UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of Starch Metabolism and Guard Cell Photosynthesis in CO_2 Regulation of Stomatal Conductance

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

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2014

The Thesis of Andisheh Bagheri is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2014

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ACKNOWLEDGMENTS

I would like to thank Dr. Julian Schroeder for this remarkable opportunity of allowing me to be a part of his research laboratory for the last two years. The knowledge, skills, and memories I have experienced in the last two years would not be possible without him. I am thankful for his support as being the chair of my committee. I would also like to thank the rest of my committee members, Dr. Clifford Kubiak and Dr. Eduardo Macagno for their time and support in being a part of my committee.

My greatest gratitude goes out to Dr. Tamar Azoulay-Shemer. Her experience, patience, kindheartedness, and advice were essential in allowing me to complete my projects. Without her help, I would have not been able to accomplish my M.S. degree. I am forever indebted to everything she has taught me.

I would also like to thank Bianca Manalansan for teaching me laboratory techniques during my undergraduate time in the laboratory. I am very appreciative for Drs. Fernando Aleman Guillen, Benjamin Brandt, Ulrich Deinlein, Cawas Engineer, Felix Hauser, Hans-Henning Kunz, Aaron Stephan, and the rest of the Schroeder lab members for all their expertise in guiding me in my experiments and answering my questions. Being part of the Schroeder lab for the past two years has been an unforgettable experience.

I would like to thank the University of California San Diego and the Department of Biological Sciences for allowing me to use their facilities and materials.

Finally I am truly appreciative of the utmost support from my family and friends for being able to complete my M.S. degree. Thank you.

ABSTRACT OF THE THESIS

The role of Starch Metabolism and Guard Cell Photosynthesis in CO_2 Regulation of Stomatal Conductance

by

Andisheh Bagheri

Master of Science in Biology

University of California, San Diego, 2014

Professor Julian Schroeder, Chair

Professor Eduardo Macagno, Co-Chair

Carbon dioxide (CO_2) is one of the main regulators of stomatal aperture. Few *Arabidopsis* mutants that are insensitive to CO_2 have been previously studied and have revealed parts of the mechanisms involved in CO_2 regulation of guard cell signal transduction. However, CO_2 regulation of stomatal conductance still requires further investigation. Here, an innovative stomatal movement analysis in response to CO_2 shifts was developed in order to study the role of starch metabolism and guard cell photosynthesis

in CO_2 regulation of stomatal conductance. These analyses revealed that high [CO_2]-induced stomatal closure was impaired in *Arabidopsis* mutants that cannot produce starch in general (*adg1*), but not in *pgi1-1 Arabidopsis* mutants that accumulate starch specifically in their guard cells, implying that functional starch metabolism solely in guard cells is sufficient for CO_2 regulation of stomatal conductance. Additionally, kidney-shaped stomata of all three *Arabidopsis GC-Chlase*Δ*N* transgenic lines (*#4*, *#5*, and *#8*) that featured severe chlorophyll deficiency specifically in their guard cells, responded to high [CO_2]-induction by stomatal closure, while their chlorophyll-less thin-shaped and collapsed stomata remained constitutively closed, suggesting that wild-type chlorophyll levels in guard cells are not required for CO_2 regulation of stomatal conductance.

1. INTRODUCTION

Plants need to assimilate CO_2 for photosynthesis while simultaneously preventing excessive loss of water. The plant cuticle is covered with wax, making it impermeable to water and CO_2. Plants use stomatal pores that control water loss and CO_2 influx by regulating their aperture. Stomata are formed by two specialized guard cells that can be found on both adaxial (upper) and abaxial (lower) leaf surfaces in most plants (Ticha, 1982). Stomatal aperture is regulated by many endogenous and environmental factors such as abscisic acid (ABA), drought, humidity, light, pathogens, ozone, and CO_2 (Kim, Böhmer, Hu, Nishimura, & Schroeder, 2010; Schroeder, Allen, Hugouvieux, Kwak, & Waner, 2001). CO_2 is one of the main factors that regulates stomatal aperture. The continuous rise in atmospheric CO_2 has been shown to increase plant intercellular CO_2 levels (C_i), inducing stomatal aperture decrease, and therefore, having considerable affects on global gas exchange, water use efficiency, and leaf heat stress.

