIS/T7 | - | AGB1 | 47 |

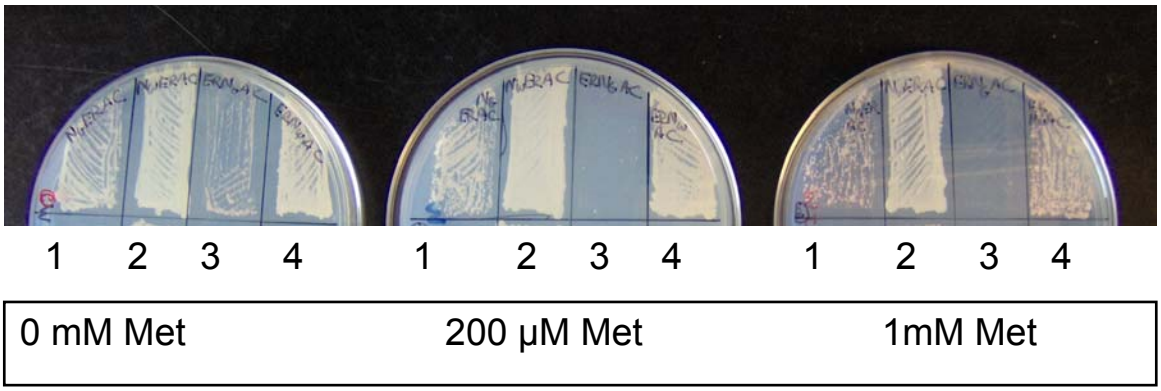
* clone provided by Dr. Zhixin Zhao, # clone provided by Dr. Jaebok Heo.

**Table 5.** Growth scores of diploid yeast expressing the Nub and Cub fusions at different Met concentrations on –His/-Ade/-Trp/-Leu media. – indicates no growth, + some growth, ++ moderate growth, and +++ prolific growth. Shaded box indicates the sole positive test interaction at 1 mM Met.

| | | Interactions with G protein subunit/receptor-Cub-PLV | | | |
|---|---|---|---|---|---|
| Cub-PLV fusions | Met conc. | $Nub_{wt}$-ERECTA | NubG-ERECTA | ERECTA-$Nub_{wt}$ | ERECTA-$Nub_G$ |
| GPA1 | 0 mM | +++ | +++ | +++ | + |
| GPA1 | 200 µM | +++ | +++ | +++ | - |
| GPA1 | 1 mM | +++ | ++ | +++ | - |
| AGB1 | 0 mM | +++ | ++ | ++ | - |
| AGB1 | 200 µM | + | - | - | - |
| AGB1 | 1 mM | - | - | - | - |
| GCR1 | 0 mM | +++ | +++ | ++ | - |
| GCR1 | 200 µM | +++ | - | + | - |
| GCR1 | 1 mM | + | - | - | - |

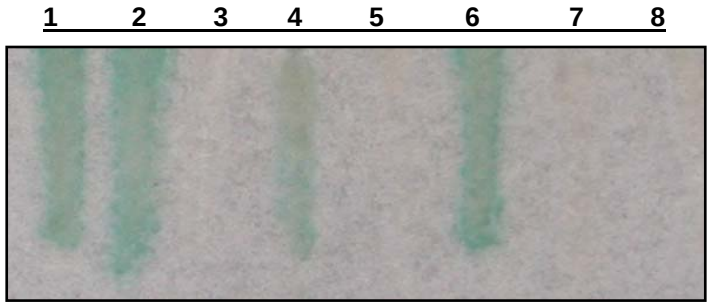
**Table 6.** Growth scores of diploid yeast expressing the Nub fusions with ERECTA- Cub at different Met concentrations on -His/-Ade/-Trp/-Leu media. – indicates no growth, + some growth, ++ moderate growth, and +++ prolific growth.

| | | Interactions with ERECTA-Cub-PLV | | | |
|---|---|---|---|---|---|
| Protein | Met conc. | Nubwt-Protein | NubG-Protein | Protein-$Nub_{wt}$ | Protein-$Nub_G$ |
| GPA1 | 0 mM | +++ | - | + | - |
| GPA1 | 200 µM | ++ | - | - | - |
| GPA1 | 1 mM | + | - | - | - |
| AGB1 | 0 mM | +++ | ++ | - | - |
| AGB1 | 200 µM | + | - | - | - |
| AGB1 | 1 mM | - | - | - | - |
| GCR1 | 0 mM | +++ | ++ | - | - |
| GCR1 | 200 µM | ++ | - | - | - |
| GCR1 | 1 mM | + | - | - | - |




1. GPA1-Cub + $Nub_G$-ERECTA (test)
2. GPA1-Cub + $Nub_{wt}$-ERECTA (positive control)
3. GPA1-Cub + ERECTA-$Nub_G$ (test)
4. GPA1-Cub + ERECTA-$Nub_{wt}$ (positive control)

**Figure 1.** GPA1-Cub and $Nub_G$-ERECTA interact in yeast. Yeast growth of GPA1-Cub and ERECTA Nub fusions on -His/-Ade/-Trp/-Leu media at 0 mM , 200 µM, and 1 mM Met.




1. GPA1-Cub + ERECTA-$Nub_{wt}$ (+ control)
2. GPA1-Cub + $Nub_{wt}$-ERECTA (+ control)
3. GPA1-Cub + ERECTA-$Nub_G$ (test)
4. GPA1-Cub + $Nub_G$-ERECTA (test)
5. ERECTA-Cub + GPA1-$Nub_{wt}$ (+ control)
6. ERECTA-Cub + $Nub_{wt}$-GPA1 (+ control)
7. ERECTA-Cub + GPA1- $Nub_G$ (test)
8. ERECTA-Cub + $Nub_G$-GPA1 (test)

**Figure 2.** The *lacZ* reporter gene is active when GPA1-Cub and $Nub_G$-ERECTA are coexpressed in yeast. X-gal filter assay for *lacZ* reporter assay; blue color indicates activation of *lacZ* gene.

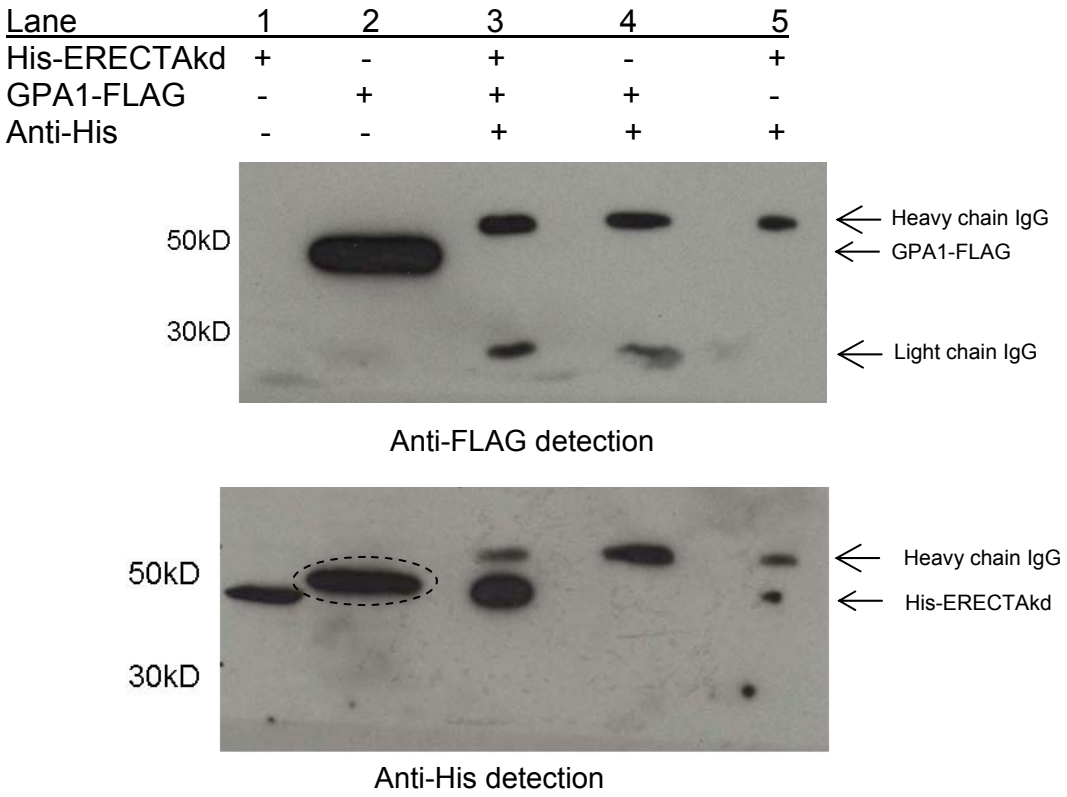



**Figure 3.** Anti-His antibody did not co-precipitate GPA1-FLAG with His-ERECTAkd (lane 3). Top panel shows western blot using anti-FLAG, bottom panel shows stripped membrane probed with anti-HIS. The circled band is from residual anti-FLAG antibody that remained on the blot after stripping.

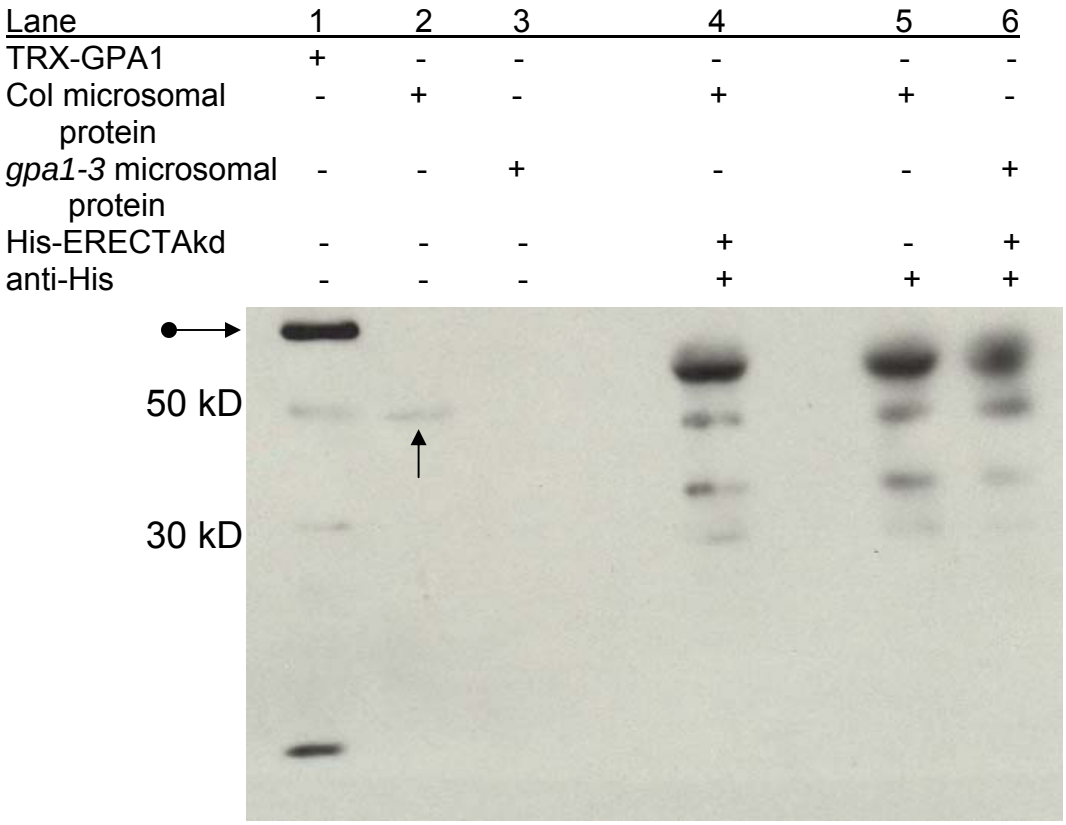
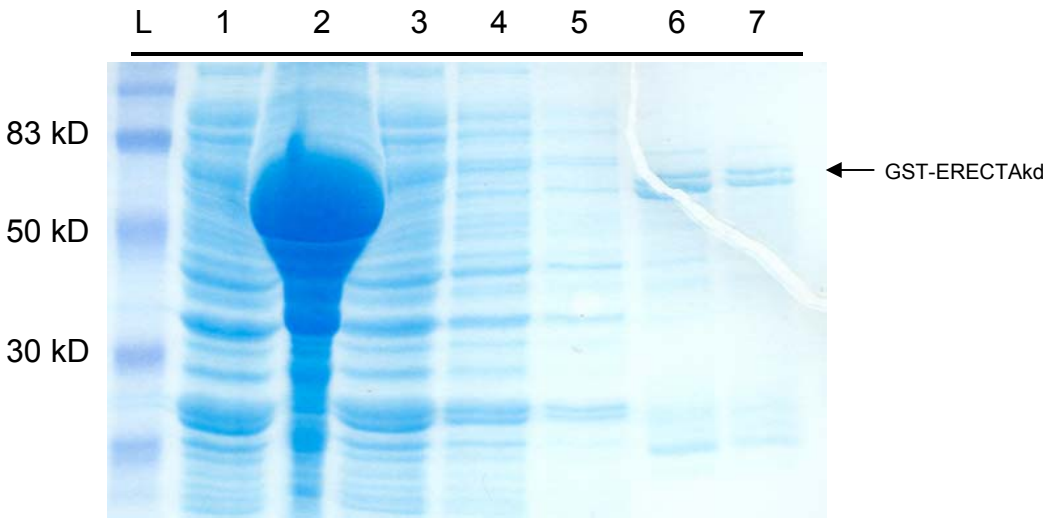
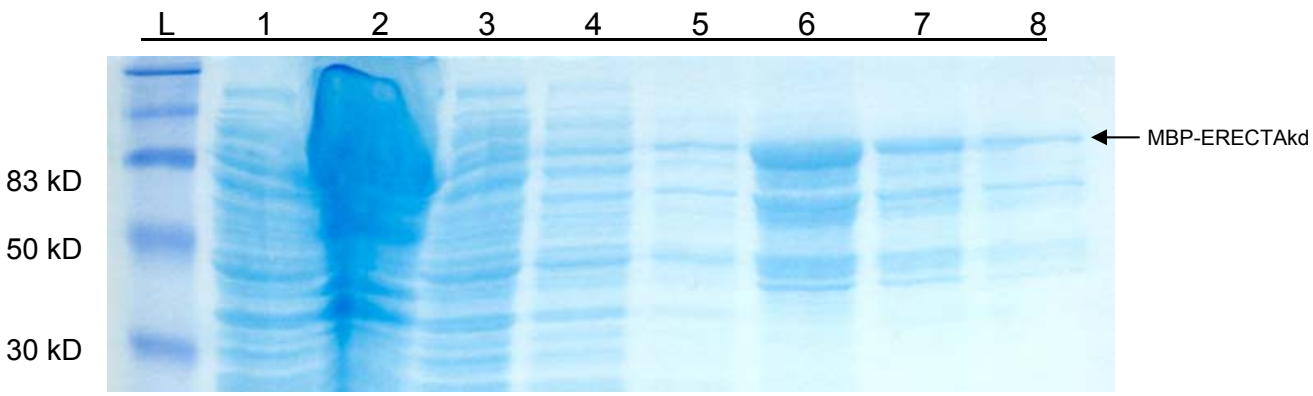



**Figure 4.** Anti-His did not coimmunoprecipate GPA1 from a mixture of Col microsomal protein and recombinant His-ERECTAkd. Western used anti-GPA1 antibody. Dotted arrow indicates TRX-GPA1 (65 kD); smaller bands in lane 1 are likely TRX-GPA1 protein degradation products. Arrow in lane 2 indicates Arabidopsis GPA1 (45 kD). Bands in lanes 4-6 are non-specific.




L. Protein ladder
1. Soluble protein
2. Insoluble protein
3. Wash 1
4. Wash 2
5. GST-ERECTAkd Elution 1
6. GST-ERECTAkd Elution 2
7. GST-ERECTAkd Elution 3

**Figure 5.** Coomassie stain of an SDS-PAGE gel showing the purification of GST-ERECTAkd. GST-ERECTAkd is approximately 68 kD.




L. Protein ladder
1. Soluble protein
2. Insoluble protein
3. Unbound protein
4. Wash 1
5. Wash 2
6. MBP-ERECTAkd Elution 1
7. MBP-ERECTAkd Elution 2
8. MBP-ERECTAkd Elution 3

**Figure 6.** Coomassie stain of an SDS-PAGE gel showing the purification of MBP-ERECTAkd fusion protein. MBP-ERECTAkd is approximately 83 kD.

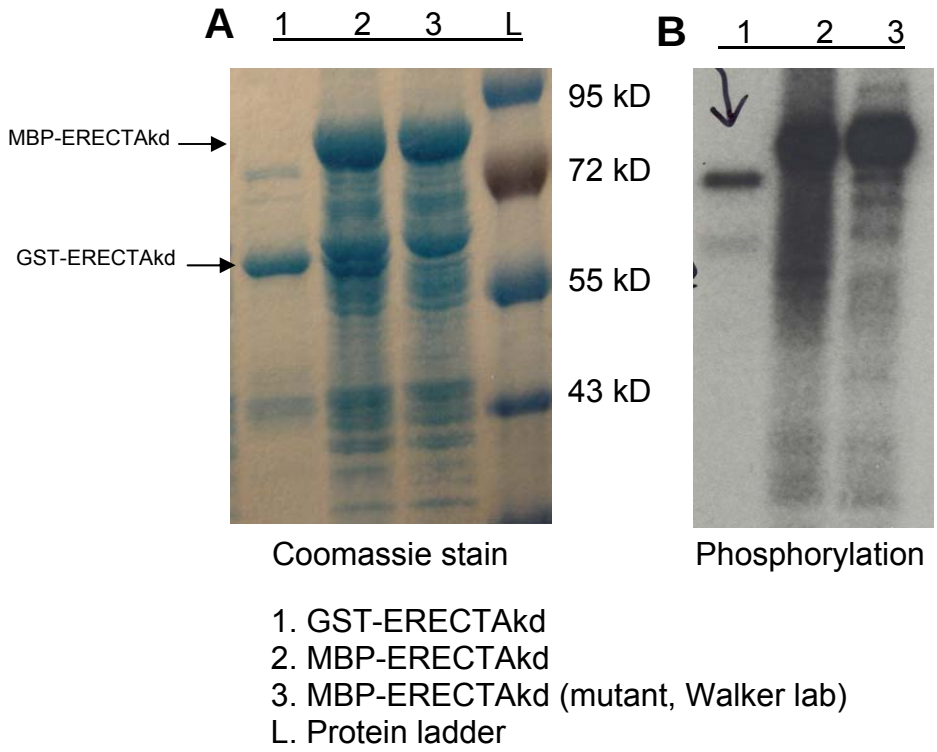
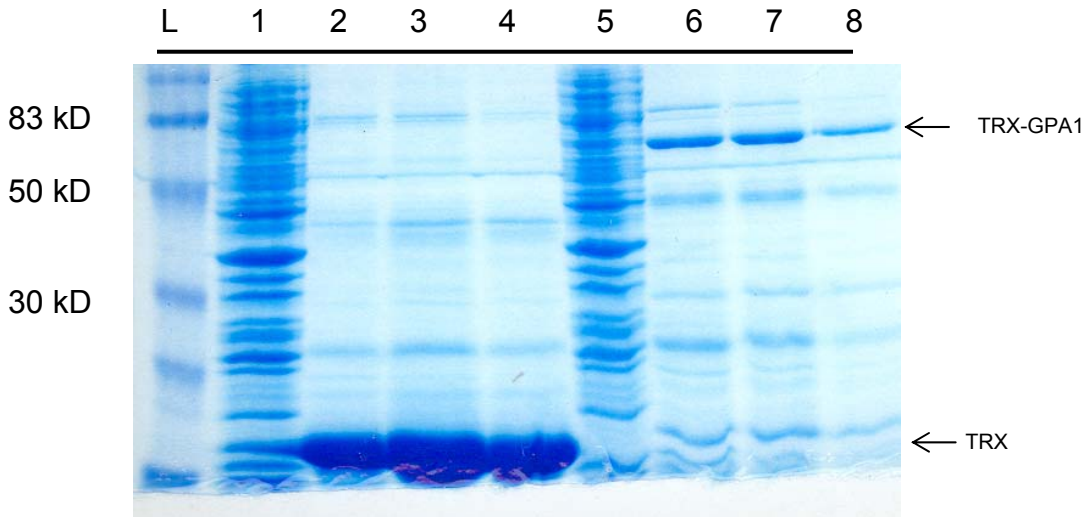



**Figure 7.** Coomassie stain (A) and autophosphorylation (B) of purified ERECTAkd fusion proteins. Arrows show positions of GST-ERECTAkd (68 kD) and MBP-ERECTAkd (83 kD).




L. Protein ladder
1. Insoluble TRX induced
2. Elution 1 TRX
3. Elution 2 TRX
4. Elution 3 TRX
5. Insoluble TRX-GPA1
6. Elution 1 TRX-GPA1
7. Elution 2 TRX-GPA1
8. Elution 3 TRX-GPA1

**Figure 8.** Coomassie stain of TRX and TRX-GPA1 fusion protein purification. TRX is approximately 15 kD and TRX-GPA1 is approximately 65 kD.

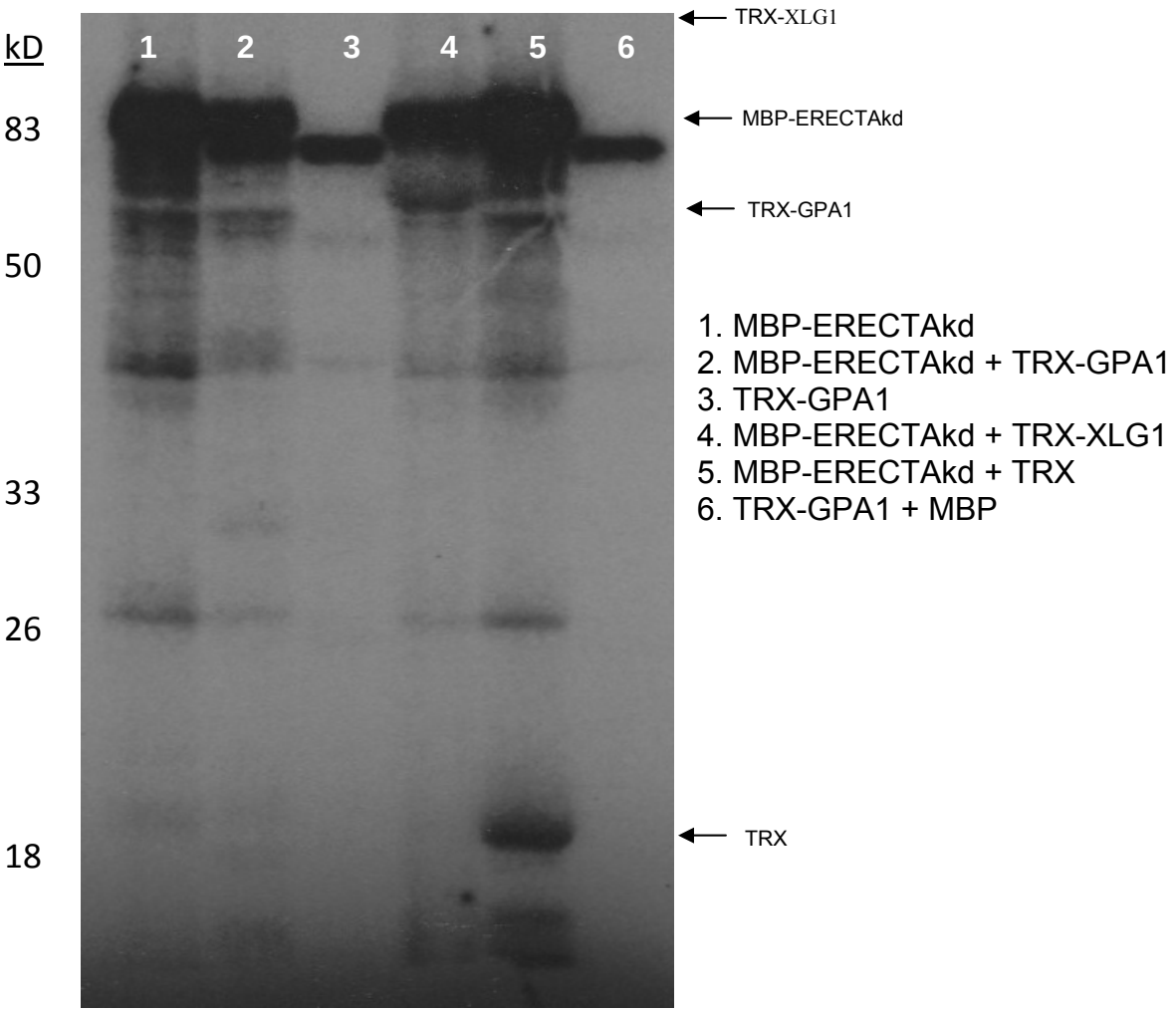
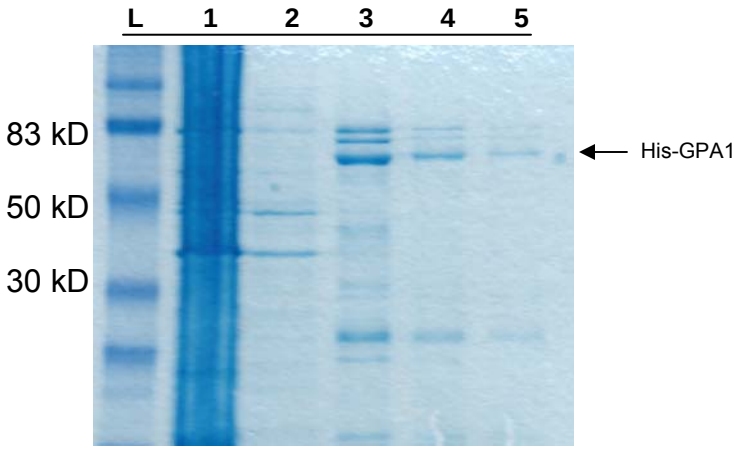



**Figure 9.** Kinase assay showing no phosphorylation of TRX-GPA1(lane 2) and phosphorylation of TRX protein (lane 5). The arrows indicate the locations of MBP-ERECTAkd (83 kD), TRX-GPA1(65 kD) if phosphorylated (lane 2), TRX-XLG (118 kD) if phosphorylated (lane 4) and TRX (15 kD).




L. Protein ladder
1. Insoluble protein
2. Unbound protein
3. His-GPA1 elution 1
4. His-GPA1 elution 2
5. His-GPA1 elution 3

**Figure 10.** Coomassie stain of SDS-PAGE gel showing His-GPA1 protein purification. His-GPA1 is approximately 52 kD and is indicated by the arrow.

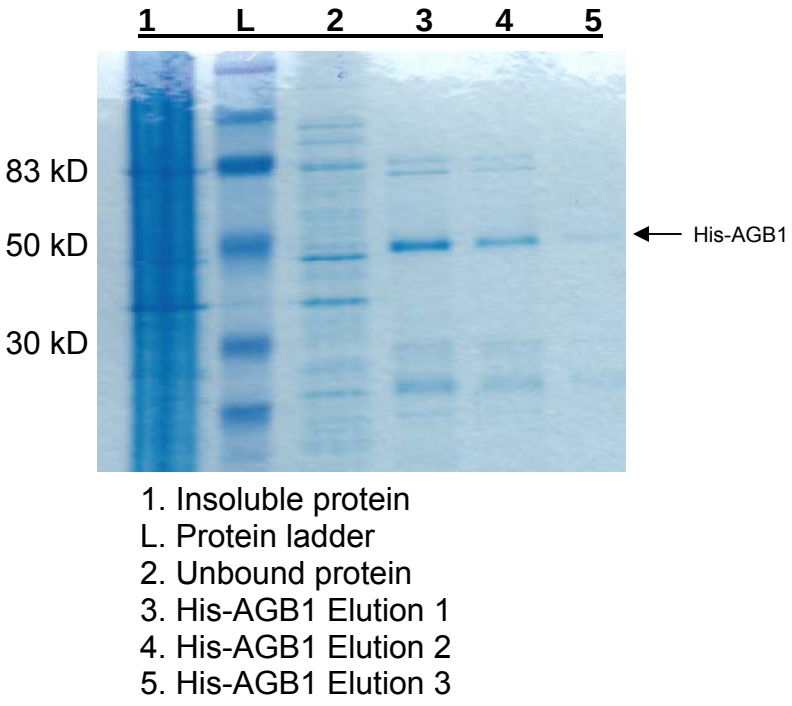
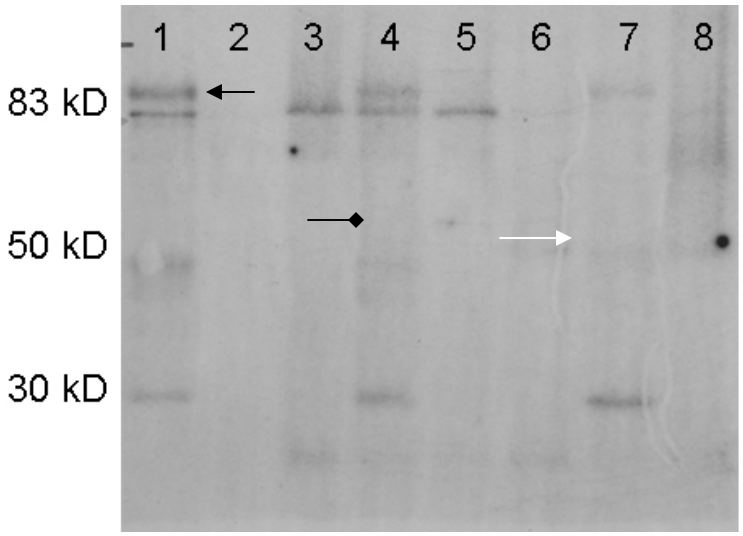



**Figure 11.** Coomassie stain of SDS-PAGE gel showing the purification of His-AGB1 fusion protein. His-AGB1 is approximately 47 kD and is indicated by the arrow.




1. MBP-ERECTAkd
2. GST-ERECTAkd
3. His-GPA1
4. MBP-ERECTAkd + His-GPA1
5. GST-ERECTAkd + His-GPA1
6. His-AGB1
7. MBP-ERECTAkd + His-AGB1
8. GST-ERECTAkd + His-AGB1

**Figure 12.** Kinase assay showing no phosophorylation of His-GPA1 or His-AGB1 by ERECTAkd. Black arrow indicates location of MBP-ERECTAkd, diamond arrow indicates where His-GPA1 should be in lanes 4 and 5 if phosphorylated, and white arrow indicates where His-AGB1 should be in lanes 7 and 8 if phosphorylated.

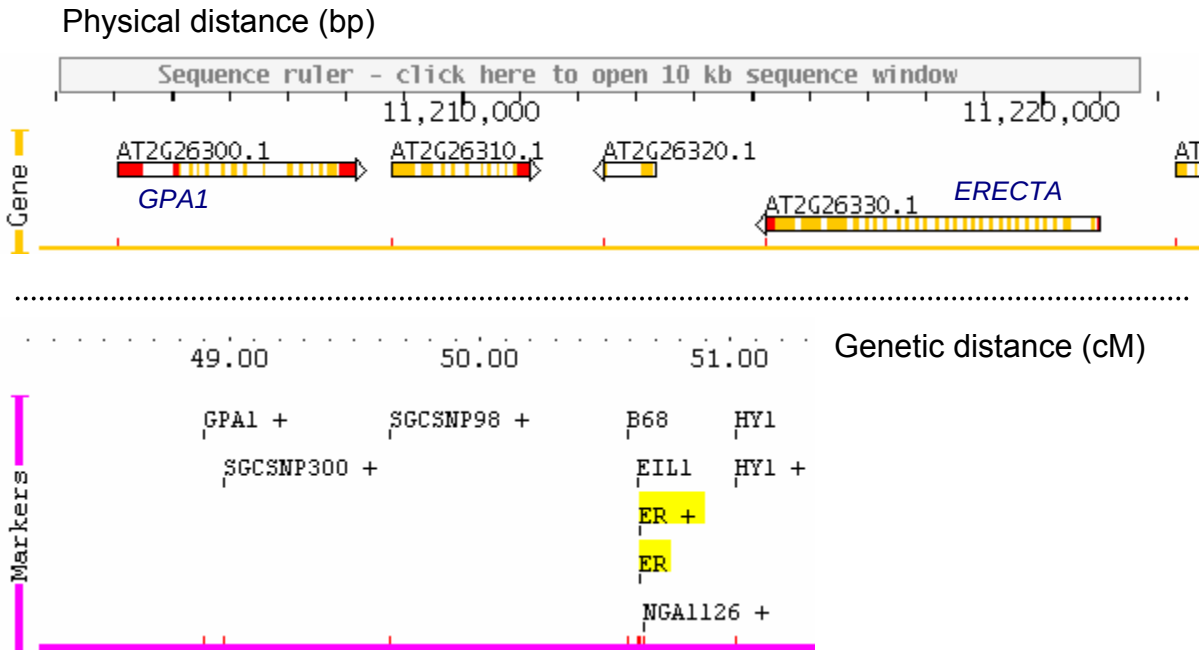



**Figure 13.** Physical (A) and genetic (B) distances between *GPA1* and *ERECTA* (from TAIR, www.arabidopsis.org).

**Table 7.** Growth scores of diploid yeast expressing the GPA1 Cub fusions with stomatal development regulator Nub fusions at the minimum Met concentration (50 µM) on –His/-Ade/-Trp/-Leu media. – indicates no growth, + some growth, ++ moderate growth, and +++ prolific growth.

| | | Interactions with GPA1-Cub-PLV | | | |
|---|---|---|---|---|---|
| Protein | Met conc. | $Nub_{wt}$-Protein | $Nub_G$-Protein | Protein-$Nub_{wt}$ | Protein-$Nub_G$ |
| ERECTA | 50 µM | ++ | - | - | - |
| ERL1 | 50 µM | + | - | +++ | - |
| ERL2 | 50 µM | - | - | + | - |
| SDD1 | 50 µM | - | - | + | - |
| TMM | 50 µM | - | - | ++ | - |

**Table 8.** Growth scores of diploid yeast expressing the GPA1 Nub fusions with stomatal development regulator Cub fusions at the minimum Met concentration (50 µM) on –His/-Ade/-Trp/-Leu media. – indicates no growth, + some growth, ++ moderate growth, and +++ prolific growth.

| | | Interactions with Protein-Cub-PLV fusions | | | |
|---|---|---|---|---|---|
| Protein | Met conc. | $Nub_{wt}$-GPA1 | $Nub_G$-GPA1 | GPA1-$Nub_{wt}$ | GPA1-$Nub_G$ |
| ERECTA | 50 µM | ++ | - | - | - |
| ERL1 | 50 µM | +++ | + | - | - |
| ERL2 | 50 µM | - | - | - | - |
| SDD1 | 50 µM | + | - | - | - |
| TMM | 50 µM | - | - | - | - |

**Table 9.** Matrix of interaction tests performed among known stomatal development regulators. X indicates interaction was not tested. Shown are growth scores of diploid yeast expressing the Nub and Cub fusions on –His/-Ade/-Trp/-Leu media. – indicates no growth at the lowest Met concentration, + some growth, ++ moderate growth, maximum Met concentration growth was observed is specified.

| Cub | ERECTA Nubs | ERL1 Nubs | ERL2 Nubs | SDD1 Nubs | TMM Nubs |
|---|---|---|---|---|---|
| ERECTA | X | - | - | - | - |
| ERL1 | $Nub_G$ERECTA 150 µM, + | X | ERL2$Nub_G$ 150 µM, ++ | X | X |
| ERL2 | - | - | X | X | X |
| SDD1 | - | X | X | X | - |
| TMM | - | X | X | - | X |

# Chapter 6: Heterotrimeric G proteins regulate reproductive trait plasticity in response to water availability


## ABSTRACT

Phenotypic plasticity is the ability for one genotype to display different phenotypes under different environmental conditions. While variation for phenotypic plasticity has been documented in numerous species, little is known about the genetic mechanisms underlying phenotypic plasticity. Given their widespread roles in hormonal and environmental signaling we examined whether genes which encode heterotrimeric G proteins are plasticity genes. We grew multiple alleles of heterotrimeric G protein mutants along with wild type *Arabidopsis thaliana* under different watering regimes to determine the contributions of G protein genes to phenotypic plasticity for a number of developmental and reproduction-related traits. G protein mutations did not significantly affect the amount of phenotypic variation within an environment for any trait, but the mutations significantly affected the mean trait values and/or the amount of phenotypic plasticity for most traits measured. *AGB1*, which encodes the β subunit of the heterotrimeric G protein in Arabidopsis, is a plasticity gene and regulates reproductive trait plasticity in response to water availability, resulting in increased fitness (defined as seed production) under drought stress.


## INTRODUCTION

Heterotrimeric G proteins are multi-subunit GTP binding proteins that function in the transduction of external signals into cellular responses in both animals and plants. According to the paradigm of G protein signaling, the G protein is activated following the binding of a ligand to an associated membrane-bound G protein coupled receptor (GPCR). This binding results in a conformational change in the alpha subunit (Gα) and

the subsequent exchange of GTP for GDP by Gα, resulting in the dissociation of Gα from the beta gamma dimer (Gβγ). Gα and/or Gβγ are then free to interact with downstream signal effectors until the intrinsic GTPase activity of Gα results in the reassembly of the inactive trimer (Assmann, 2002).

In mammals, there are a number of genes which encode heterotrimeric G protein subunits and hundreds of GPCRs have been predicted, resulting in a large, diverse assortment of potential G protein signaling pathways in humans (Fredriksson & Schioth, 2005; McCudden *et al.*, 2005). Numerous ligands have also been identified for mammalian GPCRs including light, sensory molecules including odors and tastes, hormones, neurotransmitters, and bacterial toxins (Civelli, 2005), and mutations in mammalian G protein subunits often result in genetic disorders (Spiegel & Weinstein, 2004; Weinstein *et al.*, 2006).

Plants possess few genes which encode G protein subunits and mutations in these genes, despite their broad expression throughout the plant body, do not result in lethality or in extreme phenotypes under "ideal" laboratory growth conditions (Perfus-Barbeoch *et al.*, 2004). The *Arabidopsis thaliana* genome contains single genes encoding Gα (*GPA1*) (Ma *et al.*, 1990) and Gβ (*AGB1*) (Weiss *et al.*, 1994) subunits, and two known Gγ (*AGG1* and *AGG2*) (Mason & Botella, 2000; Mason & Botella, 2001) genes. GPCR diversity is also reduced in Arabidopsis; only one putative GPCR, GCR1, has been functionally characterized (Pandey & Assmann, 2004) although several dozen additional genes have been predicted to function as GPCRs based on topology prediction (Moriyama *et al.*, 2006; Gookin *et al.*, 2008) and coupling to GPA1 in yeast (Gookin *et al.*, 2008). Recently, two novel GPCR-type G proteins, GTG1 and GTG2, have been identified which interact with GPA1 and possess GTP-binding and GTPase activities. These two proteins bind ABA and are proposed to be a newly identified class of ABA receptors (Pandey *et al.*, 2009). The reduced genetic diversity in heterotrimeric G subunits as well as the identification of a novel class of G proteins in Arabidopsis, suggest that heterotrimeric G protein signaling in plants may not always operate according to the mammalian paradigm (Temple & Jones, 2007).

Despite the paucity of heterotrimeric G protein subunits in the Arabidopsis genome, functional studies of G protein mutants show diverse roles for heterotrimeric G

proteins in germination, development, phytohormone responses (ABA, auxin, brassinosteroids, gibberellins), stress responses (ozone, ROS, pathogens) and stomatal aperture regulation (Ullah *et al.*, 2001; Wang *et al.*, 2001; Ullah *et al.*, 2002; Ullah *et al.*, 2003; Pandey & Assmann, 2004; Joo *et al.*, 2005; Llorente *et al.*, 2005; Pandey *et al.*, 2006; Wang *et al.*, 2007; Fan *et al.*, 2008; Zhang, L *et al.*, 2008; Zhang, W *et al.*, 2008). How a limited number of heterotrimeric G protein subunits can transduce such a large number of hormonal and environmental signals is a fundamental question in plant G protein signaling (Assmann, 2004). Further characterization of GTGs and other unconventional G proteins such as the XLGs (Lee & Assmann, 1999; Ding *et al.*, 2008) and the identification of GPCR ligands, potential tissue specific GPCRs, and G protein signaling effectors in plants may help to elucidate this question. An additional model which has been proposed is that G proteins may serve as signal modulators instead of direct transducers of signals (Fig. 1). By functioning as "cross-talk hubs" G proteins could fine tune a phenotype or physiological response based on multiple signals/environmental inputs (Assmann, 2004). This model also addresses another paradox in plant G protein signaling: why, if G proteins are important to plant physiology are G protein mutations not lethal it plants? A mutation may not be lethal if additional copies or similar versions of the gene exist in the genome or if the expression of the gene is specific to a certain stress (e.g., cold shock) or to a less-vital plant tissue or cell type (e.g., trichomes). However, neither of these situations applies to Arabidopsis since Gα and Gβ are both encoded by single, canonical genes which are widely expressed throughout the plant (Ma *et al.*, 1990; Huang *et al.*, 1994; Lease *et al.*, 2001; Anderson & Botella, 2007). Alternatively, if G proteins function in directing hormonal and environmental cross-talk, they may be required only in production of the "optimal" phenotype and the direct transducers of the signal would still function in the absence of functional G protein subunits.

Plants, being sessile organisms, are hypothesized to have evolved increased phenotypic plasticity, the ability of one genotype to display different phenotypes under different environmental conditions, compared to their mobile animal counterparts (Bradshaw, 1972; Schlichting, 1986; Sultan, 1987; Huey *et al.*, 2002). Heightened plasticity in plants would allow plants to compensate for inescapable and inhospitable

environments. While variation for plasticity has been documented in numerous plant and animal species and many theories have been proposed concerning the ecological and evolutionary significance of this variation, very little is known concerning the explicit genetic machinery which underlies phenotypic plasticity. Two not mutually exclusive models have been proposed concerning the genetic basis of plasticity: allelic sensitivity and gene regulation (Via *et al.*, 1995). The allelic sensitivity model proposes that phenotypic plasticity is the result of the environment directly affecting the expression of genes underlying a trait, whereas the gene regulation model suggests that plasticity is a result of regulatory genes which perceive environmental cues and affect the expression of other genes which underlie the trait. Since both of these mechanisms can result in phenotypic plasticity, genes which exhibit either allelic sensitivity or act as regulatory loci are considered to be plasticity genes.

The phenotypic plasticity (or lack there of) of a trait can be graphically represented by a reaction norm, which is a plot of the mean phenotypic value of the trait in different environmental conditions (Fig. 2). A horizontal reaction norm indicates that the trait lacks plasticity whereas a line with a non-zero slope or a curved line is indicative of phenotypic plasticity. Since plasticity genes control the shape or slope of the reaction norm of a trait, when these genes are mutated it is expected that the shape/slope of the reaction norm will diverge from that of wild type. Differences in reaction norm shapes (plasticities) among genotypes can be detected and tested using ANOVA-based statistical methods. Specifically, a significant genotype x environment/treatment interaction term for a trait indicates that there is variation for plasticity among the genotypes i.e., that the response curves have different shapes/slopes.

The genetic manipulation via mutation approach has been previously applied only in a few instances. For example, in Arabidopsis this approach has been employed to study the genetic basis of phenotypic plasticity in photomorphogenetic responses (Pigliucci & Schmitt, 1999; Pigliucci & Schmitt, 2004). Arabidopsis *hy1* and *hy2* photoreception mutants, which display constitutively active shade avoidance responses, had reduced fitness under some environmental conditions compared to the more plastic wild type (Pigliucci & Schmitt, 1999). This result suggests that *HY1* and *HY2* (which encode a plastid heme oxygenase and a phytochromobolin synthase, respectively) (Muramoto *et*

*al.*, 1999; Kohchi *et al.*, 2001) are plasticity genes. Genetic variation for plasticity has also been documented among wild type accessions of Arabidopsis (Pigliucci & Kolodynska, 2002; Schmuths et al. 2006; Brock & Weinig, 2007) and naturally occurring variation for plasticity within plant species has been assessed in QTL-based phenotypic plasticity studies. Using recombinant inbred lines in lieu of genetic mutants, these studies have lent additional support for the existence of plasticity genes. Studies on recombinant inbred lines of Arabidopsis (Kliebenstein *et al.*, 2002; Ungerer *et al.*, 2003), barley (Lacaze *et al.*, 2009) and poplar (Wu, 1998) found significant QTL x environment interaction for a number of traits and have supported both the allelic sensitivity (Wu, 1998; Kliebenstein *et al.*, 2002; Lacaze *et al.*, 2009) and gene regulation models (Wu, 1998) of phenotypic plasticity

The signaling cross-talk mechanism discussed above may be an important component of phenotypic plasticity, since phenotypes could be tweaked based on multiple environmental inputs. A mutation in a G protein subunit might decrease cross-talk and therefore reduce the plant's ability to adjust a phenotype or response to changing environments based on multiple signals. Specifically, a G protein mutation might affect the degree of plasticity of a trait (Fig. 1 B), the amount of variation within an environment for a trait (a.k.a. the "noisiness" of the trait), or a mutation might simply shift the mean value of the trait away from the wild type value (Fig. 1 C) (Assmann, 2004). Therefore, by studying populations of G protein mutants under multiple environments and under non-optimal conditions we might reveal whether G protein genes are plasticity genes as well as the importance of G proteins to plant fitness. Additionally, previous studies of heterotrimeric G proteins have, to a large extent, focused on guard cell physiology (Wang *et al.*, 2001; Pandey & Assmann, 2004; Fan *et al.*, 2008) and cell division (Ullah *et al.*, 2001; Ullah *et al.*, 2003; Chen *et al.*, 2006). By studying whole-plant phenotypic plasticity responses of G protein mutants we might reveal novel functions of G proteins that could not be identified by previous cell-centered approaches.

Since G protein signaling has been implicated in stomatal aperture regulation (Wang *et al.*, 2001; Pandey & Assmann, 2004; Fan *et al.*, 2008), stomatal density (Zhang, L *et al.*, 2008) and seed and seedling ABA responses (Pandey *et al.*, 2006), we chose G

protein regulation of phenotypic plasticity and plant fitness under drought as the focus of the present study. The following questions were asked:

1. Do G protein mutations alter the shape/slope of reaction norms in response to water availability i.e. are G protein genes "plasticity genes"?
2. Do G protein mutations alter the heights of the reaction norms?
3. Does a G protein mutation affect the level of phenotypic variation within an environment?
4. What are the fitness consequences of G protein mutations in both optimal and non-optimal environments?

Using multiple alleles of *gpa1*, *agb1*, and *gcr1* mutants we found significant variation for plasticity for a number of reproduction-related traits in response to water availability. *agb1* mutants had significantly reduced plasticity for number of fruits and seed number per fruit. Additionally, for flowering time, bolting time, and number of lateral branches, G protein mutations resulted in shifted phenotypic response curves. Interestingly, *agb1* mutants had enhanced fitness under drought stress compared to wild type, but all G protein mutants had reduced fitness under ample water conditions. These data support the hypothesis that heterotrimeric G proteins genes are indeed plasticity genes in plants.

## MATERIALS AND METHODS

### Plant growth conditions and water treatments

All seed used in this experiment were collected from parent plants that were grown together under uniform conditions. *gpa1-3*, *gpa1-4*, *agb1-1*, *agb1-2*, *gcr1-1*, *gcr1-2* and *gpa1-4agb1-2* alleles have all been previously described and were generated using the ecotype Col (Lease *et al.*, 2001; Jones *et al.*, 2003; Ullah *et al.*, 2003; Chen *et al.*, 2004). All mutants are T-DNA insertional mutants with the exception of *agb1-1* which is an EMS generated point mutation (Lease *et al.*, 2001). Genotypes of parent plants were

confirmed via PCR of genomic DNA and seed storage was identical for all seed lots. Cold stratified seeds (stratified at 4°C for 48 hours in darkness on wet filter paper) were directly sown on the surface of a soil mix composed of Miracle-Gro potting mix, Turface Greens Grade fritted clay, and perlite in a 16:8:1 volume ratio. The plants were grown in Kord 90 mm press-fit pots in walk-in a Conviron growth chamber. Photoperiod was 12 hours light (140 $\mu molm^{-2}s^{-1}$, 21°C) and 12 hours dark (19°C) and the relative humidity was 60%.

Three weeks after sowing, plants were treated with one of three watering regimes: ample water, moderate drought, or extreme drought. Plants were individually watered using a bottle-top volumetric dispenser. Plants subjected to ample watering had continually moist soil (≈ 95% of the soil water carrying capacity) and weekly water applications ranging from 55 mL to 170 mL depending on plant age. Severe drought-treated plants had soil which dried completely between watering (≈ 20% of the soil water carrying capacity) and had weekly water applications ranging from 10 mL to 50 mL. Plants receiving the moderate drought treatment received approximately twice the volume of water applied to the severe drought-treated plants (≈ 40% of the soil water carrying capacity). Water application was adjusted for treatment and plant age, and all plants within a treatment received the same amount of water. Relative water content measurements of 5 week-old fully expanded leaves from three blocks showed no significant differences between the genotypes for any watering regime indicating that the levels of drought stress were consistent across genotypes (data not shown). The average leaf relative water content for each treatment was 76% for ample water, 65% for moderate drought and 61% for severe drought.

**Experimental design and response variables**

Plants were arranged in a split-plot design. Three trays, each representing one water level, were clustered in a block, and 12 blocks were placed on separate shelves in the growth chamber. Genotypes were randomly assigned a position within a tray with two genotype replicates per tray. There were 2 replicate plants x 8 genotypes x 3 treatments x

12 blocks for a total population of 576 plants. The transition from vegetative to reproductive growth was assessed by recording bolting time (days from sowing), when 1 cm of inflorescence had emerged, and flowering time (days from sowing), when the first open flower was visible, for each plant. Because of the large population size and the fact that bolting and flowering times were affected by treatment and genotype each plant was individually harvested four weeks after the plant began to flower and the following variables were recorded: inflorescence height (cm), number of primary lateral branches, and number of fruits plus any pistil which showed elongation or swelling. Excised rosettes were dried at 70°C until a constant mass was achieved and dry mass was determined. For three blocks of plants, five fruits were harvested (two from the main inflorescence and three from the lateral branches) and seed number per fruit was determined using a dissecting scope. Aborted or shriveled seeds were excluded from the seeds per fruit measurements. Seed production was estimated for each plant in the 3 blocks which had seeds per fruit measurements as (total fruit number x seeds per fruit).

**Statistical analysis**

Experiment-wide variances for genotype means under ample water and drought stress were calculated using Minitab 15. F-test equal variance tests were performed in Minitab between mutant and wild type trait variances from ample water and severe drought stress treatments. 84 F-tests were performed and the sequential-Bonferroni correction was applied to keep a table-wide $\alpha$ of 0.05 (Rice, 1989).