The mechanism of regulating stomatal aperture is based on ions and osmolytes such as K^+, Cl^-, malate, and sucrose accumulating in the guard cell, increasing its turgor, resulting in stomatal opening, while stomatal closure occurs from the reverse process (Franks & Farquhar, 2007; Shimazaki, Doi, Assmann, & Kinoshita, 2007). CO_2 levels below ambient conditions have shown to stimulate stomatal opening, while CO_2 levels above ambient conditions induces stomatal closure (Mansfield, Hetherington, & Atkinson, 2003; Young et al., 2006).

Few *Arabidopsis* signaling mutants that are insensitive to CO_2 such as *ßca* (Hu et al., 2010), *ost1* (Xue et al., 2011), *ht1* (Hashimoto et al., 2006), *slac1* (Negi et al., 2008), and *gca2* (Young et al., 2006) have been studied in order to gain a better understanding of

CO_2 regulation of stomatal conductance. However, the mechanisms that control CO_2 regulation of guard cell signal transduction still require further investigation.

Atmospheric CO_2, respiration and photosynthesis are three main regulators of C_i. These important processes affect carbon fixation and starch metabolism in both guard cell and mesophyll cell chloroplasts. However, the specific functions of these chloroplasts and to what degree they participate in stomatal responses are still up for debate; specifically, how guard cell photosynthesis and starch metabolism regulate stomatal conductance.

The main goals of my research include:

1. Developing an innovative stomatal movement analysis in response to CO_2 changes

2. Studying the role of guard cell starch metabolism in CO_2 regulation of stomatal conductance.

3. Studying the role of guard cell photosynthesis in CO_2 regulation of stomatal conductance.

1. Developing an innovative stomatal movement analysis in response to CO_2 changes

Previously, analyses of stomatal movements in response to CO_2 changes have been utilized in many labs and published in the literature. The most common method involved placing detached leaves into a physiological solution and then bubbling CO_2 into this solution. This method however, has a few limitations. Detaching leaves may alter their condition and wound them, while bubbling CO_2 into a solution is a difficult task. Thus, there was a need in developing a new stomatal movement analysis in response to CO_2 changes in order to improve the analysis. In this new "whole plant method,"

whole plants were induced in different CO_2 concentrations using [CO_2]-controlled growth chambers and stomata were imaged in epidermal peels under the microscope. This whole plant method was employed in the rest of my research experiments.

2. The role of guard cell starch metabolism in CO_2 regulation of stomatal conductance

So far little research has been done on starch metabolism and CO_2 regulation of stomatal conductance. The only research conducted in this field includes analyses of stomatal conductance in response to red and blue light (Talbott & Zeiger, 1993) but not to CO_2 shifts. Starch is a key carbohydrate in plant photosynthetic processes. CO_2 is fixed into triose phosphates (TP), which are exported to the cytosol to produce sucrose; or converted into starch through sugar intermediates (Kunz et al., 2010) and metabolized into malate. Malate can be further metabolized or back-converted into starch. Data have shown a quantitative relationship between guard cell starch concentration and stomatal aperture (Outlaw & Manchester, 1979). In addition, malate accumulation has also been correlated with stomatal aperture (Allaway, 1973; Cyanea & Faba, 1973; Pearson & Milthorpe, 1974; Vavasseur & Raghavendra, 2005). The K^+-malate theory suggests that K^+ uptake results in water intake, which increases guard cell turgor. In this process, malate is believed to act as a counter ion (Fischer, 1968). On the other hand, apoplastic malate has been linked to stomatal closure by leading to Cl^- and malate efflux through specific anion channels in guard cells (Rainer Hedrich et al., 1994). Guard cell starch metabolism and its role in CO_2 regulation of stomatal conductance is still a subject of debate.

Starch degradation has been shown to occur in guard cells and mesophyll cells but the extent to which each plays on regulating stomatal conductance still requires further investigation. *adg1* mutants lack the starch biosynthesis key enzyme, ADP-glucose pyrophosphorylase (AGPase). This enzyme is localized to the chloroplast stroma and catalyzes the conversion of Glc1P to ADPG, which is the substrate for starch synthesis. ADGase is composed from LSU and SSU. Mutations in its SSU, *adg1-1,* results in a lack of starch in all parts of the plants (Kunz et al., 2010; Wang et al., 1998). On the other hand, mutation in the (PGI) phospho-glucose isomerase enzyme, which produces Glc6P from the Calvin Cycle intermediate Fru6P, leads to a low starch phenotype in the photoautotrophic parts of the plants, whereas heterotrophic tissues, as roots and guard cells contain a wild-type (WT) starch phenotype (Kunz et al., 2010; Tsai et al., 2009). Starch in *pgi1-1* guard cells is accounted for by GPT2 (glucose-6-phosphate transmembrane transporter 2) activity, which imports Glc6P from the cytosol into the chloroplast, bypassing the PGI reaction for starch synthesis (Kunz et al., 2010). Therefore, in order to investigate how starch in guard cells and mesophyll cells affects CO_2 regulation of stomatal conductance, *Arabidopsis thaliana* mutants that cannot produce starch in general (*adg1-1 and adg1-2*), or specifically accumulate starch in guard cells (*pgi1-1*) were exposed to CO_2 shifts and their stomatal responses were examined and compared to WT (Columbia-0).

3. The role of guard cell photosynthesis in CO_2 regulation of stomatal conductance

Photosynthetic CO_2 assimilation that occurs in guard cells and mesophyll cells has been proposed to regulate stomatal conductance. Yet, the extent to which guard cell

photosynthesis is involved in CO_2 regulation of stomatal conductance is still a matter of debate. Photosynthesis takes place in chloroplasts, which use chlorophyll as the main pigment to absorb light photons and use this energy to fix CO_2 into organic compounds via the Calvin Cycle (Eduardo Zeiger, Talbott, Frechilla, Srivastava, & Zhu, 2002). Although photosynthesis primarily occurs in mesophyll cells, which contain 3-4 times more chloroplasts than guard cells (Humble & Raschke, 1971), guard cells still have chloroplasts, suggesting an important photosynthesis function in these specialized organelles (Outlaw, Mayne, Zenger, & Manchester, 1981; Shimazaki, Gotow, & Kondo, 1982; E Zeiger, Armond, & Melis, 1981). Several studies have shown that guard cell chloroplasts are not required for CO_2-regulated stomatal responses. In two studies, plants were treated with nonflurazon to inhibit carotenoid synthesis. Yet, these plants showed normal stomatal responses to CO_2, although lacking functional chloroplasts (Hu et al., 2010; Roelfsema et al., 2006). However, a different study showed that *Paphiopedilum*, an Orchid genus that does not contain guard cell chloroplasts, showed a weaker stomatal conductance response to CO_2 when compared to *Phragmipedium*, the orchid genus that contained chlorophyll in its guard cells (Assmann & Zeiger, 1985).

In order to study the role of guard cell photosynthesis in stomatal responses to CO_2, transgenic plants that contained severely reduced chlorophyll in their guard cells were examined and compared to WT (E1728 – Columbia-0 ecotype). Chlorophyllase (Chlase) enzyme degrades chlorophyll into chlorophyllide and phytol. Dr. Tamar Azoulay-Shemer engineered transgenic plants (*GC-Chlase*Δ*N*) that over-expressed Chlase under a guard cell specific promoter. Characterization of the *GC-Chlase*Δ*N* plants (Supplementary Information) by Dr. Azoulay-Shemer revealed that although these

transgenic plants displayed similar chlorophyll and starch levels in mesophyll cells and no developmental impairments, they showed severe reductions in their starch and chlorophyll levels exclusively in guard cells. Thus, in order to understand the involvement of guard cell photosynthesis in CO_2 regulation of stomatal conductance, these transgenic plants were exposed to CO_2 shifts and their stomatal movements were analyzed.

My research focused on **a)** Developing a new innovative stomatal movement analysis in response to CO_2 shifts, which was then used to study **b)** The role of starch metabolism in guard cells in CO_2 regulation of stomatal conductance and **c)** The role of guard cells photosynthesis in CO_2 regulation of stomatal conductance.

2. MATERIALS AND METHODS

2.1. Plant Growth Procedures

Arabidopsis thaliana seeds of the Columbia-0 ecotype were sterilized in 0.5% SDS in 75% ethanol (EtOH), 75% EtOH, and 100% EtOH, respectively, and left to completely air dry in the laminar flow hood. The seeds were then sowed onto 0.5 MS media (Murashige and Skoog basal medium – Sigma Aldrich) with pH=5.8, 0.02% MES hydrate, and 0.8% Phyto Agar and then stratified in the dark at 4°C for 2-3 days. Seedlings matured in a Conviron growth chamber (21°C, 60-80% humidity, 16 h light/8 h dark photoperiod regime at 80 μmol m^{-2} s^{-1}). 7- to 9-day-old seedlings were transferred to pots of soil (with supplemental fungicide – *Mighty Myco*, mycorrhizal inoculant, Medford, Oregon). Pots were cultivated in Conviron growth chamber (21°C, 60-80% humidity, 12 h light/12 h dark photoperiod regime at 90-100 μmol m^{-2} s^{-1}) and watered 2-3 times per week.