ANOVA and ANCOVA analyses were performed in SAS 9.1 in order to identify significant sources of phenotypic variation for each trait. A reduced ANOVA model was run for each trait using the Mixed procedure and included fixed effects of genotype, treatment, and genotype x treatment interaction, and block was included in the model as a random effect. A full ANCOVA model was performed using Proc Mixed and included all the reduced model effects and interactions plus rosette mass as a covariat and rosette mass x genotype interaction. Log rosette mass did not account for more variation in the model so untransformed rosette mass was used as the covariat. The covariat was

included in the full model in order to account for possible effects of plant size on the traits measured since plant size has been shown to correlate with fecundity in Arabidopsis (Aarssen & Clauss, 1992). However, it is worth noting that in this weedy annual, plant size does not strictly correlate with fecundity-related traits such as flowering time. For example, flowering time in the Col ecotype of Arabidopsis is dictated by a number of external and genetic parameters (Michaels, 2009) and is not strictly a size-based phenomenon: the Col ecotype will flower at a small size when grown under long day conditions (Sanda *et al.*, 1997).

In accordance with the split-plot experimental design, the whole plot factor, water level, was tested over the whole-plot random error term, water level x block. Genotype and genotype x water level interaction were tested over the residual error. Data and residuals were examined to ensure that all ANOVA assumptions were satisfied. Fruit number and total seed production required transformation (square root) in order to satisfy ANOVA assumptions. The full ANCOVA model was used only in instances in which rosette mass or rosette mass x genotype interaction was significant. In these instances the reported least squares means have been corrected for the effect of the covariat. The sequential Bonferroni correction was applied to minimize inflation of table-wide error from multiple tests (Rice, 1989).

To determine if G protein mutants had significantly different plasticities compared to Col, contrasts were performed using SAS 9.1 to test *a priori*-selected comparisons on all traits which had a significant genotype x water level interaction. Because two alleles of each mutant were studied the contrasts were designed to simultaneously test the plasticity of Col against the plasticities of both mutant alleles of each gene. Combining alleles limited inflation of $\alpha$ while at the same time increasing the biological validity of the experiment by the inclusion of two independent mutant alleles for each gene. If the two mutant alleles of the same gene showed divergent responses, statistically significant differences with Col would not likely be detected. However, reaction norms were examined individually to ensure that alleles of the same gene had similar plasticity responses.

The following contrasts were performed: Col against both alleles combined for *gpa1*, *agb1*, and *gcr1* mutants for ample water vs. moderate drought and ample water vs.

severe drought. The double mutant *gpa1-4agb1-2* was tested against Col, and against both alleles combined of *gpa1* or *agb1* for ample water vs. moderate drought and ample water vs. severe drought. The sequential-Bonferroni correction was applied to adjust for the inflation of type 1 error and to maintain a table-wide α of 0.05 (Rice, 1989). It has been suggested that the sequential Bonferroni correction can be overly stringent when applied to ecological experiments, therefore we also applied biological reasoning when interpreting each contrast (Moran, 2003).

## RESULTS

### Variation for plasticity among genotypes (genotype x environment interactions)

F and p-values for the fixed effects and interactions derived from either the reduced ANOVA model or the full ANCOVA model can be found in Table 1. The covariat, rosette mass, was significant only for inflorescence height and number of lateral branches. For all other traits, rosette mass was not a significant source of variation so the reduced model was used for the analysis for these traits. We found significant genotype x environment interactions for all traits (Table 1). Least squares means and standard errors are listed for all alleles and water levels in Tables S1 and S2. F-tests of specific contrasts between any of the G protein mutants and Col for ample water vs. moderate drought and ample water vs. severe drought revealed that for bolting time, flowering time, rosette mass, and number of lateral branches there were no significant differences in genotype x water level interactions (plasticities) after the application of the sequential Bonferroni correction (Table 2). However, significant differences in plasticities were observed between Col and the G protein mutants for a number of reproduction related traits (Table 2). Interestingly, *agb1* mutants had significantly reduced plasticities compared to Col for fruit number (Fig. 3) and seeds per fruit (Fig. 4) suggesting that *AGB1* functions as a plasticity gene, mediating phenotypic plasticity in the reproductive phase of plant growth in response to water availability. Plasticities of the *gpa1-4 agb1-2* double mutant resembled those of the single *agb1* mutants for all traits and no significant contrasts were

observed between the double mutant and the *agb1* alleles for plasticities relating to any trait (Table 2). *gpa1* mutants had increased plasticity for inflorescence height compared to Col (Fig. 5). *gcr1* mutants had increased plasticity for square root fruit number compared to wild type (Fig. 3).

For number of lateral branches and total seed production, no significant differences were found in plasticities of Col and any of the G protein mutants following the sequential Bonferroni correction (Table 2). However, it should be noted that prior to that statistical correction, contrasts between Col and *agb1* in ample vs. moderate drought ($p= 0.0016$ for lateral branches and $p= 0.0014$ for total seed production) and ample water vs. severe drought ($p= 0.0016$ for lateral branches and $p= 0.0020$ for total seed production) were significant for both traits. Given that the two independent alleles of *agb1*, *agb1-1* and *agb1-2* together show enhanced plasticity for lateral branches (Fig. 6) and reduced plasticity in total seed production (Fig. 7) and that the sequential Bonferroni correction can be overly conservative for the analysis of ecological experiments (Moran, 2003), we feel confident that AGB1 also mediates plasticity for lateral branch number and total seed production. All G protein mutants had reduced fitness, defined here as total seed production, under ample water conditions but the *agb1* mutants and the double mutant *gpa1agb1* had increased fitness under both moderate and severe drought stress compared to Col.

**Genotypic shifts of reaction norms**

For bolting time and flowering time G protein mutations altered the heights of the reaction norms (Figs. 8, 9) while not significantly affecting the amount of plasticity (slope of the reaction norms) compared to Col (Tables 1 and 2). During the vegetative to reproductive phase transition, *agb1* alleles showed increased bolting (Fig. 8a) and flowering (Fig. 8b) times in all environments while *gpa1* and *gcr1* generally showed decreased bolting and flowering times. The double mutant *gpa1-4 agb1-2* showed a phenotype intermediate to *gpa1* and *agb1*, resembling wild type (See Tables S1 and S2 for mean trait values of each allele x water level). While AGB1 mediated plasticity for

lateral branches, G protein mutations also affected across treatment means for number of lateral branches: lateral branching was reduced by 40-50% in *agb1* mutants and to a lesser extent in *gpa1* mutants compared to Col (Fig. 6). The double mutant resembled the single *agb1* mutants in their reduced lateral branching across all environments.

### Phenotypic variance of within genotype x treatment trait means

The within treatment experiment-wide variance of trait means for the mutants and wild type for ample water and severe drought treatments are shown in Table S3. Equal variance tests were performed to determine if the mutants had increased or decreased phenotypic variation compared to wild type within a particular environment (Table S4). After correcting for multiple F-tests using the sequential Bonferroni correction, only one null hypothesis was rejected. *agb1-2* mutants had significantly reduced variance ($p < .0005$) for seed number per fruit under ample water compared to wild type. However, this reduction in variance was not observed in the second *agb1* allele, *agb1-1* ($p=0.168$), which brings into question the biological relevance of this statistically significant observation. Overall, phenotypic variance for traits within a given environment was not significantly impacted by G protein mutations.

## DISCUSSION

### *AGB1* functions as a plasticity gene for a number of reproduction-related traits

Our data show that mutation of the sole Gβ subunit of Arabidopsis, AGB1, results in pleiotropic effects on the extent of plasticity in response to water availability. *agb1* mutants had reduced plasticity for number of fruits, seed number per fruit and total seed production and enhanced plasticity for lateral branches compared to Col. Interestingly, *agb1* mutants had increased seed production per fruit under drought stress compared to wild type and reduced seed production per fruit compared to wild type under well-

watered conditions. The reduced plasticity of *agb1* mutants resulted in enhanced fitness under drought stress but was maladaptive under well-watered conditions. These results suggest that *AGB1* is a plasticity gene since it contributes to the shape of the phenotypic response. Additionally, our findings support the allelic sensitivity model for genetic regulation of phenotypic plasticity since *AGB1* affects both the amount of plasticity and the mean values of the traits. To our knowledge, multiple alleles have not been used previously in mutant studies of phenotypic plasticity. Two independent mutant alleles of *agb1* were used in this study which strengthens our conclusion that *AGB1* functions as a plasticity gene.

AGB1 has been previously shown to function in inflorescence and fruit development. *agb1-1* was originally isolated in a screen for *erecta-like* mutations where it showed a slightly reduced inflorescence height and significantly shortened, blunt-tipped fruits compared to wild type, phenotypes which were later also observed in *agb1-2* (Lease *et al.*, 2001; Ullah *et al.*, 2003). Additionally, it was shown that *AGB1* is expressed ubiquitously throughout the plant but its expression is elevated in flowers and highest in fruits (Lease *et al.*, 2001). The shortened fruit phenotype corresponds to our findings that *agb1* has reduced seeds per fruit. This phenotype is specific to *agb1* mutants and is not observed in *gpa1*, *agg1*, and *agg2* mutants (Ullah *et al.*, 2003; Trusov *et al.*, 2008). Functional selectivity of the Gβ subunit has been reported for other G protein-mediated responses including necrotrophic pathogen resistance (Llorente *et al.*, 2005; Trusov *et al.*, 2006), sugar inhibition of seed germination and lateral root formation (Chen *et al.*, 2006).

According to the paradigm of G protein signaling, activation of GPA1 results in a confirmational change in Gα and the release of Gβγ dimer and signal propagation. Since the reduced plasticity phenotype is present in *agb1* mutants and in *gpa1 agb1* double mutants but not in *gpa1* mutants we can conclude that Gβ is responsible for signal integration or transduction resulting in wild type plasticity for number of fruits, seed number per fruit and total seed production. *gpa1* and *gcr1* mutants showed increased plasticity compared to wild type for inflorescence height and fruit number, respectively. G protein regulation of inflorescence height plasticity is seen only in the *gpa1* mutant which is inconsistent with the conventional model of heterotrimeric G protein signaling

derived from animal-based studies. However it has been argued that, in plants, GPA1 exists primarily in its active GTP-bound form (Temple & Jones, 2007), which could be one explanation for the GPA1-specific regulation of inflorescence height plasticity. Additionally, while GCR1 might function in mediating plasticity for fruit number, there are likely additional unknown GPCRs that contribute to the perception and/or integration of environmental input signals in regards to the regulation of phenotypic plasticity.

**Shifts in reaction norms indicate newly identified roles for G proteins**

Shifts in the reaction norms of G protein mutants for lateral branch number (Fig. 6) and phase change transitions (Figs. 8 and 9) indicate newly identified functions of the heterotrimeric G protein in plants. The most striking example is in primary lateral branch number where *agb1* and *gpa1* mutants display reduced branch number. Lateral branch number is most severely attenuated in *agb1* mutants and the phenotype is also observed in the *gpa1 agb1* double mutants. The classical model of G protein signaling would suggest that lateral branching is mediated via both GPA1 and AGB1, since the lateral branching phenotype is shared by both *gpa1* and *agb1* mutants but is more severe in *agb1*. The α subunit typically requires the β subunit not only for trimer reassembly but also for GPCR association. Therefore, GPA1 activation is eliminated by both the *gpa1* and *agb1* mutations but AGB1 activation is only eliminated by the *agb1* mutation. One key determinant of lateral branch number is apical dominance, the suppression of lateral branching by auxin synthesized in the apical meristem (Leyser, 2005). G proteins have been previously implicated in additional auxin-related processes including cell division (Ullah *et al.*, 2001), lateral and adventitious root formation (Ullah *et al.*, 2003) and root waving (Pandey *et al.*, 2008). It has also been reported that *AGB1* transcript is upregulated and *GPA1* transcript is downregulated in seedlings treated with auxin and microarray analysis identified 47 auxin-regulated genes which are differentially expressed in *agb1-2* vs. Col (Ullah *et al.*, 2003). The lateral branching data presented here provide additional evidence implicating G protein signaling in auxin-related processes.

Phase change transitions also appear to be mediated somewhat via heterotrimeric G proteins (Figs. 8, 9). *agb1* mutants generally had increased bolting and flowering times while *gpa1* and *gcr1* mutants had reduced bolting and flowering times compared to wild type under our conditions. The opposite phenotypes of *gpa1* and *agb1* suggest, according to the classic paradigm of G protein signaling, that phase change signaling occurs via AGB1, because free AGB1 is active and is present in the *gpa1* mutants, while AGB1 is, *de facto*, absent in the *agb1* mutants. It has been previously reported that transgenic lines overexpressing *GCR1* have an early flowering phenotype, however those plants were grown on media in closed Petri plates and under 16 hours of light, conditions dissimilar to our study (Colucci *et al.*, 2002). It should be noted that for some traits we did observe substantial differences between trait means for independent mutant alleles of the same gene (Tables S1 and S2) which reinforces the necessity for using multiple independent insertional mutants for these large studies of phenotypic responses of populations of mutant genotypes. It is important to note that inconsistent phenotypic responses for the *agb1-1* and *agb1-2* mutants have been previously reported and may be due to the nature of the mutations (Ullah *et al.*, 2003). While *agb1-2* is a T-DNA insertional mutant which does not produce a full length AGB1 transcript (Ullah *et al.*, 2003), *agb1-1* is a missense mutation in an intron splice site (Lease *et al.*, 2001). A larger, unstable RNA transcript is observed in *agb1-1* compared to Col corresponding to the presence of the intron. Hypothetical translation of the transcript would result in 20 novel amino acids attached to a truncated AGB1 protein (Lease *et al.*, 2001).

**G protein mutations do not significantly affect the level of phenotypic variation within an environment**

Within a given environment, genetically identical organisms can show divergent phenotypes due to stochasticity in gene expression. These random events among cellular molecules can modify cell status and consequently result in phenotypic changes at organismal level (often thought of as experimental “noise”). Stochasticity is generally thought to be detrimental to fitness, however, it can also be a source of heterogeneity which can provide a fitness benefit in fluctuating environments (Kaern *et al.*, 2005; Raser

& O'Shea, 2005). A G protein mutation might increase or decrease the ability of a cell to buffer itself against stochasticity and therefore it has been hypothesized that G protein mutations might affect the amount of phenotypic variation within an environment (Assmann, 2004). To test this hypothesis we compared the phenotypic variances of the G protein mutants within an environment to the phenotypic variance of wild type within the same environment. We found that the within environment phenotypic variation was not significantly altered by mutations in G protein subunit genes (Table S4) suggesting that this hypothesis is not supported: G protein mutations do not affect the range of possible phenotypes within an environment.

**_agb1_ mutants have enhanced fitness under drought stress**

Based on their previously reported altered stomatal sensitivities to ABA (Wang *et al.*, 2001; Pandey & Assmann, 2004; Fan *et al.*, 2008), it is possible to make predictions concerning the fitness benefits or costs that G protein mutants may incur when grown under drought stress conditions. However, while *gpa1* and *agb1* are hyposensitive in ABA inhibition of stomatal opening but show wild type ABA promotion of stomatal closure (Wang *et al.*, 2001; Fan *et al.*, 2008) and *gcr1* mutants are hypersensitive towards both ABA inhibition of stomatal opening and promotion of closure (Pandey & Assmann, 2004), our detailed phenotypic analysis of whole plant traits under controlled water stress conditions did not support predictions that would be made based on these stomatal response phenotypes. *gcr1* mutants, despite their stomatal hypersensitivity to ABA or their reported improved recovery following drought stress (Pandey & Assmann, 2004) showed no fitness advantage compared to wild type under drought stress or well-watered conditions. While we predicted that *gpa1* and *agb1* mutants would have reduced fitness under drought stress based on the partial ABA insensitivity of their stomatal phenotypes, *agb1* mutants (but not *gpa1* mutants) had increased fitness under drought stress and reduced fitness under well-watered conditions. The pleiotropic nature of G protein mutations may in part explain why the stomatal response phenotypes were not predictive of plant fitness under drought stress. Recently it has been shown that G proteins regulate stomatal density, GPA1 as a positive modulator and AGB1 as a negative regulator (Zhang, L *et al.*, 2008), which may have implications for water loss and carbon

assimilation under different environmental conditions. Additionally, *agb1* mutants, and to a lesser degree *gpa1* mutants, are hypersensitive to ABA inhibition of germination and root growth (Pandey *et al.*, 2006) which may contribute to survival under drought stress. Nevertheless, while the lack of plasticity of *agb1* in reproductive-related traits resulted in a fitness advantage under drought stress, lack of plasticity was maladaptive for *agb1* under well-watered conditions. Therefore G proteins do contribute to plant fitness, in part via the regulation of phenotypic plasticity. Given that climate change can lead to increased variability in environments and resources, this finding could have important agronomical implications since *agb1* mutants have reduced plasticity and therefore more stable yields across environments.

## CONCLUSION

Phenotypic analysis of G protein mutants under multiple environmental conditions has identified novel functions of plant heterotrimeric G proteins in regulating lateral branch number, vegetative to reproductive transitions, and in phenotypic plasticity of inflorescence height, seed number per fruit, fruit number and total seed production. We also found that the known altered guard cell sensitivities towards ABA did not predict the fitness outcomes of the mutants under drought stress. All G protein mutants studied had reduced fitness under well-watered as well as drought environments with the exception of the *agb1* mutants which had improved fitness under drought stress conditions. These results thus speak to one of the apparent paradoxes of plant G-protein signaling: while G-protein mutation is not lethal, it does, in fact, result in non-optimal phenotypes under some environmental conditions.

G proteins mediate responses to ABA, auxin, brassinosteroids, gibberellins, and sugars; environmental signals including ozone and pathogens; and intrinsic, unknown developmental cues mediating leaf shape, stomatal density, and fruit shape. A fundamental question in G protein signaling is how only two heterotrimeric G protein combinations (GPA1/AGB1/AGG1 and GPA1/AGB1/AGG2) can transduce such a diversity of signals. Our results, implicating heterotrimeric G proteins in mediating phenotypic plasticity responses, support the model that G proteins function, at least in

part, as cross-talk hubs; integrating signals, rather than directly transducing them (Assmann, 2004), thereby tweaking a phenotype relative to the environment at hand. More studies are warranted in which G protein regulation of plasticity is examined across additional environmental gradients as well as across generations in order to further elucidate this novel contribution of heterotrimeric G proteins to plant development and fitness. While QTL-based studies have supported the existences of plasticity genes, this present study is one of a few that has directly tested the regulation of phenotypic plasticity by specific genes. Additional plasticity studies with other environmental signaling mutants will be integral to our understanding of whether plasticity genes are rare or common in plant and other genomes and also to gain insight into the genetic basis of phenotypic plasticity.

## LITERATURE CITED


**Aarssen LW, Clauss MJ. 1992.** Genotypic variation in fecundity allocation in *Arabidopsis thaliana*. *Journal of Ecology* **80**: 109-114.

**Anderson DJ, Botella JR. 2007.** Expression analysis and subcellular localization of the *Arabidopsis thaliana* G-protein β-subunit AGB1. *Plant Cell Rep* **26**: 1469-1480.

**Assmann SM. 2002.** Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. *Plant Cell* **14**: S355-S373.

**Assmann SM. 2004.** Plant G proteins, phytohormones, and plasticity: Three questions and a speculation. *Sci STKE* **2004**: re20.

**Bradshaw AD. 1972.** Some of the evolutionary consequences of being a plant. *Evolutionary Biology* **5**: 25-47.

**Brock MT, Weinig C. 2007.** Plasticity and environment-specific covariances: an investigation of floral-vegetative and within flower correlations. *Evolution* **61**: 2913-2924.

**Chen JG, Gao Y, Jones AM. 2006.** Differential roles of *Arabidopsis* heterotrimeric G-protein subunits in modulating cell division in roots. *Plant Physiol* **141**: 887-897.

**Chen JG, Pandey S, Huang J, Alonso JM, Ecker JR, Assmann SM, Jones AM. 2004.** GCR1 can act independently of heterotrimeric G-protein in response to brassinosteroids and gibberellins in *Arabidopsis* seed germination. *Plant Physiol* **135**: 907-915.

**Civelli O. 2005.** GPCR deorphanizations: The novel, the known and the unexpected transmitters. *Trends in Pharmacological Sciences* **26**: 15-19.

**Colucci G, Apone F, Alyeshmerni N, Chalmers D, Chrispeels MJ. 2002.** GCR1, the putative *Arabidopsis* G protein-coupled receptor gene is cell cycle-regulated, and its overexpression abolishes seed dormancy and shortens time to flowering. *Proc Natl Acad Sci U S A* **99**: 4736-4741.

**Ding L, Pandey S, Assmann SM. 2008.** *Arabidopsis* extra-large G proteins (XLGS) regulate root morphogenesis. *Plant J* **53**: 248-263.

**Fan LM, Zhang W, Chen JG, Taylor JP, Jones AM, Assmann SM. 2008.** Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ- and RGS-deficient *Arabidopsis* lines. *Proc Natl Acad Sci U S A* **105**: 8476-8481.

**Fredriksson R, Schioth HB. 2005.** The repertoire of G-protein-coupled receptors in fully sequenced genomes. *Molecular Pharmacology* **67**: 1414-1425.

**Gookin TE, Kim J, Assmann SM. 2008.** Whole proteome identification of plant candidate G-protein coupled receptors in *Arabidopsis*, rice, and poplar: Computational prediction and *in-vivo* protein coupling. *Genome Biol* **9**: R120.

**Huang H, Weiss CA, Ma H. 1994.** Regulated expression of the *Arabidopsis* G protein α subunit gene GPA1. *International Journal of Plant Sciences* **155**: 3.

**Huey RB, Carlson M, Crozier L, Frazier M, Hamilton H, Harley C, Hoang A, Kingsolver JG. 2002.** Plants versus animals: Do they deal with stress in different ways? *Integrative and Comparative Biology* **42**: 415-423.

**Jones AM, Ecker JR, Chen JG. 2003.** A reevaluation of the role of the heterotrimeric G protein in coupling light responses in *Arabidopsis*. *Plant Physiol* **131**: 1623-1627.

**Joo JH, Wang SY, Chen JG, Jones AM, Fedoroff NV. 2005.** Different signaling and cell death roles of heterotrimeric G protein α and β subunits in the *Arabidopsis* oxidative stress response to ozone. *Plant Cell* **17**: 957-970.

**Kaern M, Elston TC, Blake WJ, Collins JJ. 2005.** Stochasticity in gene expression: From theories to phenotypes. *Nat Rev Genet* **6**: 451-464.

**Kliebenstein DJ, Figuth A, Mitchell-Olds T. 2002.** Genetic architecture of plastic methyl jasmonate responses in *Arabidopsis thaliana*. *Genetics* **161**: 1685-1696.

**Kohchi T, Mukougawa K, Frankenberg N, Masuda M, Yokota A, Lagarias JC. 2001.** The *Arabidopsis HY2* gene encodes phytochromobilin synthase, a ferredoxin-dependent biliverdin reductase. *Plant Cell* **13**: 425-436.

**Lacaze X, Hayes PM, Korol A. 2009.** Genetics of phenotypic plasticity: QTL analysis in barley, *Hordeum vulgare*. *Heredity* **102**: 163-173.

**Lease KA, Wen J, Li J, Doke JT, Liscum E, Walker JC. 2001.** A mutant *Arabidopsis* heterotrimeric G-protein β subunit affects leaf, flower, and fruit development. *Plant Cell* **13**: 2631-2641.

**Lee YR, Assmann SM. 1999.** *Arabidopsis thaliana* 'extra-large GTP-binding protein' (AtXLG1): A new class of G-protein. *Plant Mol Biol* **40**: 55-64.

**Leyser O. 2005.** The fall and rise of apical dominance. *Curr Opin Genet Dev* **15**: 468-471.

**Llorente F, Alonso-Blanco C, Sanchez-Rodriguez C, Jorda L, Molina A. 2005.** ERECTA receptor-like kinase and heterotrimeric G protein from *Arabidopsis* are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. *Plant Journal* **43**: 165-180.

**Ma H, Yanofsky MF, Meyerowitz EM. 1990.** Molecular cloning and characterization of *GPA1*, a G protein α subunit gene from *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* **87**: 3821-3825.

**Mason MG, Botella JR. 2000.** Completing the heterotrimer: Isolation and characterization of an *Arabidopsis thaliana* G protein γ-subunit cDNA. *Proc Natl Acad Sci U S A* **97**: 14784-14788.

**Mason MG, Botella JR. 2001.** Isolation of a novel G-protein γ-subunit from *Arabidopsis thaliana* and its interaction with Gβ. *Biochim Biophys Acta* **1520**: 147-153.

**McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS. 2005.** G-protein signaling: Back to the future. *Cellular and Molecular Life Sciences* **62**: 551-577.

**Michaels SD. 2009.** Flowering time regulation produces much fruit. *Curr Opin Plant Biol* **12**: 75-80.

**Moran MD. 2003.** Arguments for rejecting the sequential Bonferroni in ecological studies. *Oikos* **100**: 403-405.

**Moriyama E, Strope P, Opiyo S, Chen Z, Jones A. 2006.** Mining the *Arabidopsis thaliana* genome for highly-divergent seven transmembrane receptors. *Genome Biology* **7**: R96.

**Muramoto T, Kohchi T, Yokota A, Hwang I, Goodman HM. 1999.** The *Arabidopsis* photomorphogenic mutant *hy1* is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase. *Plant Cell* **11**: 335-348.

**Pandey S, Assmann SM. 2004.** The *Arabidopsis* putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signaling. *Plant Cell* **16**: 1616-1632.

**Pandey S, Chen JG, Jones AM, Assmann SM. 2006.** G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. *Plant Physiol* **141**: 243-256.

**Pandey S, Monshausen GB, Ding L, Assmann SM. 2008.** Regulation of root-wave response by extra large and conventional G proteins in *Arabidopsis thaliana*. *Plant J* **55**: 311-322.

**Pandey S, Nelson DC, Assmann SM. 2009.** Two novel GPCR-type G proteins are abscisic acid receptors in *Arabidopsis*. **136**: 136-148.

**Perfus-Barbeoch L, Jones AM, Assmann SM. 2004.** Plant heterotrimeric G protein function: Insights from *Arabidopsis* and rice mutants. *Curr Opin Plant Biol* **7**: 719-731.

**Pigliucci M, Kolodynska A. 2002.** Phenotypic plasticity and integration in response to flooded conditions in natural accessions of *Arabidopsis thaliana* (L.) Heynh (Brassicaceae). *Ann Bot* **90**: 199-207.

**Pigliucci M, Schmitt J. 1999.** Genes affecting phenotypic plasticity in *Arabidopsis*: Pleiotropic effects and reproductive fitness of photomorphogenic mutants. *Journal of Evolutionary Biology* **12**: 551-562.

**Pigliucci M, Schmitt J. 2004.** Phenotypic plasticity in response to foliar and neutral shade in gibberellin mutants of *Arabidopsis thaliana*. *Evolutionary Ecology Research* **6**: 243-259.

**Raser JM, O'Shea EK. 2005.** Noise in gene expression: Origins, consequences, and control. *Science* **309**: 2010-2013.

**Rice WR. 1989.** Analyzing tables of statistical tests. *Evolution* **43**: 223-225.

**Sanda S, John M, Amasino R. 1997.** Analysis of flowering time in ecotypes of *Arabidopsis thaliana*. *J Hered* **88**: 69-72.

**Schlichting C 1986.** The evolution of phenotypic plasticity in plants. *Annu Rev Ecol Syst* **17**:667-693.

**Schmuths H, Bachmann K, Weber WE, Horres R, Hoffmann MH. 2006.** Effects of preconditioning and temperature during germination for 73 natural accessions of *Arabidopsis thaliana*. *Ann Bot* **97**: 623-34.

**Spiegel AM, Weinstein LS. 2004.** Inherited diseases involving G proteins and G protein-coupled receptors. *Annu Rev Med* **55**: 27-39.

**Sultan SE. 1987.** Evolutionary implications of phenotypic plasticity in plants. *Evolutionary Biology* **21**: 127-178.

**Temple BR, Jones AM. 2007.** The plant heterotrimeric G-protein complex. *Annu Rev Plant Biol* **58**: 249-266.

**Trusov Y, Rookes JE, Chakravorty D, Armour D, Schenk PM, Botella JR. 2006.** Heterotrimeric G proteins facilitate *Arabidopsis* resistance to necrotrophic pathogens and are involved in jasmonate signaling. *Plant Physiol* **140**: 210-220.

**Trusov Y, Zhang W, Assmann SM, Botella JR. 2008.** Gγ1 + Gγ2 ≠ Gβ: Heterotrimeric G protein Gγ-deficient mutants do not recapitulate all phenotypes of Gβ-deficient mutants. *Plant Physiol* **147**: 636-649.

**Ullah H, Chen JG, Temple B, Boyes DC, Alonso JM, Davis KR, Ecker JR, Jones AM. 2003.** The β-subunit of the *Arabidopsis* G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. *Plant Cell* **15**: 393-409.

**Ullah H, Chen JG, Wang SC, Jones AM. 2002.** Role of a heterotrimeric G protein in regulation of *Arabidopsis* seed germination. *Plant Physiology* **129**: 897-907.

**Ullah H, Chen JG, Young JC, Im KH, Sussman MR, Jones AM. 2001.** Modulation of cell proliferation by heterotrimeric G protein in *Arabidopsis*. *Science* **292**: 2066-2069.

**Ungerer MC, Halldorsdottir SS, Purugganan MA, Mackay TFC. 2003.** Genotype-environment interactions at quantitative trait loci affecting inflorescence development in *Arabidopsis thaliana*. *Genetics* **165**: 353-365.

**Via S, Gomulkiewicz R, Dejong G, Scheiner SM, Schlichting CD, Vantienderen PH. 1995.** Adaptive phenotypic plasticity - consensus and controversy. *Trends in Ecology & Evolution* **10**: 212-217.

**Wang S, Narendra S, Fedoroff N. 2007.** Heterotrimeric G protein signaling in the *Arabidopsis* unfolded protein response. *Proc Natl Acad Sci U S A* **104**: 3817-3822.

**Wang XQ, Ullah H, Jones AM, Assmann SM. 2001.** G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. *Science* **292**: 2070-2072.

**Weinstein LS, Chen M, Xie T, Liu J. 2006.** Genetic diseases associated with heterotrimeric G proteins. *Trends Pharmacol Sci* **27**: 260-266.

**Weiss CA, Garnaat CW, Mukai K, Hu Y, Ma H. 1994.** Isolation of cDNAs encoding guanine nucleotide-binding protein β-subunit homologues from maize (ZGB1) and Arabidopsis (AGB1). *Proc Natl Acad Sci U S A* **91**: 9554-9558.

**Wu RL. 1998.** The detection of plasticity genes in heterogeneous environments. *Evolution* **52**: 967-977.

**Zhang L, Hu G, Cheng Y, Huang J. 2008.** Heterotrimeric G protein α and β subunits antagonistically modulate stomatal density in *Arabidopsis thaliana*. *Dev Biol* **324**: 68-75.

**Zhang W, He SY, Assmann SM. 2008.** The plant innate immunity response in stomatal guard cells invokes G-protein-dependent ion channel regulation. *Plant J* **56**: 984-996.

**Table 1** ANOVA or ANCOVA F-values and (p-values) for main effects and interactions for all traits

| Source | Bolting time | Flowering time | Inflor. height | Sqrt fruit number | Lateral branches | Rosette mass | Seeds per fruit | Sqrt total seeds |
|---|---|---|---|---|---|---|---|---|
| Genotype | 85.71 **(<.0001)** | 52.55 **(<.0001)** | 6.24 **(<.0001)** | 9.92 **(<.0001)** | 1.31 (0.2414) | 27.95 **(<.0001)** | 9.09 **(<.0001)** | 7.01 **(<.0001)** |
| Water level | 27.68 **(<.0001)** | 12.23 **(0.0003)** | 121.50 **(<.0001)** | 321.46 **(<.0001)** | 16.17 **(<.0001)** | 759.39 **(<.0001)** | 179.4 **(0.0001)** | 289.64 **(<.0001)** |
| Genotype x water level | 2.88 **(0.0003)** | 2.97 **(0.0002)** | 5.11 **(<.0001)** | 13.94 **(<.0001)** | 93.64 **(<.0001)** | 7.96 **(<.0001)** | 3.51 **(<.0001)** | 2.69 **(0.0020)** |
| Rosette mass (covariant) | N/A | N/A | 35.21 **(<.0001)** | N/A | 3.39 **(<.0001)** | N/A | N/A | N/A |
| Rosette mass x genotype | N/A | N/A | 2.37 (0.0218) | N/A | 8.27 **(<.0001)** | N/A | N/A | N/A |

N/A indicates covariant was not included in the model, underlined p-values are significant before the Bonferroni correction, bolded p-values are significant following a sequential Bonferroni correction.

**Table 2** p-values from contrasts testing for significant differences in genotype x water level interactions (plasticities) between the specified genotypes for the specified water levels: A (ample water), M (moderate drought), S (severe drought)

| Contrast | Bolting time | Flowering time | Inflor. height | Sqrt fruit number | Rosette mass | Seeds per fruit | Sqrt seed number | Lateral branches |
|---|---|---|---|---|---|---|---|---|
| Col vs. both *gpa1* A vs. M | 0.0731 | 0.551 | **0.0002** | 0.6011 | 0.3219 | 0.5651 | 0.9886 | 0.9378 |
| Col vs. both *gpa1* A vs. S | 0.0014 | 0.0011 | **<.0001** | 0.2664 | 0.0299 | 0.1319 | 0.2520 | 0.9413 |
| Col vs. both *agb1* A vs. M | 0.5329 | 0.911 | 0.3804 | **<.0001** | 0.0097 | **0.0003** | 0.0014 | 0.0016 |
| Col vs. both *agb1* A vs. S | 0.3596 | 0.7977 | 0.9071 | 0.0069 | 0.0026 | **<.0001** | 0.0020 | 0.0016 |
| Col vs. *gpa1-4agb1-2* A vs. M | 0.7913 | 0.9155 | 0.1361 | **<.0001** | 0.0040 | 0.0081 | 0.0220 | 0.1342 |
| Col vs. *gpa1-4agb1-2* A vs. S | 0.7514 | 0.3157 | 0.0208 | 0.1212 | 0.0047 | 0.0077 | 0.0572 | 0.0906 |
| Col vs. both *gcr1* A vs. M | 0.3580 | 0.5147 | 0.8188 | 0.0025 | 0.9922 | 0.8084 | 0.9326 | 0.6722 |
| Col vs. both *gcr1* A vs. S | 0.6762 | 0.0958 | 0.6926 | **<.0001** | 0.2350 | 0.2017 | 0.9715 | 0.8862 |
| Both *gpa1* vs. *gpa1-4agb1-2* A vs. M | 0.1408 | 0.6392 | 0.0202 | **<.0001** | **<.0001** | **0.0004** | 0.0091 | 0.0513 |
| Both *gpa1* vs. *gpa1-4agb1-2* A vs. S | 0.0053 | 0.0383 | 0.0346 | 0.0043 | **<.0001** | 0.1092 | 0.2794 | 0.0457 |
| Both *agb1* vs. *gpa1-4agb1-2* A vs. M | 0.3556 | 0.8155 | 0.0034 | 0.4968 | 0.4449 | 0.5572 | 0.6068 | 0.0938 |
| Both *agb1* vs. *gpa1-4agb1-2* A vs. S | 0.2063 | 0.1617 | 0.0018 | 0.3714 | 0.7898 | 0.3176 | 0.3887 | 0.1636 |

Underlined p-values are significant before application of the sequential Bonferroni correction, bolded p-values are significant after the sequential Bonferroni correction.

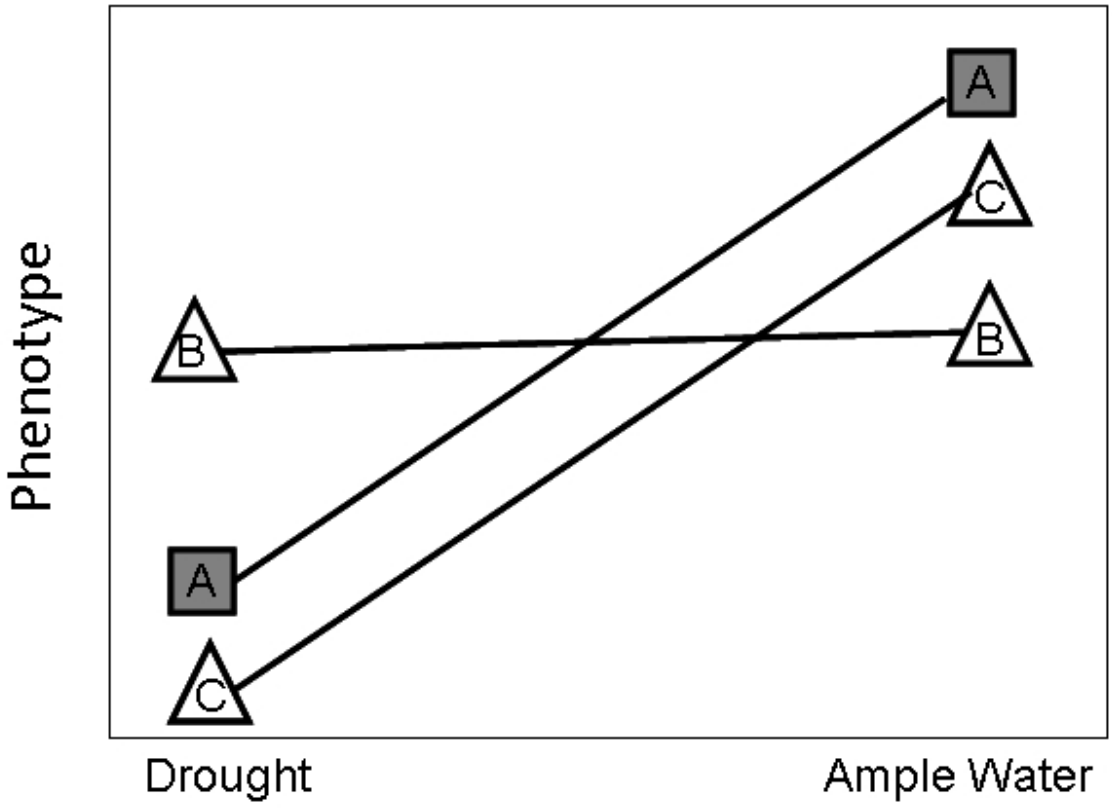



**Figure 1.** Reaction norms for a theoretical trait for wild type (A) and "mutants" (B and C). The triangles and squares represent the mean phenotypic value in a particular environment. A mutation could modify the reaction norm by affecting the shape/slope of the line (B) i.e. plasticity, or by shifting the reaction norm away from wild type mean values (C).

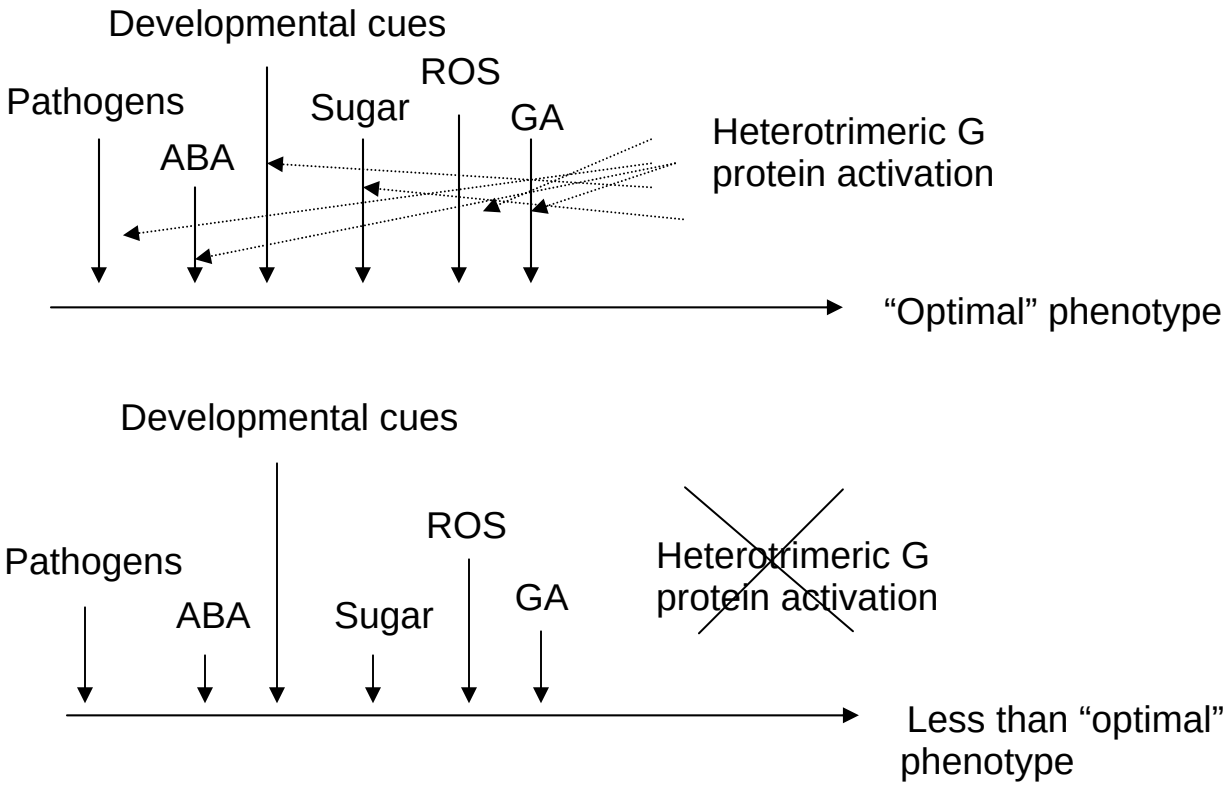






**Figure 2.** Cross-talk model for of heterotrimeric G protein signaling. Heterotrimeric G proteins might function in hormonal and environmental cross talk by integrating different signal inputs resulting in the optimal phenotype (top). In the absence of heterotrimeric G proteins, hormonal cross talk is reduced resulting in reduced signal integration and a less than optimal phenotype (bottom).

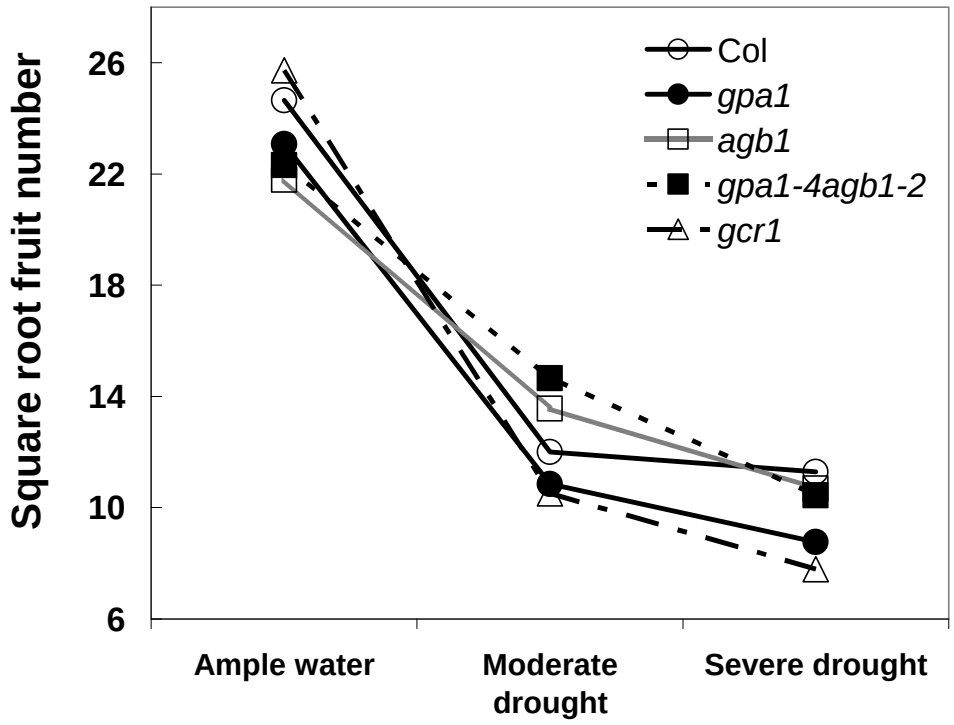



**Figure 3.** *agb1* and *gcr1* mutants have reduced and enhanced plasticity, respectively, for fruit number in response to water availibility. Reaction norms are shown for square root transformed number of fruits. *gpa1*, *agb1*, and *gcr1* means shown are combined least squares means of the individual alleles, as done in the contrasts.

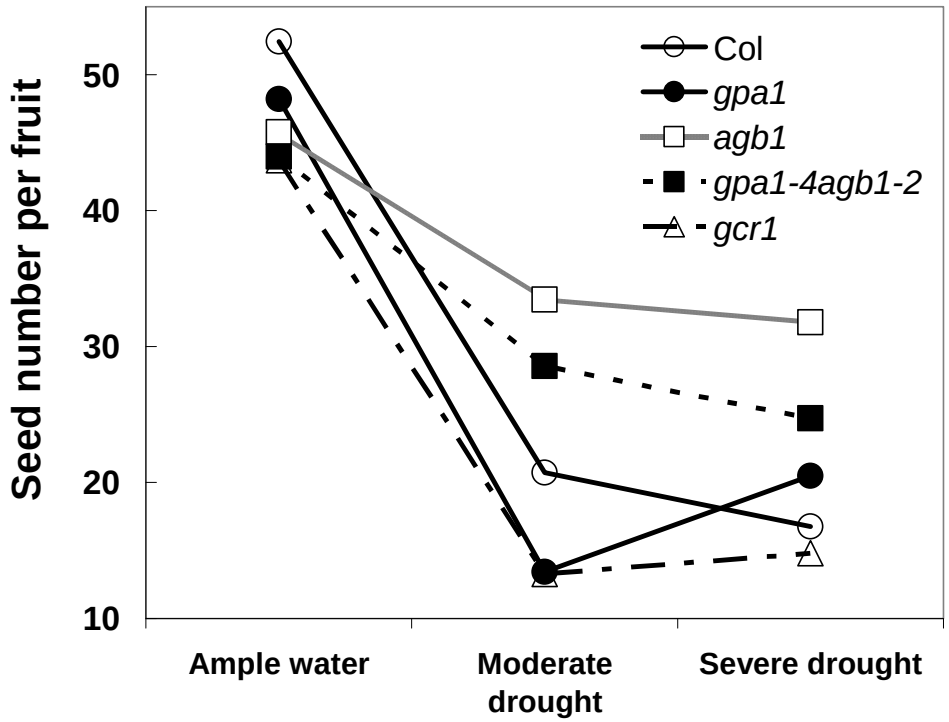



**Figure 4.** *agb1* mutants have reduced plasticity for seed number per fruit in response to water availability. Reaction norms are shown for seed number per fruit. *gpa1*, *agb1*, and *gcr1* means shown are combined least squares means of the individual alleles, as done in the contrasts.

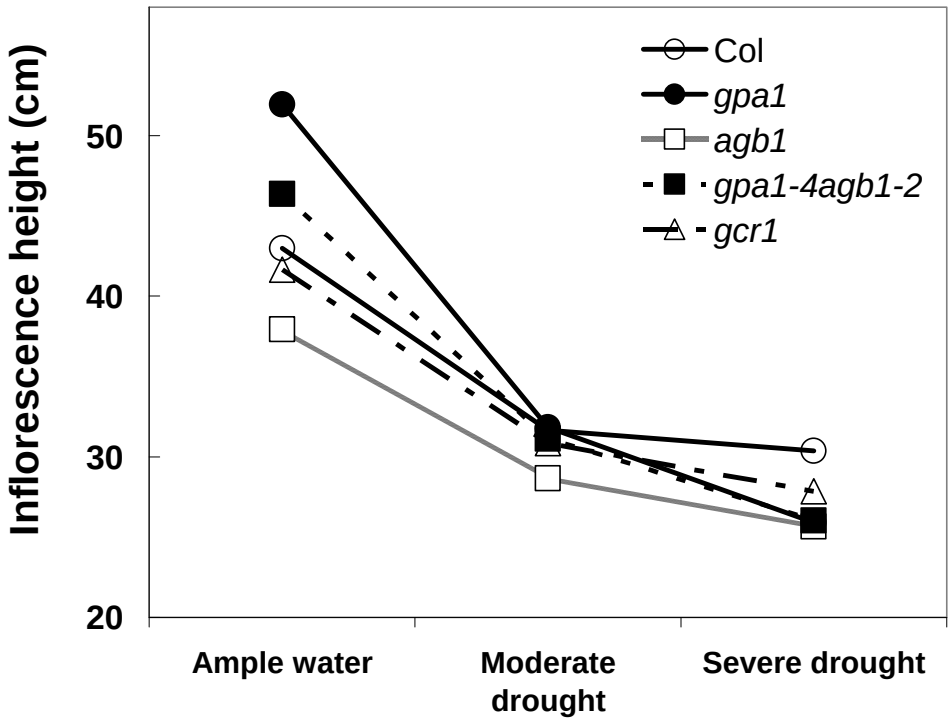



**Figure 5.** *gpa1* mutants show enhanced plasticity for inflorescence height in response to water availability. Reaction norms are shown for inflorescence height. *gpa1*, *agb1*, and *gcr1* means shown are combined means from the least squares means of the individual alleles, as done in the contrasts. Least squares means were corrected for the covariant.

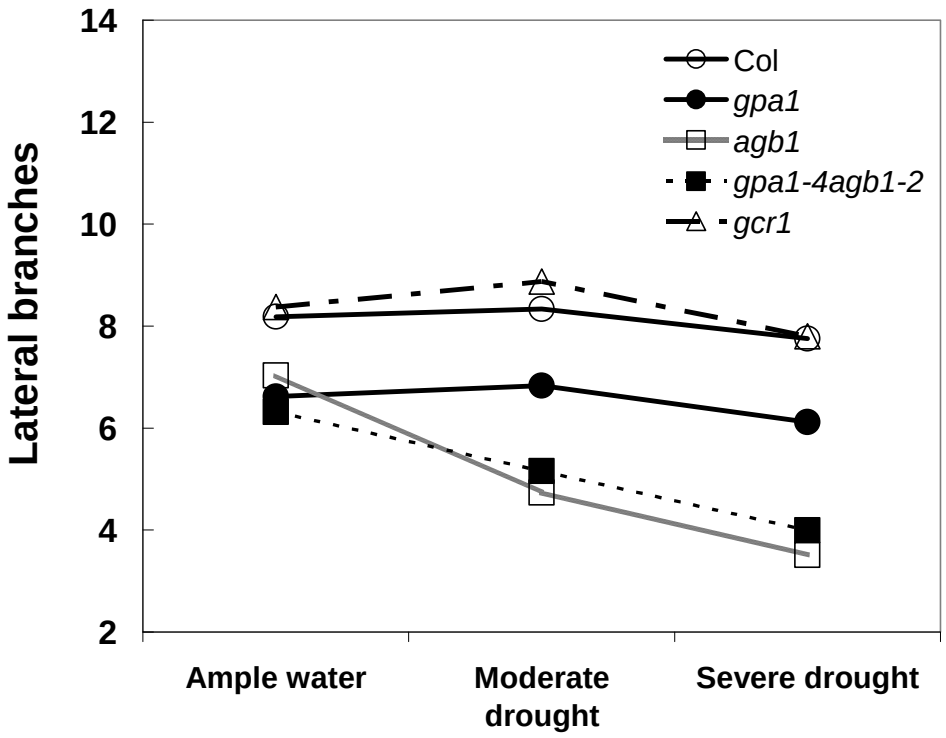



**Figure 6.** *agb1* mutants have increased plasticity for lateral branch number in response to water availability. Reaction norms are shown for number of lateral branches for all alleles. *gpa1*, *agb1*, and *gcr1* means shown are combined means from least squares means of the individual alleles, as done in the contrasts. Least squares means were corrected for the effect of the covariant.