2.2. Physiological Assays

2.2.1 Stomatal Movement Analysis in response to ABA

Stomatal movement analyses in response to ABA were performed with detached leaves of 3- to 5-week-old plants grown in an ambient CO_2 growth chamber (21°C, 60-80% humidity, 12 h light/12 h dark photoperiod regime at 90-100 μmol m^{-2} s^{-1}). Plants were misted each day for 2 days prior to the experiment. The 5th true leaf was detached and floated with its abaxial side in contact with 5 mL of opening buffer solution (5 mM KCl, 50 μM $CaCl_2$, 10 mM MES, pH=5.7 Tris-HCl) for 2 hours, 120-140 μmol m^{-2} s^{-1}. Afterward, 10 μM [ABA] (final conc. – diluted in EtOH) was added to the treated samples, while 0.1% EtOH (final conc.) was added to the opening buffer solution for the

untreated samples. The solution mixture was left to incubate for 60 or 90 min in the same light and humidity conditions.

2.2.2. Stomatal Movement Analysis in response to CO_2 changes

2.2.2.A. Whole Plant Method

Stomatal movements analyses in response to CO_2 changes were performed with whole plants of 3- to 5-week-old plants grown in an ambient CO_2 growth chamber (21°C, 60-80% humidity, 12 h light/12 h dark photoperiod regime at 90-100 µmol m^{-2} s^{-1}). Individual pots were placed in a 150-200 ppm CO_2-controlled chamber (Percival intelles control system) set to 120-140 µmol m^{-2} s^{-1}, ~60% humidity, and 21°C for 2 or 5 hours. Plants were then transferred from the 150-200 ppm CO_2-controlled chamber to an 800 ppm high CO_2-controlled chamber (Percival intelles control system – same light, humidity, and temperature) for an additional 45 or 90 min of incubation.

2.2.2.B. Bubbling Method

Stomatal movements in response to CO_2 changes were performed with detached leaves of 3- to 5-week-old plants grown in an ambient CO_2 growth chamber (21°C, 60-80% humidity, 12 h light/12 h dark photoperiod regime at 90-100 µmol m^{-2} s^{-1}). The 5th true leaf was detached and floated with its abaxial side in contact with 5 mL of opening buffer solution (5 mM KCl, 50 µM $CaCl_2$, 10 mM MES, pH=5.7 Tris-HCl) for 2 hours, 120 µmol m^{-2} s^{-1}. Afterward, 800 ppm [CO_2] was pumped through a tube into the solution for the treated samples. The untreated samples were left to sit and incubate in ambient [CO_2] with the same light and humidity conditions.

2.3. Analyzing the leaf epidermis on a microscope slide

2.3.1. Blending the epidermis

2.3.1.A. ABA – After incubation with ABA, each 5th true leaf was detached and blended with deionized water in a commercial blender (Waring commercial blender, Torrington, Connecticut) for 15-20 seconds. The epidermal tissues were collected using a 100 μm nylon-mesh filter (EMD Millipore, http://www.millipore.com). The filtered contents were placed onto a microscope slide with a cover glass on top. This slide was observed under a 40x inverted light microscope attached to a camera and at least 20-30 stomata were imaged per leaf. Later, stomatal apertures were measured using "Image J" software (NIH, http://rsbweb.nih.gov/ij/).

2.3.1.B CO_2 – After incubation with CO_2 in either the whole plant method or the bubbling method, each 5th detached leaf was blended with CO_2-equilibrated deionized water (200 or 800 ppm, correspondingly). This CO_2-equilibrated deionized water was left in the corresponding CO_2-controlled chamber 24 hours prior to the start of the experiment. The rest of the steps after blending for 15-20 seconds are the same as described in 2.3.1.A. ABA.

2.3.2. Attachment of epidermal peels to cover glass

After incubation with either ABA or CO_2, the abaxial side of the 5th leaf was adhered to a microscope cover slip slide using a medical adhesive (Hollister, Libertyville, IL). A single edge industrial blade was used to carefully excise the upper mesophyll cell layers. The epidermal layer was then gently washed with a soft sponge and water was used to eliminate the remaining mesophyll cells. The cover slip was then placed on top a microscope slide and observed under the confocal microscope.