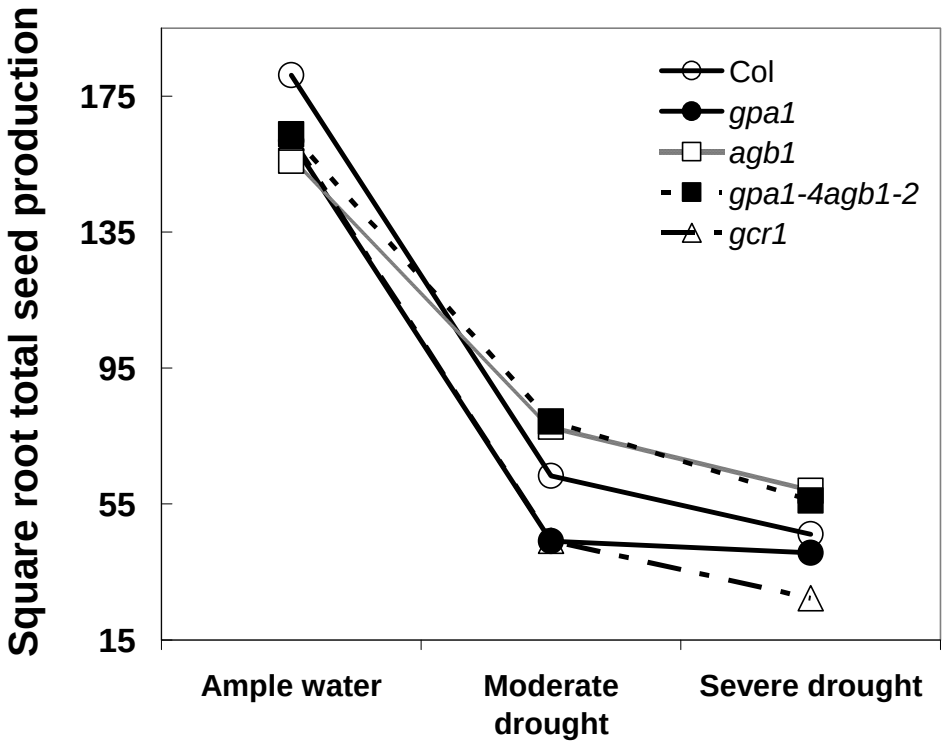



**Figure 7.** *agb1* mutants have reduced plasticity for total seed production in response to water availability and increased fitness under drought conditions compared to wild type. Reaction norms are shown for square root transformed total seed production. *gpa1*, *agb1*, and *gcr1* means shown are combined means from the least squares means of individual alleles, as done in the contrasts.

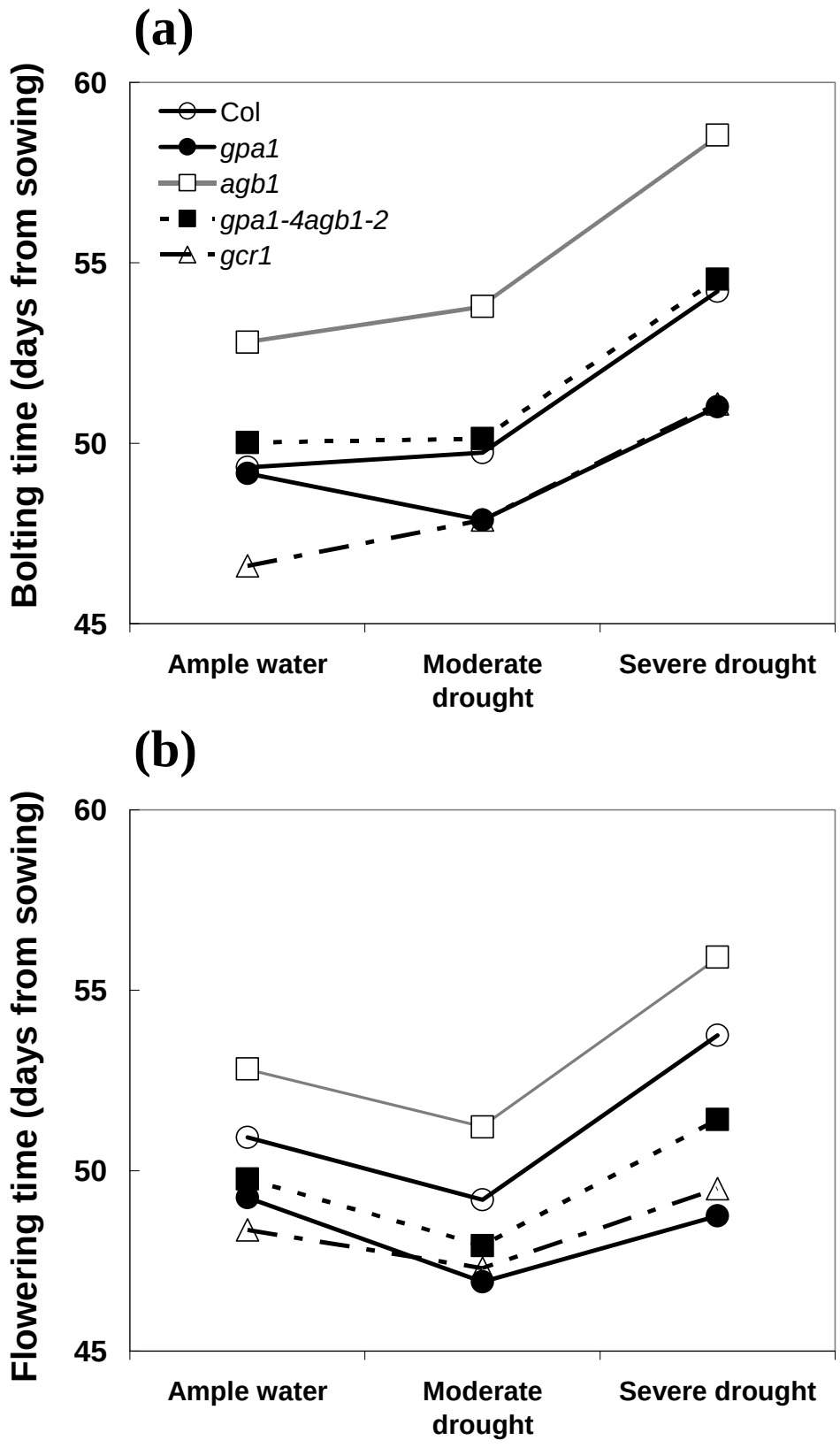



**Figure 8.** Heterotrimeric G proteins are regulators of bolting and flowering time in Arabidopsis. Reaction norms are shown for bolting time (a) and flowering time (b). *gpa1*, *agb1*, and *gcr1* means shown are combined means from the least squares means of the individual alleles, as done in the contrasts.

**Table S1** Least squares means and standard errors for traits under ample water (A), moderate drought (M), and severe drought (S)

| | | Bolting time | | Flowering time | | Lateral branches | | Rosette mass (mg) | |
|---|---|---|---|---|---|---|---|---|---|
| Genotype | Water level | Mean | S.E. | Mean | S.E. | Mean | S.E. | Mean | S.E. |
| Col | A | 49.33 | 0.91 | 50.92 | 0.92 | 8.18 | 0.56 | 606.7 | 18.92 |
| *gpa1-3* | A | 50.21 | 0.91 | 50.00 | 0.92 | 7.05 | 0.59 | 700 | 18.92 |
| *gpa1-4* | A | 48.13 | 0.91 | 48.50 | 0.92 | 6.18 | 0.44 | 573.3 | 18.92 |
| *agb1-1* | A | 54.76 | 0.91 | 54.68 | 0.93 | 6.96 | 0.46 | 600 | 19.09 |
| *agb1-2* | A | 50.83 | 0.91 | 50.96 | 0.92 | 7.10 | 0.36 | 475.6 | 18.92 |
| *gpa1-4agb1-2* | A | 50.01 | 0.92 | 49.77 | 0.94 | 6.31 | 0.41 | 513.2 | 19.27 |
| *gcr1-1* | A | 45.88 | 0.91 | 47.46 | 0.92 | 8.58 | 0.40 | 510.9 | 18.92 |
| *gcr1-2* | A | 47.33 | 0.91 | 49.25 | 0.92 | 8.17 | 0.51 | 605.5 | 18.92 |
| Col | M | 49.74 | 0.91 | 49.19 | 0.93 | 8.34 | 0.26 | 288.2 | 19.09 |
| *gpa1-3* | M | 48.79 | 0.91 | 47.63 | 0.92 | 5.83 | 0.23 | 332.1 | 18.92 |
| *gpa1-4* | M | 46.96 | 0.91 | 46.21 | 0.92 | 7.83 | 0.27 | 263 | 18.92 |
| *agb1-1* | M | 55.00 | 0.91 | 52.21 | 0.92 | 4.41 | 0.24 | 298.6 | 18.92 |
| *agb1-2* | M | 52.58 | 0.91 | 50.21 | 0.92 | 5.05 | 0.30 | 247.8 | 18.92 |
| *gpa1-4agb1-2* | M | 50.13 | 0.91 | 47.92 | 0.92 | 5.15 | 0.28 | 264.6 | 18.92 |
| *gcr1-1* | M | 47.25 | 0.91 | 46.50 | 0.92 | 9.03 | 0.31 | 234.1 | 18.92 |
| *gcr1-2* | M | 48.50 | 0.91 | 48.08 | 0.92 | 8.71 | 0.29 | 244.8 | 18.92 |
| Col | S | 54.21 | 0.91 | 53.75 | 0.92 | 7.75 | 0.38 | 190 | 18.92 |
| *gpa1-3* | S | 52.88 | 0.93 | 50.95 | 0.95 | 4.76 | 0.34 | 187.5 | 19.47 |
| *gpa1-4* | S | 49.14 | 0.91 | 46.55 | 0.93 | 7.47 | 0.38 | 162 | 19.09 |
| *agb1-1* | S | 60.29 | 0.91 | 57.55 | 0.93 | 3.24 | 0.32 | 199.9 | 19.09 |
| *agb1-2* | S | 56.79 | 0.91 | 54.28 | 0.93 | 3.78 | 0.42 | 167.7 | 19.27 |
| *gpa1-4agb1-2* | S | 54.54 | 0.91 | 51.42 | 0.92 | 4.00 | 0.41 | 164.8 | 18.92 |
| *gcr1-1* | S | 51.17 | 0.91 | 49.58 | 0.92 | 8.21 | 0.41 | 163.1 | 18.92 |
| *gcr1-2* | S | 51.01 | 0.91 | 49.41 | 0.93 | 7.40 | 0.38 | 168.9 | 19.09 |

-

**Table S2** Least squares means and standard errors for traits under ample water (A), moderate drought (M), and severe drought (S)

| | | Inflorescence height (cm) | | Square root fruits | | Seeds per fruit | | Square root total seeds | |
|---|---|---|---|---|---|---|---|---|---|
| Genotype | Water level | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Col | A | 42.98 | 1.79 | 24.66 | 0.62 | 52.44 | 3.03 | 181.21 | 7.50 |
| *gpa1-3* | A | 49.34 | 1.83 | 22.72 | 0.62 | 49.30 | 3.03 | 163.28 | 7.50 |
| *gpa1-4* | A | 54.57 | 1.41 | 23.41 | 0.62 | 47.10 | 3.03 | 160.36 | 7.50 |
| *agb1-1* | A | 37.37 | 1.45 | 20.34 | 0.62 | 46.17 | 3.03 | 147.19 | 7.50 |
| *agb1-2* | A | 38.52 | 1.15 | 23.25 | 0.62 | 45.33 | 3.03 | 164.73 | 7.50 |
| *gpa1-4agb1-2* | A | 46.36 | 1.31 | 22.31 | 0.63 | 43.97 | 3.03 | 163.80 | 8.13 |
| *gcr1-1* | A | 41.71 | 1.28 | 25.12 | 0.62 | 44.40 | 3.03 | 158.92 | 7.50 |
| *gcr1-2* | A | 41.58 | 1.61 | 26.32 | 0.62 | 43.03 | 3.03 | 166.69 | 7.50 |
| Col | M | 31.65 | 0.89 | 12.00 | 0.62 | 20.72 | 3.03 | 63.62 | 7.50 |
| *gpa1-3* | M | 32.05 | 0.83 | 11.62 | 0.62 | 12.47 | 3.32 | 44.70 | 8.13 |
| *gpa1-4* | M | 31.61 | 0.91 | 10.08 | 0.62 | 14.38 | 3.03 | 43.43 | 7.50 |
| *agb1-1* | M | 28.05 | 0.85 | 12.73 | 0.62 | 31.54 | 3.03 | 73.37 | 7.50 |
| *agb1-2* | M | 29.23 | 1.00 | 14.40 | 0.62 | 35.33 | 3.03 | 82.15 | 7.50 |
| *gpa1-4agb1-2* | M | 31.12 | 0.94 | 14.66 | 0.62 | 28.57 | 3.03 | 79.20 | 7.50 |
| *gcr1-1* | M | 31.83 | 1.03 | 10.89 | 0.62 | 9.55 | 3.03 | 32.46 | 7.50 |
| *gcr1-2* | M | 29.89 | 0.97 | 10.16 | 0.62 | 16.98 | 3.03 | 55.95 | 7.50 |
| Col | S | 30.36 | 1.22 | 11.28 | 0.62 | 16.78 | 3.03 | 46.16 | 7.50 |
| *gpa1-3* | S | 27.67 | 1.11 | 9.76 | 0.64 | 21.18 | 3.03 | 46.35 | 7.50 |
| *gpa1-4* | S | 24.14 | 1.22 | 7.77 | 0.62 | 19.82 | 3.03 | 34.81 | 7.50 |
| *agb1-1* | S | 25.36 | 1.06 | 10.22 | 0.62 | 30.87 | 3.03 | 55.72 | 7.50 |
| *agb1-2* | S | 25.96 | 1.34 | 11.15 | 0.62 | 32.70 | 3.03 | 62.12 | 7.50 |
| *gpa1-4agb1-2* | S | 26.02 | 1.30 | 10.45 | 0.62 | 24.73 | 3.03 | 56.03 | 7.50 |
| *gcr1-1* | S | 28.14 | 1.32 | 7.62 | 0.62 | 14.53 | 3.03 | 26.01 | 7.50 |
| *gcr1-2* | S | 27.56 | 1.23 | 7.97 | 0.62 | 15.05 | 3.03 | 28.65 | 7.50 |

**Table S3** Variance and (n) of genotype means in ample water and severe drought treatments

| **Genotype** | Col | *gpa1-3* | *gpa1-4* | *agb1-1* | *agb1-2* | *gcr1-1* | *gcr1-2* | *gpa1-4 agb1-2* |
|---|---|---|---|---|---|---|---|---|
| **Bolting time** | | | | | | | | |
| Ample water | 15.45 (24) | 11.91 (24) | 11.33 (24) | 9.78 (23) | 10.75 (24) | 7.24 (24) | 8.49 (24) | 9.56 (22) |
| Severe drought | 18.96 (24) | 12.85 (21) | 16.09 (23) | 17.78 (23) | 23.15 (23) | 25.01 (24) | 18.86 (23) | 13.39 (24) |
| **Flowering time** | | | | | | | | |
| Ample water | 14.60 (24) | 11.39 (24) | 10.09 (24) | 11.02 (23) | 11.78 (24) | 9.13 (24) | 10.02 (24) | 8.52 (22) |
| Severe drought | 24.02 (24) | 16.60 (21) | 16.43 (23) | 13.70 (23) | 32.86 (23) | 33.47 (24) | 22.89 (23) | 17.04 (24) |
| **Rosette dry mass** | | | | | | | | |
| Ample water | 7.6 (24) | 9.9 (24) | 10.2 (24) | 12.2 (23) | 8.0 (24) | 7.0 (24) | 10.0 (24) | 7.3 (22) |
| Severe drought | 2.1 (24) | 3.9 (21) | 4.2 (23) | 3.4 (23) | 1.3 (22) | 2.6 (24) | 2.7 (24) | 2.1 (24) |
| **Inflorescence height** | | | | | | | | |
| Ample water | 9.37 (24) | 7.94 (24) | 12.91 (24) | 10.53 (23) | 21.13 (24) | 12.01 (24) | 20.67 (24) | 15.40 (22) |
| Severe drought | 4.87 (24) | 15.24 (21) | 21.19 (23) | 7.12 (23) | 10.33 (23) | 12.53 (24) | 5.75 (24) | 19.71 (24) |
| **Lateral branches** | | | | | | | | |
| Ample water | 1.51 (24) | 0.85 (24) | 1.85 (24) | 1.17 (23) | 1.43 (24) | 1.56 (24) | 1.28 (24) | 1.17 (22) |
| Severe drought | 0.96 (24) | 0.85 (21) | 4.15 (23) | 2.29 (23) | 0.26 (23) | 2.81 (24) | 3.09 (23) | 0.49 (24) |
| **Fruit number** | | | | | | | | |
| Ample water | 20545 (24) | 13367 (24) | 4713 (24) | 14840 (23) | 21323 (24) | 17308 (24) | 13620 (24) | 11970 (22) |
| Severe drought | 2107 (24) | 1517 (24) | 1687 (23) | 1014 (23) | 508 (23) | 2069 (24) | 2224 (23) | 1874 (24) |
| **Seeds per silique** | | | | | | | | |
| Ample water | 26.92 (6) | 23.88 (6) | 9.76 (6) | 7.05 (6) | 0.411 (6) | 76.69 (6) | 182.5 (6) | 3.799 (6) |
| Severe drought | 127.74 (6) | 15.43 (6) | 69.78 (6) | 4.04 (6) | 25.84 (6) | 73.14 (6) | 23.88 (6) | 3.259 (6) |

**Table S4** F-values and (p-values) from equal variance tests of single mutant and wild type mean variances under ample water and severe drought treatments

| **Bolting time** | *gpa1-3* | *gpa1-4* | *agb1-1* | *agb1-2* | *gcr1-1* | *gcr1-2* | *gpa1-4 agb1-2* |
|---|---|---|---|---|---|---|---|
| Ample water | 1.30 (0.538) | 1.36 (0.463) | 1.58 (0.289) | 1.44 (0.389) | 2.13 (0.076) | 1.82 (0.159) | 1.62 (0.273) |
| Severe drought | 1.48 (0.358) | 1.18 (0.697) | 1.07 (0.880) | 0.82 (0.636) | 0.76 (0.512) | 1.00 (0.991) | 1.42 (0.411) |
| **Flowering time** | | | | | | | |
| Ample water | 1.28 (0.556) | 1.45 (0.382) | 1.32 (0.505) | 1.24 (0.611) | 1.60 (0.267) | 1.46 (0.373) | 1.71 (0.219) |
| Severe drought | 1.45 (0.407) | 1.46 (0.397) | 1.75 (0.193) | 0.73 (0.461) | 0.72 (0.432) | 1.05 (0.913) | 1.41 (0.416) |
| **Rosette dry mass** | | | | | | | |
| Ample water | 0.77 (0.531) | 0.75 (0.486) | 0.62 (0.267) | 0.95 (0.903) | 1.09 (0.845) | 0.76 (0.516) | 1.04 (0.931) |
| Severe drought | 0.53 (0.145) | 0.49 (0.099) | 0.61 (0.245) | 1.56 (0.312) | 0.80 (0.589) | 0.77 (0.531) | 1.00 (0.991) |
| **Inflorescence height** | | | | | | | |
| Ample water | 1.18 (0.694) | 0.73 (0.448) | 0.89 (0.781) | 0.44 (0.057) | 0.75 (0.492) | 0.45 (0.064) | 0.61 (0.247) |
| Severe drought | *0.32* (0.008) | *0.23* (0.001) | 0.68 (0.373) | 0.47 (0.080) | *0.39* (0.028) | 0.85 (0.694) | 0.25 (0.001) |
| **Lateral branches** | | | | | | | |
| Ample water | 1.76 (0.181) | 0.81 (0.622) | 1.28 (0.563) | 1.05 (0.907) | 0.96 (0.929) | 1.18 (0.694) | 1.29 (0.567) |
| Severe drought | 1.13 (0.794) | *0.23* (0.001) | *0.42* (0.041) | *3.72* (0.003) | *0.34* (0.012) | *0.31* (0.007) | 1.94 (0.120) |
| **Fruit number** | | | | | | | |
| Ample water | 1.54 (0.310) | 4.36 (0.001) | 1.38 (0.449) | 0.96 (0.928) | 1.19 (0.684) | 1.51 (0.331) | 1.72 (0.218) |
| Severe drought | 1.39 (0.462) | 1.25 (0.605) | 2.08 (0.091) | *4.15* (0.001) | 1.02 (0.969) | 0.95 (0.896) | 1.12 (0.782) |
| **Seeds per silique** | | | | | | | |
| Ample water | 1.13 (0.899) | 2.76 (0.290) | 3.82 (0.168) | ***65.5*** **(<.0005)** | 0.35 (0.275) | 0.15 (0.056) | 7.09 (0.051) |
| Severe drought | *8.28* (0.037) | 1.83 (0.523) | *31.6* (0.002) | 4.94 (0.104) | 1.75 (0.555) | 1.07 (0.943) | 39.2 (0.001) |

Bold font indicates significant difference after the sequential Bonferroni correction, underlines show significant difference before the correction

# Chapter 7 Heterotrimeric G proteins regulate cross-generational plasticity in *Arabidopsis thaliana*


## ABSTRACT

Phenotypic plasticity is the ability for one genotype to display different phenotypes under different environmental conditions. Cross-generational plasticity is a form of phenotypic plasticity in which the environment of the parent affects the phenotype of the offspring. Genetic variation for cross-generational plasticity has been documented in both plant and animal species but very little is known concerning the specific genes and genetic mechanisms underlying this phenomenon. Heterotrimeric G proteins are eukaryotic proteins that function in signal transduction and development in both animals and plants. We have previously shown that heterotrimeric G proteins of the model plant *Arabidopsis thaliana* regulate within-generation phenotypic plasticity in response to water availability for a number of fitness-associated traits. Therefore, we examined whether G proteins also regulate Arabidopsis cross-generational plasticity in response to water availability. We found significant genotype x parent treatment interactions, i.e. genetic variation for cross-generational plasticity, for bolting time, flowering time, lateral branch number, and fruit number among G protein mutant and wild type plants. Genotypes harboring mutations in the sole Gα (GPA1) and the sole Gβ (AGB1) subunit had significantly reduced or absent cross-generational plasticity compared to Col. This loss of cross-generational plasticity had differential effects on plant fitness depending on the environment of the offspring. Our data demonstrate that G proteins are required for specific cross-generational plasticity responses in Arabidopsis, and are, to our knowledge, among the first gene products to be implicated in regulating this fascinating source of phenotypic variation.

## INTRODUCTION

Phenotypic plasticity is the ability for one genotype to display different phenotypes when grown under different environmental conditions. It has been hypothesized that plants, due to their sessile natures, may have heightened plasticity compared to animals since plasticity may contribute to plant survival in inhospitable or resource-poor environments, from which plants cannot escape (Bradshaw, 1972; Sultan, 1987; Huey *et al.*, 2002). One specific form of phenotypic plasticity is cross-generational plasticity, in which the environment of the parent affects the offspring phenotype (Donohue and Schmitt, 1998; Sultan, 2004).

Cross-generational plasticity in response to different parental environmental factors including nutrients (Wulff and Bazzaz, 1992; Sultan, 1996), $CO_2$ (Andalo et al., 1998), light (Sultan, 1996; Andalo et al., 1999; Galloway, 2005), drought (Sultan, 1996; Riginos et al., 2007), photoperiod (Munir et al., 2001), temperature (Lacey et al., 1997; Andalo et al., 1999; Blodner et al., 2007), and herbivory (Agrawal et al., 1999; Agrawal, 2001) has been reported in a number of different plants species, including Arabidopsis (Andalo et al., 1998; Andalo et al., 1999; Munir et al., 2001; Blodner et al., 2007). Cross-generational plasticity may be adaptive in plants species which have rapid generation times and limited dispersal ranges, since the parent environment is potentially highly correlated with the offspring environment (Donohue and Schmitt, 1998). Such is the case with Arabidopsis, which has a rapid generation time and a pod-shattering seed dispersal system, resulting in parental and offspring environments that are temporally and spatially similar. Though not widely understood, possible mechanisms for cross-generational plasticity include but are not limited to: alterations in resource provisioning to seed or pollen, gene expression changes via inheritance of parental mRNA transcripts or proteins, environmentally induced megaspore or pollen selection, alteration of seed coat properties resulting in changes in dormancy and germination timing, and changes to dispersal systems (Mazer and Gorchov, 1996; Donohue and Schmitt, 1998; Donohue, 2009). Epigenetic effects such environmentally induced alterations in DNA methylation or histone modification (Kakutani, 2002; Meaney and Szyf, 2005; Henderson and Jacobsen, 2007) may also contribute to cross-generational plasticity, although this has yet to be

specifically established. Many of the cross-generational plasticity studies in plants cited above and others (see, Donohue and Schmitt, 1998) report significant genetic variation for cross-generational plasticity when natural populations or recombinant inbred lines (RILs) are evaluated, suggesting that cross-generational plasticity is under genetic control, however the genes underlying cross-generational plasticity, especially with regard to offspring adult traits, have not been determined. Arabidopsis is an ideal study system to explore the genetic mechanisms of cross-generational plasticity because it has a sequenced genome and single gene knockout mutants of Arabidopsis are widely available. Additionally, as mentioned above, genetic variation for cross-generational plasticity has been observed in Arabidopsis and its life history and dispersal strategy suggest that cross-generational plasticity may be adaptive in this species.

Heterotrimeric G proteins are GTP-binding proteins that function in the transduction of extracellular ligands into intracellular responses. G proteins are integral to eukaryotic cell function and physiology as defects in heterotrimeric G protein signaling in mammals have been linked to numerous diseases (Simonds, 2003; Spiegel and Weinstein, 2004; Weinstein et al., 2006). In the basic paradigm of heterotrimeric G protein signaling, the inactive protein exists as a trimer consisting of a Gα subunit bound to GDP, a Gβ subunit, and a Gγ subunit. When a ligand binds to a G protein coupled receptor (GPCR) associated with the trimer, a conformational changes occurs in the G protein and Gα exchanges GDP for GTP. The protein then dissociates into a Gα-GTP subunit and Gβγ dimer, which propagate the signal via interactions with downstream signal effectors. The G protein stays in the active state until the intrinsic GTPase activity of Gα results in the hydrolysis of GTP to GDP and subsequent reassociation of the inactive trimer.

The Arabidopsis genome contains one Gα gene (*GPA1*) (Ma *et al.*, 1990), one Gβ gene (*AGB1*) (Weiss *et al.*, 1994), and two genes which encode Gγ subunits (*AGG1* and *AGG2*) (Mason and Botella, 2000, 2001). Approximately a dozen putative GPCRs have also been identified in Arabidopsis, via bioinformatics (Moriyama et al., 2006; Gookin et al., 2008) and interaction assays with GPA1 (Pandey and Assmann, 2004; Gookin et al., 2008). While heterotrimeric G protein diversity is greatly reduced in plant (Assmann, 2002) as compared to animal genomes (Fredriksson and Schioth, 2005; McCudden et al.,

2005), analysis of Arabidopsis heterotrimeric G protein mutants has identified numerous functions for G proteins in plants, including environmental and hormonal signaling, cell division, development, and stomatal aperture regulation (Perfus-Barbeoch *et al.*, 2004).

Recently we have shown that heterotrimeric G proteins regulate phenotypic plasticity in response to water availability for a number of fecundity-related traits (Chapter 3). *agb1* mutants exhibited reduced plasticity for fruit number and seeds per fruit compared to wild type plants, resulting in greater seed production under drought stress, suggesting the *AGB1* functions as a "plasticity gene". Therefore, we investigated whether G proteins may also function in the regulation of cross-generational plasticity. Using heterotrimeric G protein mutants of Arabidopsis we found that heterotrimeric G proteins do regulate cross-generational plasticity for bolting and flowering time, lateral branch number, and fruit number and are, to our knowledge, the first proteins to be directly implicated in mediating cross-generational plasticity responses of offspring adult developmental and reproductive traits.

## MATERIALS AND METHODS

### Parental plant growth conditions and water treatments

All seed used to generate parent plants were collected from plants that were grown together under uniform conditions. The mutant alleles used have been previously described and are T-DNA insertional mutants (Jones et al., 2003; Ullah et al., 2003) except for *agb1-1* which is a point mutation (Lease *et al.*, 2001). Parent plant growth conditions and treatments were previously reported in Nilson and Assmann (submitted, Chapter 6). Briefly, following cold stratification (4°C for 48 hours in darkness on wet filter paper), seeds were directly sown on the surface of a soil mix composed of Miracle-Gro potting mix, Turface Greens Grade fritted clay, and perlite in a 16:8:1 volume ratio. The plants were grown in Kord 90 mm press-fit pots in walk-in Conviron growth chamber. Photoperiod was 12 hours light (140 $\mu molm^{-2}s^{-1}$, 21°C) and 12 hours dark (19°C) and the relative humidity was 60%. Plants were arranged in 12 blocks according to a split-plot experimental design. Three weeks after sowing, plants were treated with

one of two watering regimes: ample water or moderate drought. Plants were individually watered using a bottle-top volumetric dispenser. Plants subjected to ample watering had continually moist soil (≈ 95% of the soil water carrying capacity) and weekly water applications ranging from 55 mL to 170 mL depending on plant age. Plants receiving the moderate drought treatment received 20 mL to 100 mL of water weekly (≈ 40% of the soil water carrying capacity). The drought treatment of the parent plants is termed moderate drought because the plants received double the quantity of water of severely drought treated plants which showed wilting and had dry soil in between waterings. The severe drought treatment is described in Nilson and Assmann (submitted, Chapter 6) but those plants were not used as parent plants in this study. Water application was adjusted for treatment and plant age, and all plants within a treatment received the same amount of water. Relative water content measurements of 5 week-old fully expanded leaves showed no significant differences between the genotypes for either watering regime indicating that the levels of drought stress were consistent between genotypes (data not shown). The average leaf relative water content was 76% for ample water and 65% for moderate drought. The inflorescence of each plant was individually harvested four weeks after the onset of flowering. All seed was harvested within a three week period. Seeds were dried at room temperature under uniform conditions for six months, then threshed and stored at room temperature in plastic microcentrifuge tubes for three months before they were used for the cross-generational plasticity experiments.

**Cross-generational plasticity of offspring adult traits: offspring growth conditions and water treatments**

Seeds from replicate parent plants were combined together from one block and this seed was used for assessment of cross-generational plasticity of offspring plants. Seed was stratified and sown exactly as described for the parent plants. Resulting offspring plants were subjected to either ample water or moderate drought watering regimes exactly as described above, i.e. regimes identical to those applied to their parents.

**Cross-generational plasticity of offspring adult traits: experimental design and response variables**

Plants were arranged in 12 blocks in a split-plot experimental design. Each block consisted of 4 trays on one shelf of the growth chamber. Two adjacent trays received the moderate drought treatment, and the other two trays received the ample water treatments. Because two alleles of the Gα and Gβ mutants were used, replicate number for Col was doubled and the plants were randomly assigned to one of two Col groups. For the experiment there were 3 replicate plants x 6 genotypes x 2 parent treatments x 2 offspring treatments x 12 blocks for a total initial population size of 864 individual plants, however plant mortality resulted in a final population size of 842 offspring plants.

Days from sowing to bolting (defined as 1 cm of inflorescence emergence) and days from sowing to flowering (when the first open flower was visible) were recorded for each plant. Each plant was individually harvested four weeks after its onset of flowering date; this accommodated the large population size as well as treatment effects on flowering time. At harvest the following traits were measured: inflorescence height (cm), number of primary lateral branches, and total fruit number (mature fruits plus pistils which showed swelling and elongation). Rosettes were harvested and dried at 70°C until a constant mass was achieved for rosette dry mass measurement. For three blocks, five fruits were randomly sampled and seed length (mm) and seed number per fruit was obtained. For the three blocks which had seeds per fruit measurements, total seed production was calculated by multiplying total number of fruits x seed number per fruit.

**Statistical analysis**

ANOVA and ANCOVA analyses were performed using SAS 9.1 Proc Mixed. The reduced ANOVA model included genotype, parent treatment, and offspring treatment as fixed main effects and block as a random effect. The following interaction terms were also included in the reduced model: genotype x parent treatment and genotype x offspring treatment. Parent treatment x offspring treatment, and genotype x offspring treatment x parent treatment interactions were originally included in the model but were

later removed from the model because they were not significant for any traits. The full ANCOVA model included all the effects and interactions of the reduced model plus a covariant, log rosette mass and log rosette mass x genotype interaction. This covariant was included in the full model to account for possible effects of plant size on fecundity-related traits. Plant size has been shown to correlate with fitness in Arabidopsis (Aarssen and Clauss, 1992) however not all fecundity-associated traits are highly correlated with plant size. For example, the Col ecotype of Arabidopsis will flower early, at a small size, when grown under long day conditions (Sanda *et al.*, 1997). The split-plot experimental design dictated that the whole plot factor, offspring treatment, be tested over the whole-plot random error term, offspring treatment x block. All other effects and interactions were tested over the residual error. For all ANOVA and ANCOVA analyses, data and residuals were examined to ensure that the statistical assumptions were met. Fruit number and total seed number required square root transformation in order to meet the statistical assumptions of ANOVA. The full ANCOVA model was used only for traits where log rosette mass or log rosette mass x genotype interaction was significant (bolting time, flowering time and seed number per fruit). The reduced model was used in all other instances (number of lateral branches, square root fruit number, inflorescence height, fruit length and square root total seed production). To minimize inflation of table-wide statistical error due to multiple tests, the sequential Bonferroni correction (Rice, 1989) was applied to the table of p-values. Contrasts were performed on traits which showed significant genotype x offspring treatment (variation for phenotypic plasticity) or significant genotype x parent treatment (variation for cross-generational phenotypic plasticity) interactions, in order to test *a priori*-selected hypotheses. If the ANCOVA model was used for a trait, the corrected LSMs were used for the contrasts. Both alleles of *gpa1* were tested against both groups of Col and both alleles of *agb1* were tested against both groups of Col. Again, the Bonferroni correction was applied to reduce the inflation of type 1 error.

## RESULTS

### Within-generation and across -generation plasticity of offspring traits: overview

Table 1 shows the ANOVA/ANCOVA results for all traits. There were no significant parent treatment x genotype x offspring treatment (parent environment affects genotypic variation for offspring phenotypic plasticity) or parent treatment x offspring treatment (parent environment affects offspring phenotypic plasticity) interactions for any trait so these higher order interactions were removed from the models. Significant genotype x offspring treatment interactions (genetic variation for within-generation plasticity in response to offspring environment) were observed for number of lateral branches, inflorescence height, seed number per fruit, and fruit length. Genotype x offspring treatment interaction for square root total seed production was significant before application of the sequential Bonferroni correction ($p=0.0027$) however the p-value was slightly larger than the cut-off ($p=0.0023$) after application of the correction. Note that the sequential Bonferroni correction can be overly cautious for ecological experiments (Moran, 2003) and therefore we are interpreting this result as significant. Interestingly, for bolting time, flowering time, lateral branch number, and square root of fruit number, genotype x parent treatment interaction was significant indicating that the genotypes showed variation for cross-generational plasticity for these traits.

### With in generation plasticity: genetic variation for phenotypic plasticity in response to offspring environment

To determine if *gpa1* or *agb1* plants had significantly different plasticities in response to offspring environment compared to Col plants, contrasts were performed on genotype x offspring LSMs (and therefore averaged across parent treatment) and the results are shown in Table 2. For number of lateral branches both *gpa1* and *agb1* had plasticities which differed significantly from Col. *agb1* mutants had reduced plasticity for lateral branch number while *gpa1* had enhanced plasticity (Fig. 1). For inflorescence height, *agb1* mutants had significantly reduced plasticity compared to Col ($p = 0.0011$, Fig. 2). *agb1* mutants also had significantly reduced phenotypic plasticity for fruit length

($p < .0001$) and seed number per fruit ($p < .0001$) compared to Col (Figs. 3-4). Significant variation for plasticity was observed for square root total seed production, with *agb1* have significantly less plasticity ($p = 0.0009$) compared to Col (Fig. 5). The reduction in plasticity for total seed production resulted in *agb1* having increased fitness under drought stress compared to Col.

Our results are similar to a previous study where we reported that *AGB1* functions as a plasticity gene and mediates phenotypic plasticity in response to water availability for a number of fitness related traits (Nilson and Assmann, submitted, Chapter 6). In both experiments, we found that *agb1* mutants had reduced plasticity for seed number per fruit as well as total seed production, resulting in increased seed production (fitness) by *agb1* plants under drought stress. Though not measured in the previous study, the present study revealed that *agb1* mutants also had reduced plasticity for fruit length, which is consistent with the seeds per fruit data. For inflorescence height and lateral branch number the results of this experiment are not consistent with our previous findings. In the previous experiment, *agb1* mutants showed significantly enhanced plasticity compared to Col for lateral branch number (Nilson and Assmann, submitted, Chapter 6) but in the present experiment *agb1* mutants showed reduced plasticity for lateral branching. However, as discussed below, there was also significant genotype x parent treatment interaction for lateral branch number, with *agb1* having reduced plasticity for lateral branching. The confounding effect of variation for cross-generational plasticity may have reduced the amount of measurable phenotypic plasticity for lateral branches in the offspring generation. It is important to note that the present study is not an exact replication of the previous experiment because the dual parental environments (ample water and moderate drought) in which the seeds developed likely contributed additional variation to most traits when examined across offspring treatments.

**Variation for cross-generational plasticity: G proteins regulate cross-generational plasticity of offspring traits**

Contrasts were performed on the LSMs for genotype x parent treatment in order to determine which genotypes significantly differed from wild type for cross-generational plasticity (the means were therefore averaged across offspring environment). Both *gpa1*

alleles were compared against both Col alleles for parent treatment ample water vs parent treatment moderate drought and both *agb1* alleles were compared against both Col alleles for parent treatment ample water vs. parent treatment drought. The contrast results are shown in Table 3. All contrasts were significant, indicating that *gpa1* and *agb1* mutants have altered cross-generational plasticity compared to Col for bolting time, flowering time, lateral branch number, and square root transformed fruit number. Examination of the reaction norms reveals that cross-generational plasticity was nearly abolished in the mutants compared to Col for all traits that showed significant genetic variation for cross-generational plasticity. For Col, moderate drought treatment of the parent reduced bolting (Fig. 6) and flowering (Fig. 7) times in offspring by four days while mean bolting and flowering times for *gpa1* and *agb1* were not significantly affected by parental treatment. Col also showed cross-generational plasticity for number of lateral branches (Fig. 8); moderate drought treatment of parents reduced lateral branch number in offspring. Conversely, *gpa1* and *agb1* mutants show no cross-generational plasticity for lateral branch number. Finally, for Col, moderate drought treatment of parents significantly reduced fruit number compared to ample water treatment of parents (Fig. 9). For *gpa1* and *agb1* mutants, there is a lack of cross-generational plasticity for fruit number. *gpa1* and *agb1* mutants have significantly less fruit production than Col when parents are grown under ample water conditions, however, moderate drought treatment of parents increases fruit production in *gpa1* and *agb1* relative to Col.

## DISCUSSION

### G proteins are required for cross-generational plasticity of offspring developmental and reproductive traits

Despite the fact that naturally occurring genetic variation for cross-generational plasticity has been observed in a number of studies for seed, seedling, and adult traits (Wulff and Bazzaz, 1992; Sultan, 1996; Lacey et al., 1997; Andalo et al., 1998; Donohue and Schmitt, 1998; Andalo et al., 1999; Munir et al., 2001), very little is known about the genetic mechanisms underlying phenotypic plasticity within a generation, let alone cross-

generational phenotypic plasticity. One exception is the phytochrome family of photoreceptors which have been implicated in mediating both phenotypically plastic shade avoidance responses within a generation (Pigliucci and Schmitt, 1999) and parent environment effects on seed germination and dormancy (Donohue et al., 2007; Donohue et al., 2008). Additionally, *NIA1* and *NIA2* which encode the nitrogen reductase apoenzyme have been shown to regulate maternal nitrogen effects on seed dormancy (Alboresi et al., 2005). Here, using mutant genotypes of Arabidopsis, we have implicated heterotrimeric G proteins in mediating cross-generational plasticity in response to parental water treatment. GPA1 and AGB1 regulate cross-generational plasticity for bolting and flowering times, lateral branch number, and fruit number and are therefore the first proteins that have been directly linked to regulating cross-generational plasticity of offspring adult reproductive traits.

One strategy which some plant species employ to deal with drought stress is drought escape, when plants hasten their life cycle, e.g. flower earlier, in response to drought in order to reproduce prior to desiccation (Ludlow, 1989). Interestingly, we found that for bolting time and flowering time, Col offspring of drought treated parents bolted and flowered significantly earlier across offspring environments than plants whose parents were grown under ample water conditions. G protein mutants however, showed no or reduced cross-generational plasticity for bolting and flowering times (Figs. 6 & 7). Our data suggest that the parental environment contributes to whether or not a plant will display a drought escape strategy but that this cross-generational effect is dependent on the presence of functional GPA1 and AGB1. As expected, offspring treatment also significantly affected bolting and flowering time (Table 1). This suggests that bolting and flowering times are regulated by both the parental environment and the offspring environment. However, there was no significant genotype x offspring treatment interaction for bolting and flowering time following the sequential Bonferrroni correction suggesting that G proteins are involved primarily in mediating the parental environmental component of this drought escape strategy.

We also found significant genetic variation for cross-generational plasticity for lateral branch number. In response to parental drought stress, Col offspring had reduced lateral branching across environments while lateral branching in *gpa1* and *agb1* mutants

was not affected by parental environment (Fig. 8). This effect is not likely to be attributed to allometric differences, since log rosette mass was not a significant source of variation. Fruit number also showed significant genetic variation for cross-generational plasticity. Col showed plasticity for fruit number in response to parental treatment, but *gpa1* and *agb1* mutants lacked cross-generational plasticity for fruit number (Fig. 9). This resulted in Col producing more fruits than *gpa1* and *agb1* when parent plants were amply watered, however, when parents experienced drought, *gpa1* and *agb1* offspring produced more fruits than Col offspring. In terms of the cross-generational plasticity for fruit number and lateral branching displayed by Col, this may be a conservative bet-hedging strategy (Seger and Brockmann, 1987; Crean and Marshall, 2009) to produce less fruits in order to maintain or increase the quality of the fruits that are produced in potentially resource-limited environments. Reduced fruit production and lateral branch number in response to parental drought treatment may also be a method to reduce offspring competition in potentially resource-scarce environments. On the other hand, Col offspring of drought-treated parents may simply be less fit than offspring of well-watered parents.

A question that emerges is what are the fitness consequences to *gpa1* and *agb1* offspring which lacked cross-generational plasticity? We observed that under ample water conditions *gpa1* and *agb1* mutants had reduced total seed production (Fig. 5) suggesting that their lack of cross-generational plasticity may have at least in part contributed to this reduction in fitness and supports the hypothesis that cross-generational plasticity is adaptive, at least under certain environmental conditions. Interestingly though, *agb1* offspring were more fit in terms of their total seed production under drought stress compared to wild type (Fig. 5). The differential effects on fitness caused by the *gpa1* and *agb1* mutations may result from fitness trade-offs relating to the pleiotropic nature of the mutations. *gpa1* and *agb1* mutations affect multiple developmental and physiological processes (Perfus-Barbeoch et al., 2004) in addition to cross-generational plasticity. Antagonistic pleiotropic effects on fitness have also been reported for the *FRIGIDA* gene within a generation; mutant alleles of this gene flower early but display no fitness benefit because of reduced inflorescence node formation and branching (Scarcelli et al., 2007).

The observation that heterotrimeric G proteins regulate cross-generational plasticity begs the question, what is the explicit mechanism by which GPA1 and AGB1 regulate this phenomenon? *AGB1* is expressed in stratified seeds prior to germination. However neither *GPA1* transcript nor GPA1 protein is expressed in stratified seeds (Pandey et al., 2006) or mature seed (Weiss et al., 1993) prior to germination. It is not known whether or not *GPA1* is expressed in stratified seeds from drought-treated plants. The lack of *GPA1* expression in stratified seed suggests that G protein regulation of cross-generational plasticity is not caused directly by the presence of *GPA1* or *AGB1* transcript or protein in the seed, but rather is the result of some form of parental or epigenetic effect (Pandey et al., 2006). GPA1 and AGB1 may function in parental effects that regulate seed hormone, nutrient, or metabolite composition (reviewed in, Donohue and Schmitt, 1998), or affect seed coat properties in response to drought stress (Sultan, 1996; Lacey et al., 1997). Additionally, *gpa1* and *agb1* mutant seed developed under parental drought stress could have altered mRNA or protein contents (Donohue, 2009) and/or DNA or histone modifications which could alter gene expression and thus impair the manifestation of cross-generational plasticity. Given the small size of Arabidopsis seed and the limited quantities of seed that were recovered from drought-treated parent plants, even though 24 replicate plants of each genotype were used for each of the two water treatments (ample water and moderate drought) in that experiment (240 plants in total), we were unable to test these hypotheses in the context of the present experiment.

Since *agb1* mutants had increased seed production under drought stress, heterotrimeric G proteins may be useful targets for biotechnological crop improvement or conventional breeding programs focusing on increasing crop yield in low water agricultural systems. *agb1* mutants also had more stable seed yields across environments and more stable fruit production between generations. The enhanced stability of reproductive traits due to *agb1* mutation may be of agronomical benefit given the potentially volatility of resources and environments due to climate change. Additionally, evidence is emerging that cross-generational plasticity can contribute to human disease (Bateson et al., 2004). Given that disruptions in mammalian heterotrimeric G protein signaling pathways lead to morbidity (Simonds, 2003; Spiegel and Weinstein, 2004; Weinstein et al., 2006) and that the ubiquitously expressed mammalian $G_s\alpha$ encoding

locus is epigenetically regulated via imprinting (Peters and Williamson, 2008; Plagge et al., 2008), an assessment of whether G proteins regulate cross-generational plasticity in humans, as we have shown they do in plants, could have significant medical and public health implications.

## CONCLUSION

We have found that heterotrimeric G proteins not only regulate phenotypic plasticity in response to water availability within a generation but also function in the regulation of cross-generational plasticity in response to parental water level for germination rate, bolting and flowering time, lateral branch number, and fruit production and are thus the first proteins identified which regulate cross-generational plasticity of offspring developmental and reproductive traits. *gpa1* and *agb1* mutants showed reduced or no cross-generational plasticity for these traits and had reduced offspring fitness under ample water growth conditions, although *agb1* mutants had heightened fitness under drought stress. The explicit mechanisms through which G proteins function in cross-generational plasticity, whether it is via changes in seed provisioning, seed quality, seed hormone or metabolite content, seed coat properties, parental inherited mRNAs or proteins or some form of epigenetic mechanism will require future research.

## LITERATURE CITED


**Aarssen LW, Clauss MJ** (1992) Genotypic variation in fecundity allocation in *Arabidopsis thaliana*. J Ecol **80:** 109-114

**Agrawal AA** (2001) Transgenerational consequences of plant responses to herbivory: an adaptive maternal effect? Am Nat **157:** 555-569

**Agrawal AA, Laforsch C, Tollrian R** (1999) Transgenerational induction of defences in animals and plants. Nature **401:** 60-63

**Alboresi A, Gestin C, Leydecker MT, Bedu M, Meyer C, Truong HN** (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. Plant Cell Environ **28:** 500-512

**Andalo C, Mazer SJ, Godelle B, Machon N** (1999) Parental environmental effects on life history traits in *Arabidopsis thaliana* (Brassicaceae). New Phytol **142:** 173-184

**Andalo C, Raquin C, Machon N, Godelle B, Mousseau M** (1998) Direct and maternal effects of elevated $CO_2$ on early root growth of germinating *Arabidopsis thaliana* seedlings. Annals of Botany **81:** 405-411

**Assmann SM** (2002) Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. Plant Cell **14 Suppl:** S355-373

**Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, Gluckman P, Godfrey K, Kirkwood T, Lahr MM, McNamara J, Metcalfe NB, Monaghan P, Spencer HG, Sultan SE** (2004) Developmental plasticity and human health. Nature **430:** 419-421

**Blodner C, Goebel C, Feussner I, Gatz C, Polle A** (2007) Warm and cold parental reproductive environments affect seed properties, fitness, and cold responsiveness in *Arabidopsis thaliana* progenies. Plant Cell Environ **30:** 165-175

**Bradshaw AD** (1972) Some of the evolutionary consequences of being a plant. Evol Biol **5:** 25-47

**Crean AJ, Marshall DJ** (2009) Coping with environmental uncertainty: dynamic bet hedging as a maternal effect. Philos Trans R Soc Lond B Biol Sci **364:** 1087-1096

**Donohue K** (2009) Completing the cycle: maternal effects as the missing link in plant life histories. Philos Trans R Soc Lond B Biol Sci **364:** 1059-1074

**Donohue K, Heschel MS, Butler CM, Barua D, Sharrock RA, Whitelam GC, Chiang GCK** (2008) Diversification of phytochrome contributions to germination as a function of seed-maturation environment. New Phytol **177:** 367-379

**Donohue K, Heschel MS, Chiang GC, Butler CM, Barua D** (2007) Phytochrome mediates germination responses to multiple seasonal cues. Plant Cell Environ **30:** 202-212

**Donohue K, Schmitt J** (1998) Maternal environmental effects in plants. *In* TA Mousseau, CW Fox, eds, Maternal effects as adaptations. Oxford University Press, New York, pp 137-158

**Fredriksson R, Schioth HB** (2005) The repertoire of G-protein-coupled receptors in fully sequenced genomes. Mol Pharmacol **67:** 1414-1425

**Galloway LF** (2005) Maternal effects provide phenotypic adaptation to local environmental conditions. New Phytol **166:** 93-99

**Gookin TE, Kim J, Assmann SM** (2008) Whole proteome identification of plant candidate G-protein coupled receptors in *Arabidopsis*, rice, and poplar: computational prediction and in-vivo protein coupling. Genome Biol **9:** R120

**Henderson IR, Jacobsen SE** (2007) Epigenetic inheritance in plants. Nature **447:** 418-424

**Huey RB, Carlson M, Crozier L, Frazier M, Hamilton H, Harley C, Hoang A, Kingsolver JG** (2002) Plants versus animals: Do they deal with stress in different ways? Integr Comp Biol **42:** 415-423

**Jones AM, Ecker JR, Chen JG** (2003) A reevaluation of the role of the heterotrimeric G protein in coupling light responses in *Arabidopsis*. Plant Physiol **131:** 1623-1627

**Kakutani T** (2002) Epi-alleles in plants: inheritance of epigenetic information over generations. Plant Cell Physiol **43:** 1106-1111

**Lacey EP, Smith S, Case AL** (1997) Parental effects on seed mass: Seed coat but not embryo/endosperm effects. Am J Bot **84:** 1617-1620

**Lease KA, Wen J, Li J, Doke JT, Liscum E, Walker JC** (2001) A mutant *Arabidopsis* heterotrimeric G-protein β subunit affects leaf, flower, and fruit development. Plant Cell **13:** 2631-2641

**Ludlow MM** (1989) Strategies of response to water stress. *In* KH Kreeb, H Richter, TM Hinckley, eds, Structural and functional responses to environmental stresses. SPB Academic, The Hague

**Ma H, Yanofsky MF, Meyerowitz EM** (1990) Molecular cloning and characterization of GPA1, a G protein α subunit gene from *Arabidopsis thaliana*. Proc Natl Acad Sci USA **87:** 3821-3825

**Mason MG, Botella JR** (2000) Completing the heterotrimer: isolation and characterization of an *Arabidopsis thaliana* G protein γ-subunit cDNA. Proc Natl Acad Sci USA **97:** 14784-14788

**Mason MG, Botella JR** (2001) Isolation of a novel G-protein γ subunit from *Arabidopsis thaliana* and its interaction with Gβ. Biochim Biophys Acta **1520:** 147-153

**Mazer SJ, Gorchov DL** (1996) Parental effects on progeny phenotype in plants: Distinguishing genetic and environmental causes. Evolution **50:** 44-53

**McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS** (2005) G-protein signaling: back to the future. Cell Mol Life Sci **62:** 551-577

**Meaney MJ, Szyf M** (2005) Maternal care as a model for experience-dependent chromatin plasticity? Trends Neurosci **28:** 456-463

**Moran MD** (2003) Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos **100:** 403-405

**Moriyama E, Strope P, Opiyo S, Chen Z, Jones A** (2006) Mining the *Arabidopsis thaliana* genome for highly-divergent seven transmembrane receptors. Genome Biol **7:** R96

**Munir J, Dorn LA, Donohue K, Schmitt J** (2001) The effect of maternal photoperiod on seasonal dormancy in *Arabidopsis thaliana* (Brassicaceae). Am J Bot **88:** 1240-1249

**Pandey S, Assmann SM** (2004) The *Arabidopsis* putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signaling. Plant Cell **16:** 1616-1632

**Pandey S, Chen JG, Jones AM, Assmann SM** (2006) G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. Plant Physiol **141:** 243-256

**Perfus-Barbeoch L, Jones AM, Assmann SM** (2004) Plant heterotrimeric G protein function: insights from *Arabidopsis* and rice mutants. Curr Opin Plant Biol **7:** 719-731

**Peters J, Williamson CM** (2008) Control of imprinting at the *Gnas* cluster. Adv Exp Med Biol **626:** 16-26

**Pigliucci M, Schmitt J** (1999) Genes affecting phenotypic plasticity in *Arabidopsis*: pleiotropic effects and reproductive fitness of photomorphogenic mutants. J Evol Biol **12:** 551-562

**Plagge A, Kelsey G, Germain-Lee EL** (2008) Physiological functions of the imprinted *Gnas* locus and its protein variants Gα(s) and XLα(s) in human and mouse. J Endocrinol **196:** 193-214

**Rice WR** (1989) Analyzing tables of statistical tests. Evolution **43:** 223-225

**Riginos C, Heschel MS, Schmitt J** (2007) Maternal effects of drought stress and inbreeding in *Impatiens capensis* (Balsaminaceae). Am J Bot **94:** 1984-1991

**Sanda S, John M, Amasino R** (1997) Analysis of flowering time in ecotypes of *Arabidopsis thaliana*. J Hered **88:** 69-72

**Scarcelli N, Cheverud JM, Schaal BA, Kover PX** (2007) Antagonistic pleiotropic effects reduce the potential adaptive value of the *FRIGIDA* locus. Proc Natl Acad Sci USA **104:** 16986-16991

**Seger J, Brockmann HJ** (1987) What is bet-hedging? *In* P Harvey, L Partridge, eds, Oxford Surveys in Evolutionary Biology. Oxford University Press

**Simonds WF** (2003) G protein-regulated signaling dysfunction in human disease. J Investig Med **51:** 194-214

**Spiegel AM, Weinstein LS** (2004) Inherited diseases involving G proteins and G protein-coupled receptors. Annu Rev Med **55:** 27-39

**Sultan SE** (1987) Evolutionary implications of phenotypic plasticity in plants. Evol Biol **21:** 127-178

**Sultan SE** (1996) Phenotypic plasticity for offspring traits in *Polygonum persicaria*. Ecology **77:** 1791-1807

**Sultan SE** (2004) Promising directions in plant phenotypic plasticity. Perspect Plant Ecol Evol Syst **6:** 227-233

**Ullah H, Chen JG, Temple B, Boyes DC, Alonso JM, Davis KR, Ecker JR, Jones AM** (2003) The β-subunit of the *Arabidopsis* G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell **15:** 393-409

**Weinstein LS, Chen M, Xie T, Liu J** (2006) Genetic diseases associated with heterotrimeric G proteins. Trends Pharmacol Sci **27:** 260-266

**Weiss CA, Garnaat CW, Mukai K, Hu Y, Ma H** (1994) Isolation of cDNAs encoding guanine nucleotide-binding protein β-subunit homologues from maize (ZGB1) and Arabidopsis (AGB1). Proc Natl Acad Sci USA **91:** 9554-9558

**Weiss CA, Huang H, Ma H** (1993) Immunolocalization of the G protein α subunit encoded by the *GPA1* gene in *Arabidopsis*. Plant Cell **5:** 1513-1528
**Wulff RD, Bazzaz FA** (1992) Effect of the parental nutrient regime on growth of the progeny in *Abutilon-theophrasti* (Malvaceae). Am J Bot **79:** 1102-1107

**Table 1.** p-values from the ANOVA/ANCOVA for all traits. Bold font indicates significant differences after application of the sequential Bonferroni correction. Underlined p-values are significant before applicationof the correction. N/A specifies that the ANOVA and not the ANCOVA model was used for the trait. * Cut-off for the Bonferroni correction was 0.0023, so this p-value is nearly significant following the correction.

| Source | Bolting time | Flowering time | Lateral branches | Sq. rt. fruit number | Inflor. height | Seeds per fruit | Fruit length | Sq. rt. total seeds | Shoot mass |
|---|---|---|---|---|---|---|---|---|---|
| Genotype | **0.0001** | **0.0001** | **<.0001** | 0.25 | **<.0001** | **0.0024** | **<.0001** | 0.4844 | **<.0001** |
| Offspring treatment | **<.0001** | **<.0001** | **<.0001** | **<.0001** | **<.0001** | 0.0211 | 0.0322 | **0.0022** | 0.0317 |
| Parent treatment | **<.0001** | **<.0001** | 0.0085 | 0.0236 | 0.6599 | 0.5438 | 0.3656 | 0.1025 | 0.3971 |
| log rosette mass (covariant) | **<.0001** | **<.0001** | N/A | N/A | N/A | 0.1579 | N/A | N/A | N/A |
| Genotype x offspring treatment | 0.0261 | 0.0294 | **<.0001** | 0.0565 | **<.0001** | **<.0001** | **<.0001** | 0.0027 * | 0.5976 |
| Genotype x parent treatment | **<.0001** | **<.0001** | **0.0008** | **0.001** | 0.3835 | 0.3965 | 0.2879 | 0.5018 | 0.4519 |
| Genotype x log rosette mass | **0.0008** | **0.0007** | N/A | N/A | N/A | **0.0023** | N/A | N/A | N/A |

**Table 2.** F-values and (p-values) from contrasts of genotype x offspring treatment LSMs for traits which showed significant genotype x offspring treatment interaction. Bold font indicated significant p-values following the sequential Bonferroni correction.

| Contrast | Lateral branches | Inflor. height | Seeds per fruit | Fruit length | Sq. rt. total seeds |
|---|---|---|---|---|---|
| Both Col vs. both *gpa1*, control offspring treatment vs. ample water offspring treatment | 9.62 **(0.002)** | 0.01 (0.9374) | 0.15 (0.6987) | 0.39 (0.5346) | 0.24 (0.6224) |
| Both Col vs. both *agb1*, control offspring treatment vs. ample water offspring treatment | 16.79 **(<.0001)** | 10.71 **(0.0011)** | 71.53 **(<.0001)** | 57.19 **(<.0001)** | 11.37 **(0.0009)** |

**Table 3**. F-values and (p-values) from contrasts of genotype x parent treatment LSMs for traits which showed significant genotype x parent treatment interaction. Bold font indicated significant p-values following the sequential Bonferroni correction

| Contrast | Bolting time | Flowering time | Lateral branches | Sq. rt. fruit number |
|---|---|---|---|---|
| Both Col vs. both *gpa1*, control parent treatment vs. ample water parent treatment | 29.39 **(<.0001)** | 27.18 **(<.0001)** | 9.62 **(0.002)** | 14.66 **(0.0001)** |
| Both Col vs. both *agb1*, control parent treatment vs. ample water parent treatment | 15.13 **(0.0001)** | 13.4 **(0.0003)** | 15.73 **(<.0001)** | 8.48 **(0.0037)** |

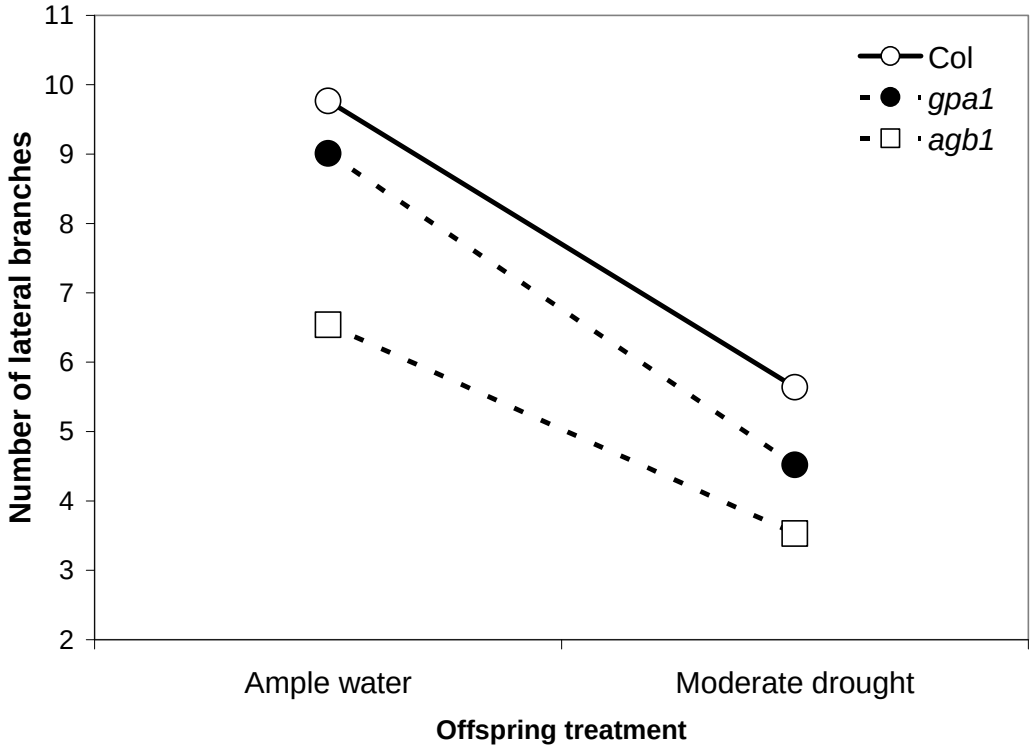



**Figure 1**. *agb1* mutants have reduced plasticity for number of lateral branches in response to offspring water treatment. Shown are combined allele least squares means as done in the contrasts.

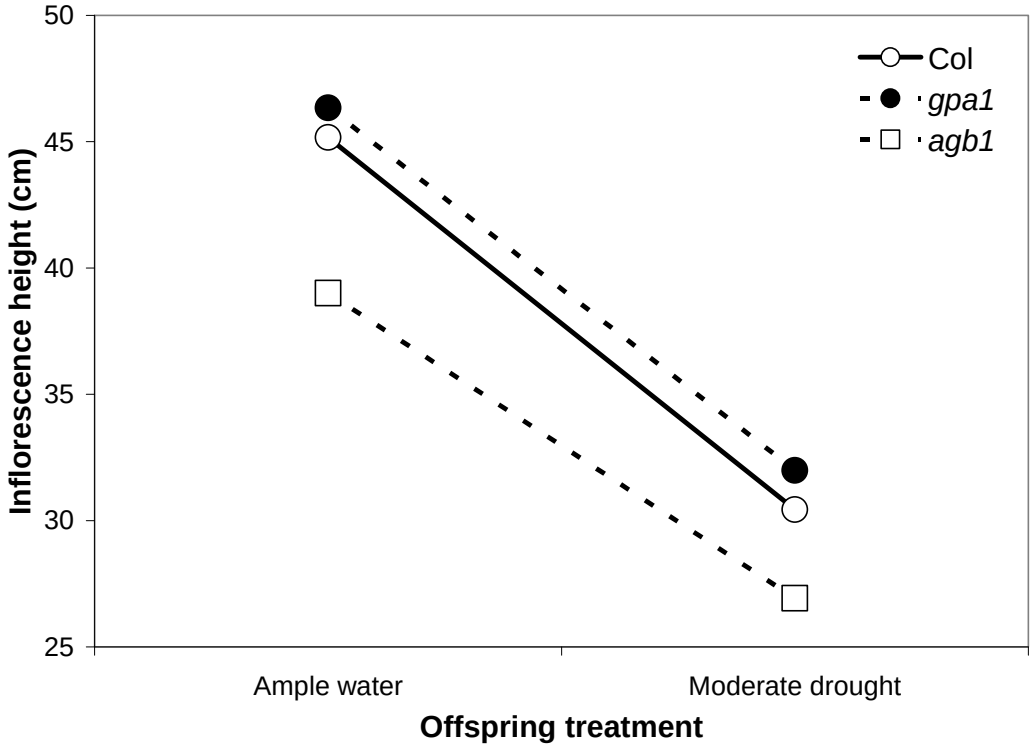



**Figure 2**. *agb1* mutants have reduced plasticity for inflorescence height in response to offspring water treatment. Shown are combined allele least squares means as done in the contrasts.

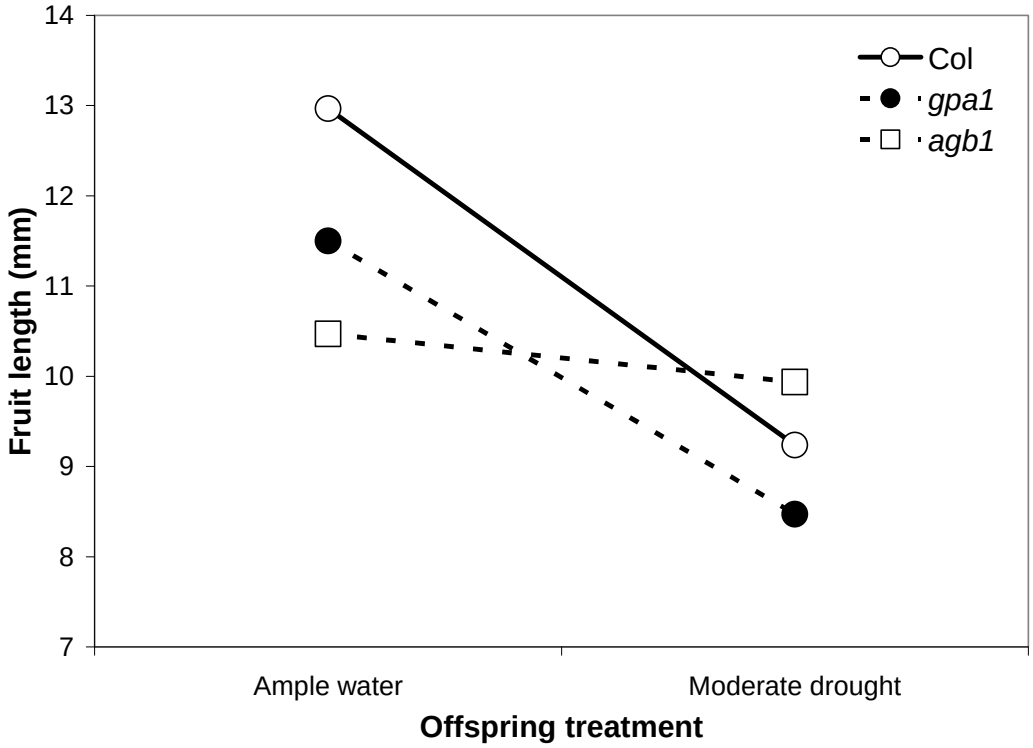



**Figure 3**. *agb1* mutants have reduced plasticity for fruit length in response to offspring water treatment. Shown are combined allele least squares means as done in the contrasts.

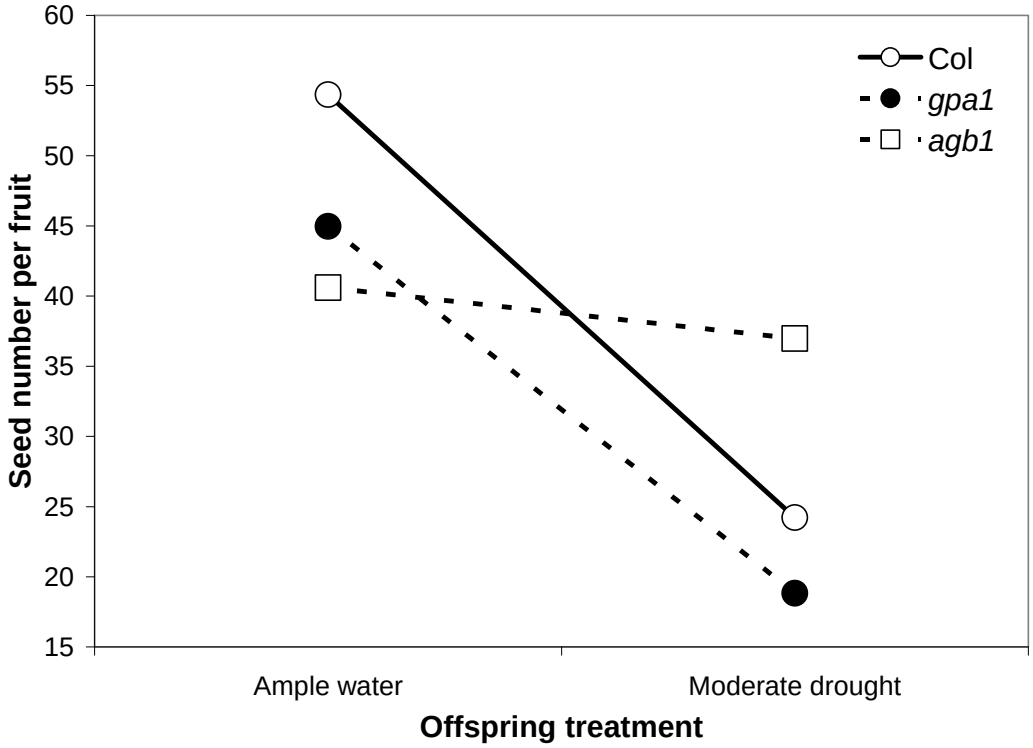



**Figure 4.** *agb1* mutants have reduced plasticity for seed number per fruit in response to offspring water treatment. Shown are combined allele least squares means as done in the contrasts.

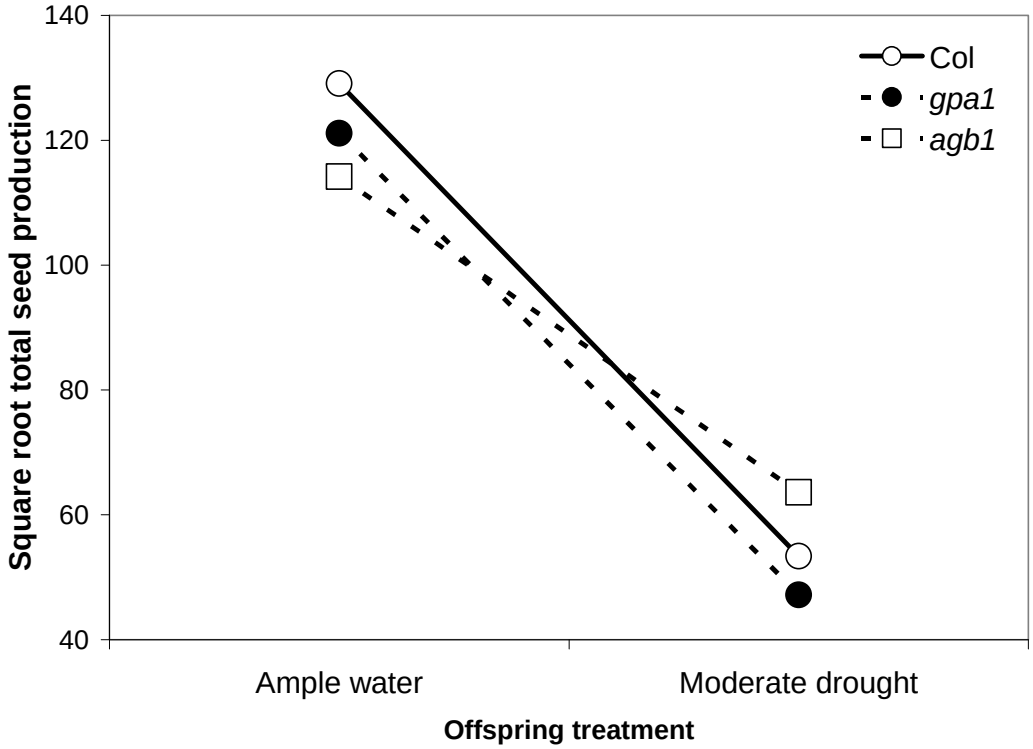



**Figure 5**. *agb1* mutants have reduced plasticity for total seed production in response to offspring water treatment. Shown are combined allele least squares means as done in the contrasts.

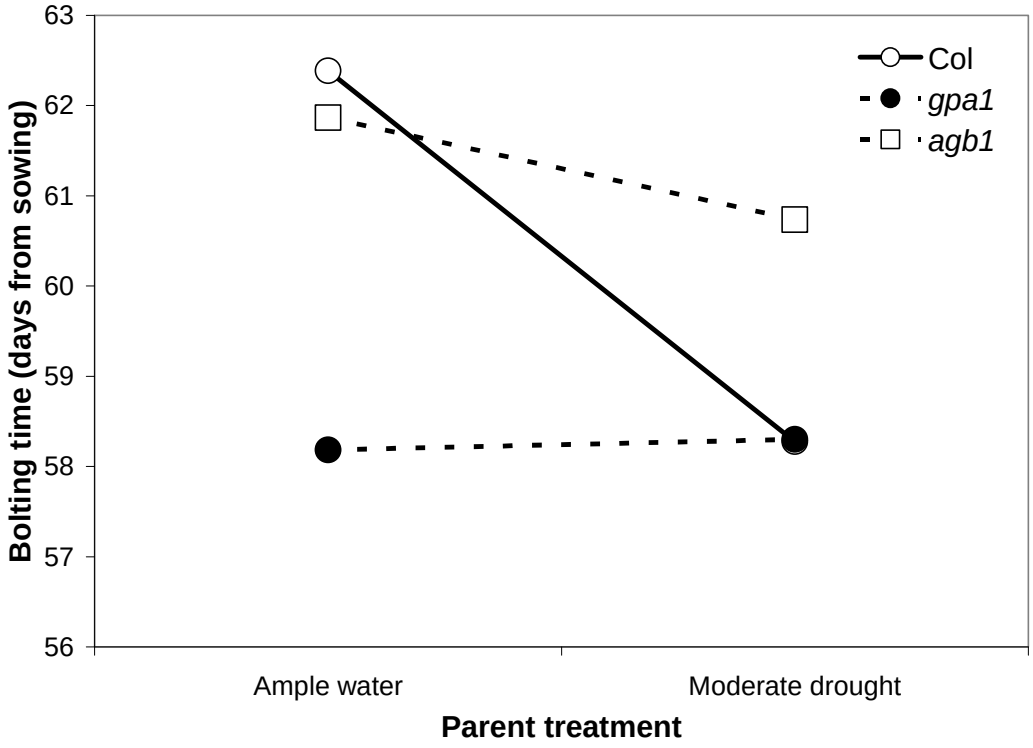



**Figure 6**. *gpa1* and *agb1* mutants lack cross-generational plasticity for bolting time. Shown are combined allele least squares means as done in the contrasts.

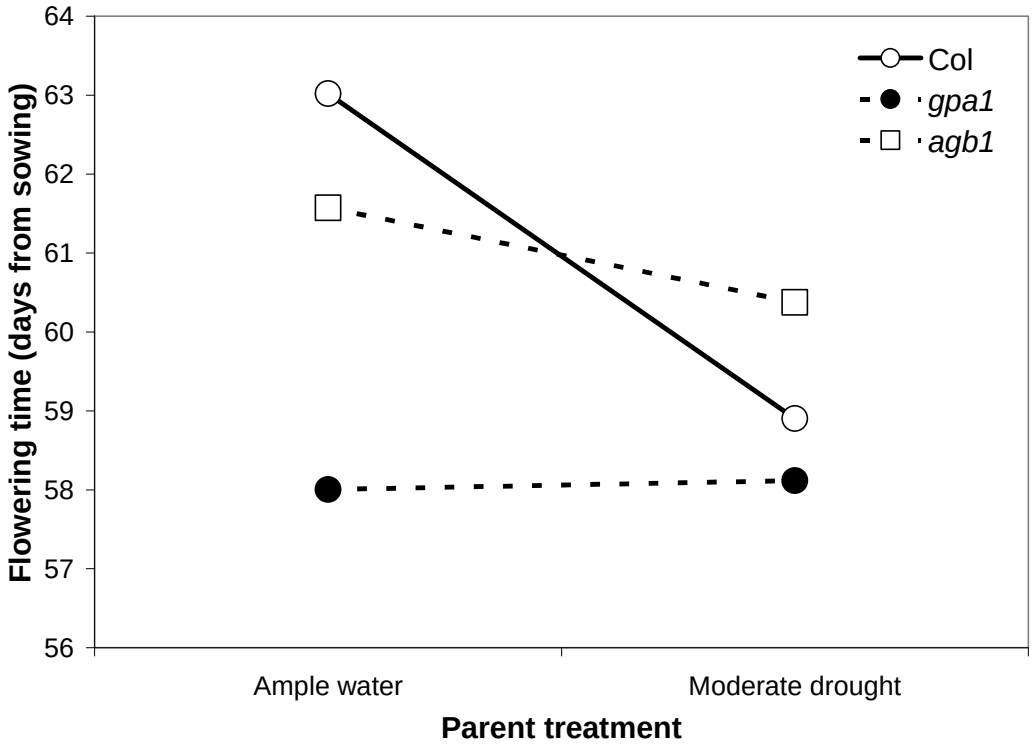



**Figure 7**. *gpa1* and *agb1* mutants lack cross-generational plasticity for flowering time. Shown are combined allele least squares means as done in the contrasts.

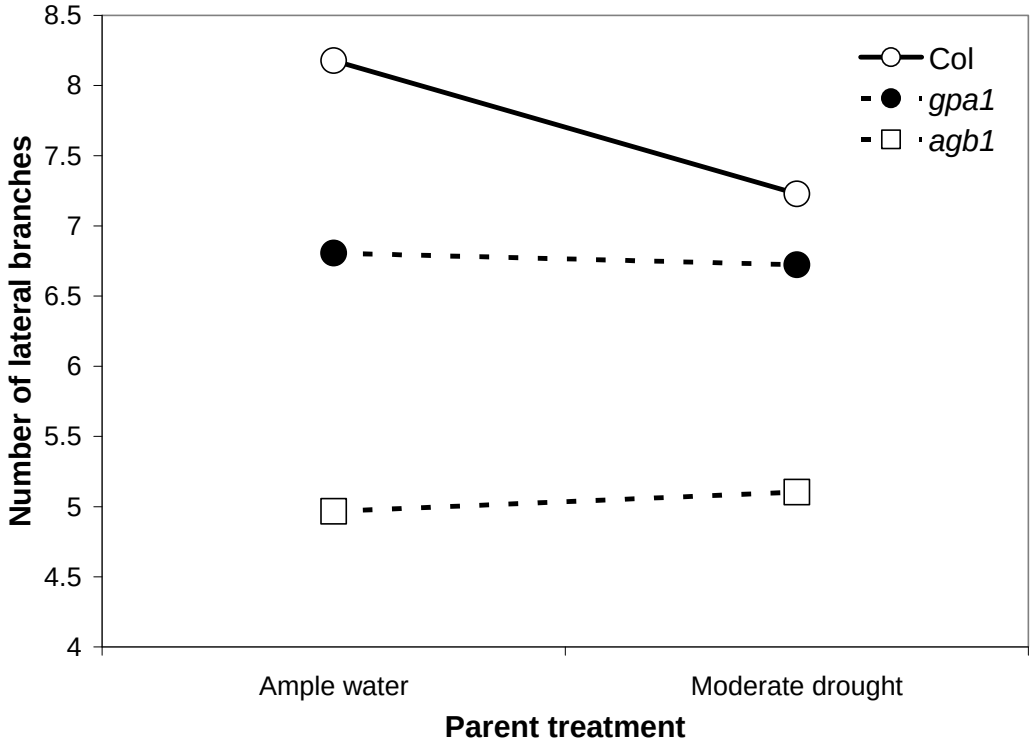



**Figure 8**. *gpa1* and *agb1* mutants lack cross-generational plasticity for lateral branch formation. Shown are combined allele least squares means as done in the contrasts.

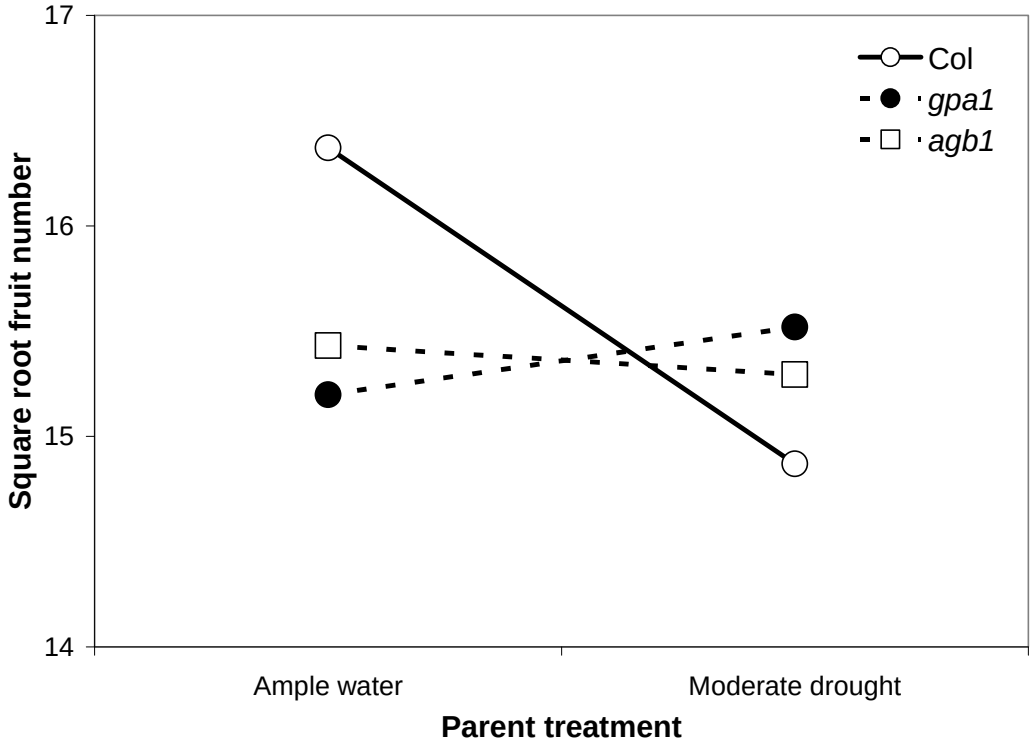



**Figure 9**. *gpa1* and *agb1* mutants lack cross-generational plasticity for fruit number. Shown are combined allele least squares means as done in the contrasts.

## Chapter 8: Suggestions for future research and significance of thesis research

## SUGGESTIONS FOR FUTURE RESEARCH

### Extensions of Chapter 4

In an initial screen of all the G protein mutants for transpiration efficiency (TE) I found that one allele of *agb1*, *agb1-1*, had reduced TE compared to Col (Fig. 1). However, this was not observed in an additional allele of *agb1*, *agb1-2*. *agb1* mutants had increased stomatal densities in rosette leaves compared to Col, however the difference was not statistically significant (Fig. 2). It has been reported that *agb1* mutants have increased stomatal density in cotyledons, but, the allele used for this experiment was never specified (Zhang, L *et al.*, 2008) . It would be beneficial to repeat the TE experiments with additional alleles of *agb1* which could be acquired from the stock centers. Additionally, phenotyping putative GPCR mutants and/or Gγ mutants (if novel Gγs or *agg* mutants are isolated) would aid in understanding how heterotrimeric G proteins regulate transpiration efficiency and stomatal density. *agg1-1c* mutants did show increased stomatal density (Fig. 2) however, this could be a residual effect of the introgression of *agg1-2* into the Col background from Ws, since this phenotype was not observed in *agg1-2* (*agg1-2* showed reduced stomatal density). Gas exchange analysis should also be performed on the other G protein mutants, *agb1* mutants especially, with the whole-plant Arabidopsis gas exchange chamber newly available from Licor which has been recently acquired by the Assmann lab. The altered rosette architecture (small size and short petioles) of the *agb1* mutants limited my ability to take reliable measurements of *agb1* using the leaf chamber fluorometer but this obstacle could be overcome with the whole-plant chamber. Finally, given the altered $CO_2$ sensitivities and stomatal densities of the *gpa1* mutants (Chapter 4), it would be interesting to examine if G proteins regulate environmentally-induced changes to stomatal density, namely, $CO_2$-induced changes. I was unable to find space at facilities that had the capacity for both $CO_2$-limited conditions and $CO_2$-enriched conditions (Duke University, McGill

University) but I still think this would be a worth while experiment to do if space could be obtained.

**Extensions of Chapter 5**

Understanding how G proteins regulate stomatal density via epidermal cell expansion and stomata formation is a very interesting question that should be pursued further. Genetic analysis of *gpa1tmm, gpa1sdd1*, *gpa1fama*, and other *gpa1* stomatal development double mutants will be useful in understanding where G proteins might function in the stomatal development pathway (Nadeau, 2009). Additional screening of *gpa1erecta* recombinants or the creation of *GPA1* RNAi lines in the *erecta* background could be used to produce *gpa1erecta* double mutants. I tested a number of stomatal development-related proteins for physical interaction with GPA1 using yeast-based or *in vitro* techniques (Chapter 3). My results were inconclusive and I believe at least the potential for GPA1 interaction with ERECTA should be examined using a different technique such as biomolecular fluorescence complementation or fluorescence energy resonance transfer.

**Extensions of Chapter 6**

In Chapter 6 the data suggest that AGB1 regulates phenotypic plasticity in response to water availability. This finding begs the question; do G proteins regulate phenotypic plasticity in response to other environmental gradients? While I examined plasticity in response to drought stress, plasticity in response to flooding would be an interesting extension since the G protein mutants had reduced fitness under ample water treatment (Chapter 6). G proteins have also been implicated in functioning in pathogen (Llorente *et al.*, 2005; Trusov *et al.*, 2006; Trusov *et al.*, 2008; Zhang, W *et al.*, 2008), ozone (Joo *et al.*, 2005), and $CO_2$ (Chapter 4) signaling so these environments may be of interest for additional plasticity studies. Eventually, characterization of GPCR and G protein signaling effector mutants for phenotypic plasticity responses will also be useful to better understand the mechanism behind G protein regulation of plasticity.

**Extensions of Chapter 7**

I identified heterotrimeric G proteins as regulators of cross-generational plasticity in response to parental water level in Chapter 7. While this is an interesting observation, I believe an even more interesting question is what is the mechanism? Possible mechanisms include differential inheritance of mRNAs in seed, altered seed coat properties, altered biochemical composition of seeds (e.g., proteins, hormones, nutrients) or changes in the methylation state of genomic DNA (epigenetic mechanisms). Unfortunately, my ability to address these hypotheses was limited by the small amount of seed I recovered from the drought-treated plants. With additional seed a number of experiments could be performed. Microarray or proteomic analysis of G protein mutant and wild type seed from drought-treated or ample water parents may help to pin point specific proteins or mRNA transcripts that contribute to cross-generational plasticity. Detailed biochemical analysis of seed could be performed to determine if seed hormone or nutrient contents differ due to parental environment. Finally, extraction of genomic DNA from seed which would be subjected to global methylation assays would show if there are any differences in methylation in the genomes of seed from moderate drought vs. amply watered parents of the different genotypes. All these techniques would require growing a large number of Col, *gpa1*, *agb1* plants under conditions similar to my cross-generational study in order to bulk large quantities of seed from different parental environments. I recommend including at least three times the number of drought-treated plants in the experimental design since seed yield is greatly reduced by drought treatment. It will also be crucial to plant some of the seed to make sure the cross-generational plasticity effects are present before investing time in the mechanism studies.

**SIGNIFICANCE OF THESIS RESEARCH**

In this thesis I researched the function of heterotrimeric G proteins at multiple levels of biological complexity, including the whole-leaf and the whole-plant. In the past, heterotrimeric G protein function in Arabidopsis has been studied mainly via cell

and molecular biology techniques. These approaches revealed that G proteins regulate stomatal movements (reviewed in Chapter 2) which can have important implication for whole-plant water status. One goal of plant molecular biology is to understand the genetic basis of phenotypes in order to use this information to aid in conventional breeding or biotechnological crop improvement. I was able to address how G proteins contribute to water status regulation and plant fitness which gives us a better idea of the potential utility of G proteins as biotechnological targets that could be useful for the production of drought-tolerant plants. I examined TE, which is an important agronomical trait that has been used in the past by plant breeders to develop more drought-tolerant crops (Condon *et al.*, 2004; Slafer *et al.*, 2005; Richards, 2006; Monneveux *et al.*, 2007). Therefore this research has significant implications for biotechnology applications since GPA1 (Chapter 4) and ERECTA (Masle *et al.*, 2005) are the only known genetic regulators of TE. Interestingly, the finding, that *gpa1* mutants have increased TE and reduced stomatal conductance is opposite of our predictions based on single-cell assays of stomatal physiology (Wang *et al.*, 2001; Fan *et al.*, 2008). I believe this result reinforces the importance of combining molecular and cell biology with whole-plant approaches if a gene's contribution to plant physiology is to be truly understood.

My research was also significant because I was able to use molecular biology tools to address ecological questions concerning the genetic basis of phenotypic plasticity. One reason for the slow progress in understanding the genetic basis of phenotypic plasticity is the lack of overlap and collaboration between molecular biologists who can study individual genes and ecologists who are interested in understanding phenotypic plasticity. By combining these two types of approaches I was able to show that heterotrimeric G proteins regulate phenotypic plasticity in response to water availability both within and between generations. While genetic variation for phenotypic plasticity is abundant within species and QTL for phenotypic plasticity have been isolated, only a very few loci have been directly linked to phenotypic plasticity regulation. The discovery that heterotrimeric G proteins regulate phenotypic plasticity has important implications concerning the adaptive nature of plasticity and has increased our understanding of the role of heterotrimeric G proteins in plant physiology.

## LITERATURE CITED


**Condon AG, Richards RA, Rebetzke GJ, Farquhar GD. 2004.** Breeding for high water-use efficiency. J Exp Bot **55**: 2447-2460

**Fan LM, Zhang W, Chen JG, Taylor JP, Jones AM, Assmann SM. 2008.** Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ- and RGS-deficient *Arabidopsis* lines. Proc Natl Acad Sci USA **105**: 8476-8481

**Joo JH, Wang S, Chen JG, Jones AM, Fedoroff NV. 2005.** Different signaling and cell death roles of heterotrimeric G protein α and β subunits in the *Arabidopsis* oxidative stress response to ozone. Plant Cell **17**: 957-970

**Llorente F, Alonso-Blanco C, Sanchez-Rodriguez C, Jorda L, Molina A. 2005.** ERECTA receptor-like kinase and heterotrimeric G protein from *Arabidopsis* are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Plant J **43**: 165-180

**Masle J, Gilmore SR, Farquhar GD. 2005.** The *ERECTA* gene regulates plant transpiration efficiency in *Arabidopsis*. Nature **436**: 866-870

**Monneveux P, Sheshshayee MS, Akhter J, Ribaut JM. 2007.** Using carbon isotope discrimination to select maize (*Zea mays l.*) inbred lines and hybrids for drought tolerance. Plant Science **173**: 390-396

**Nadeau JA. 2009.** Stomatal development: New signals and fate determinants. Curr Opin Plant Biol **12**: 29-35

**Richards RA. 2006.** Physiological traits used in the breeding of new cultivars for water-scarce environments. Agric Water Manage **80**: 197-211

**Slafer GA, Araus JL, Royo C, Del Moral LFG. 2005.** Promising eco-physiological traits for genetic improvement of cereal yields in mediterranean environments. Ann App Biol **146**: 61-70

**Trusov Y, Rookes JE, Chakravorty D, Armour D, Schenk PM, Botella JR. 2006.** Heterotrimeric G proteins facilitate *Arabidopsis* resistance to necrotrophic pathogens and are involved in jasmonate signaling. Plant Physiol **140**: 210-220

**Trusov Y, Sewelam N, Rookes JE, Kunkel M, Nowak E, Schenk PM, Botella JR. 2008.** Heterotrimeric G proteins-mediated resistance to necrotrophic pathogens includes mechanisms independent of salicylic acid-, jasmonic acid/ethylene- and abscisic acid-mediated defense signaling. Plant J. 58: 69-81

**Wang XQ, Ullah H, Jones AM, Assmann SM. 2001.** G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. Science **292**: 2070-2072

**Zhang L, Hu G, Cheng Y, Huang J. 2008.** Heterotrimeric G protein α and β subunits antagonistically modulate stomatal density in *Arabidopsis thaliana*. Dev Biol **324**: 68-75

**Zhang W, He SY, Assmann SM. 2008.** The plant innate immunity response in stomatal guard cells invokes G-protein-dependent ion channel regulation. Plant J **56**: 984-996

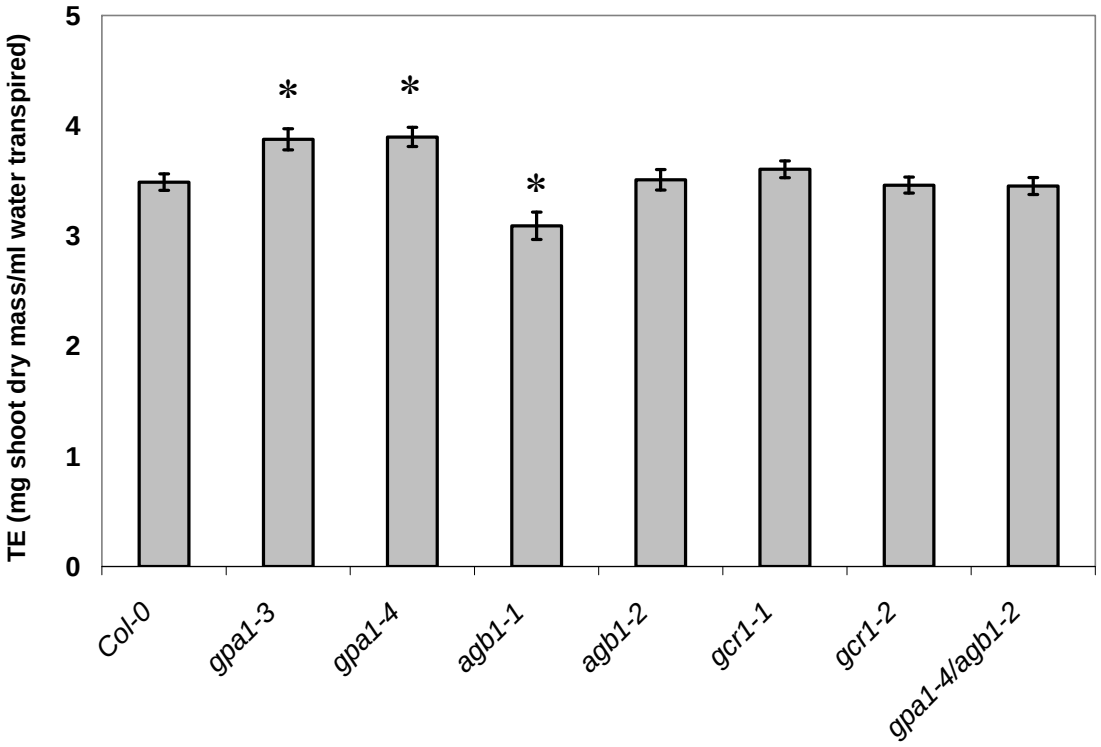



**Figure 1**. *gpa1* mutants have increased transpiration efficiency (TE) and *agb1-1* has reduced TE compared to Col, asterisks indicates mean differs significantly ($p < 0.05$) from the mean for Col. The conditions used for this experiment were similar to those described in Chapter 4 for the ample water treatment.

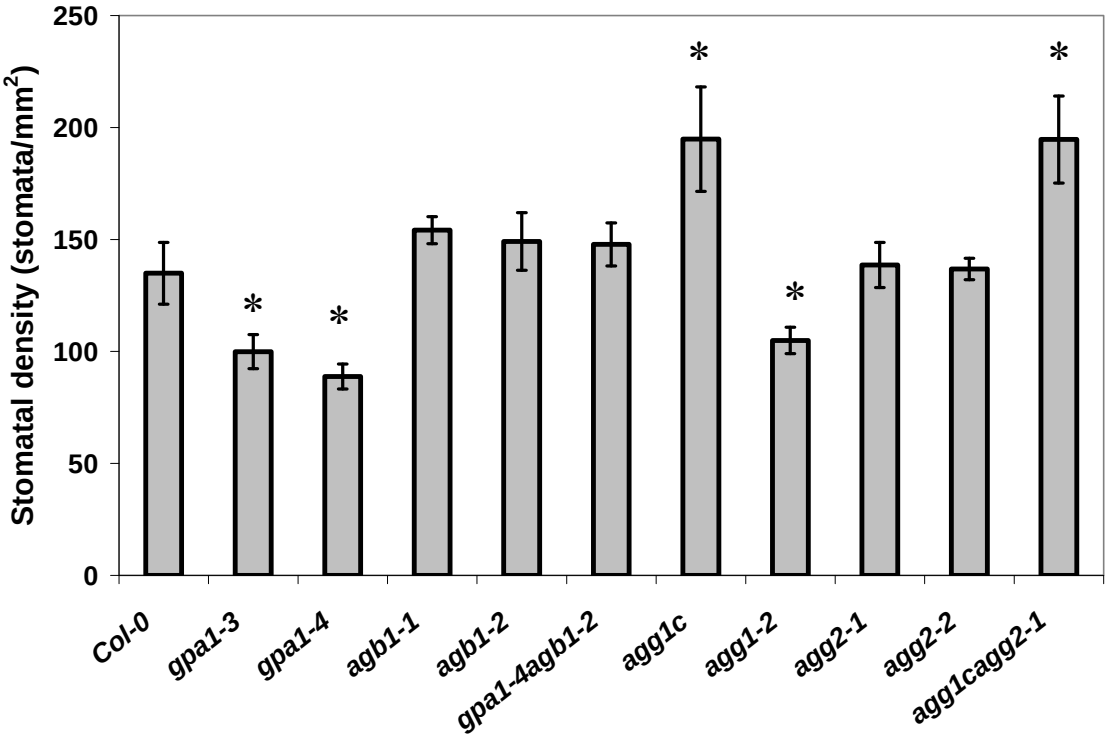



**Figure 2.** *agb1* mutants have increased stomatal densities compared to Col though not statistically significant and *agg1c* has increased and *agg1-2* has decreased stomatal densities compared to Col, asterisk indicates mean differs significantly ($p < 0.05$) from mean for Col. The experiment was performed as described in Chapter 4.

# VITA ~ Sarah E. Nilson

**Education** **THE PENNSYLVANIA STATE UNIVERSITY, UNIVERSITY PARK, PA**

Ph.D. candidate. Plant Biology Graduate Program. Advisor: Dr. Sarah Assmann. Defense date: June 12th 2009. Dissertation: Heterotrimeric G protein regulation of transpiration efficiency, stomatal density, and phenotypic plasticity in *Arabidopsis thaliana*. **Sept 2003-current.**

**UNIVERSITY OF PITTSBURGH, PITTSBURGH, PA**

Degrees: B.S. Biology (2002), *summa cum laude* and B.A. Anthropology (2002), *summa cum laude*. Minors: Chemistry and Spanish. **Jan 1999-April 2002**.

**Teaching** **PENN STATE UNIVERSITY ~ PENN STATE DEPARTMENT OF BIOLOGY**

Research mentor. Instructed and supervised undergraduate researchers pursuing independent research projects in the Assmann Lab. **May 2008-current.**

**HONORS BIOLOGY: FUNCTION AND DEVELOPMENT OF ORGANISMS (BIOL240M) ~ PENN STATE UNIVERSITY**

Invited guest lecturer. Topics: Plant diversity, anatomy, and evolution. **March 2006, 2008.**

**PLANT DEVELOPMENTAL BIOLOGY (BIOL408) ~ PENN STATE UNIVERSITY**

Invited guest lecturer. Topic: Plant biotechnology as a research tool. **March 2005, 2006, 2007.**

**PLANT DEVELOPMENTAL BIOLOGY (BIOL408) ) ~ PENN STATE UNIVERSITY**

Teaching assistant**.** Developed plant histology and microscopy labs and instructed upper-level undergraduate students. Prepared and graded lab quizzes. Graded exams and substitute lectured on plant development. **Jan-May 2005.**

**Awards & Fellowships** **NASA SPACE GRANT FELLOWSHIP ~ 2006, 2007**

**THE DEPARTMENT OF BIOLOGY GRADUATE ASSISTANT EXCELLENCE IN TEACHING AWARD ~ 2005**

**NATIONAL SCIENCE FOUNDATION GRADUATE RESEARCH FELLOWSHIP HONORABLE MENTION ~ 2005**

**Publications** **Nilson SE** and Assmann SM (submitted to New Phytologist) Heterotrimeric G proteins regulate reproductive trait plasticity in response to water availability

**Nilson SE** and Zhang W (in review, invited) Heterotrimeric G protein regulation of stomatal movements (book chapter) in **Integrated G Protein Signaling in Plants** (Baluska F, Jones A, Yalovsky S, eds.). Springer-Verlag: Berlin/Heidelberg.

Zhang W, **Nilson SE,** and Assmann SM (2008) Isolation and Whole-Cell Patch Clamping of Arabidopsis Guard Cell Protoplasts. **Cold Spring Harbor Protocols**.

**Nilson SE** and Assmann SM (2007) The control of transpiration: insights from Arabidopsis. **Plant Phys** 143:19-27.

Manzi S, Navratil JS, Ruffing MJ, Liu CC, Danchenko N, **Nilson SE**, Krishnaswami S, King DE, Kao AH, Ahearn JM (2004) Measurement of erythrocyte C4d and complement receptor 1 in systemic lupus erythematosus. **Arthritis Rheum** 50:3596-3604.