2.4. Confocal Imaging and stomatal auto-fluorescence measurements

After preparing a slide using the method described in section 2.3.2. Attachment of epidermal peels to cover glass, a Zeiss LSM 710 inverted confocal microscope (Dr. Mark Estelle lab) was used to image stomata. The microscope is equipped with a 493-571 nm filter for GFP emission, a 638-721 nm filter for chlorophyll auto-fluorescence, and an argon laser (488 nm and 633 nm) for excitation. Z-stack images were taken of the abaxial side and all layers were summed up using the Z-project function. Chlorophyll auto-fluorescence measurements were done by circling individual stomata using the freehand selection tool in Image J and calculated in terms of pixel numbers relative to WT.

2.5. Stomatal Aperture Calculations

After preparing a slide using the method described in section 2.3.1. Blending the epidermis, stomatal images were taken with a digital camera attached to an inverted light microscope (Nikon Eclipse TS100). The images were taken using the 40x magnification and saved as a tiff file. This file was opened using Image J software. The straight freehand line tool was selected to measure stomatal width and length. The following calculation was carried-out to convert this number into μm: (Image J number) * (50/2.945) = μm.

2.6. Plant Genotyping

2.6.1. Genomic DNA (gDNA) Extraction

Small leaves of 3- to 4-week old plants grown in an ambient CO_2 growth chamber (21°C, 60-80% humidity, 12 h light/12 h dark photoperiod regime at 90-100 μmol m^{-2} s^{-1})

were placed in eppendorfs containing 400 µL DNA extraction buffer (0.2 M Tris-HCl, 25 mM EDTA, 0.250 M NaCl, 0.5% SDS, pH=7.5) and grinded with pestle plastic sticks. Tubes were then centrifuged for 5 min at 13000 rpm. Supernatant was transferred to 300 µL isopropyl alcohol, vortexed, incubated for 5 min at room temperature for DNA precipitation, and centrifuged for 10 min at 13000 rpm. Excess fluid was vacuumed out using a pipet tip and DNA was washed with 70% EtOH and left to air-dry. 50 uL of deuterium depleted water (ddw) was added and gDNA was stored in -20ºC.

2.6.2. Polymerase Chain Reaction (PCR)

A mixture of 16.5 µL ddw, 2.5 µL 10x buffer, 2 µL of 2.5 mM dNTPs, 1 µL of $MgCl_2$, 0.5 µL primers, and 1 µL of Apex Taq Polymerase (used recommended company protocol) per reaction was made. 25 µL of this mixture was transferred to a PCR tube on ice with 1 µL gDNA (ddw was used as the negative control and WT as the positive control). PCR tubes were then placed in the thermal cycler for DNA segment amplification via PCR.

2.6.3. Gel Electrophoresis

DNA electrophoresis gel consisted of 1.25 g Agarose in 100 mL TAE (Tris-acetate-EDTA) buffer solution. This mixture was heated using a conventional microwave until all Agarose completely dissolved and 1.014 µg Ethidium Bromide (Sigma Aldrich, MKBG7890V) to 1 mL Agarose (final conc.) was added. The gel was poured and left to sit for 20-30 min in room temperature to solidify. Next, the gel was placed in TAE solution bath. 3.75 µg of GeneRuler DNA ladder was added to a gel well, loading dye (Fermentas, lot # 00092036) was mixed in each PCR tube, and 15 µL from each PCR

tube were added to the gel wells. 100 mV was applied to the gel for the DNA to run from the negative to positive side and the gel was imaged under UV light.

3. RESULTS

3.1. Improving methods for stomatal movement analyses in response to CO_2 changes

In order to practice and evaluate different techniques for stomatal movement analyses in response to CO_2 changes, an ABA and CO_2 insensitive mutant, *ost1-3* (Ler ecotype) (Mustilli, Merlot, Vavasseur, Fenzi, & Giraudat, 2002), was exposed to ABA and CO_2 shifts and its responses were compared to WT *Arabidopsis thaliana* plant, Columbia-0 (Col-0) ecotype. Whole plants were grown in a growth chamber for 4 weeks under ambient CO_2. The 5th true leaf was detached and pre-incubated in an opening buffer solution (5 mM KCl, 50 µM $CaCl_2$, 10 mM MES, pH=5.7 Tris-HCl), 120 µmol m^{-2} s^{-1} light for 2 hours in order to promote stomatal opening. Next, 10 µM [ABA] was added to the treated samples for 90 min (0.1% EtOH was added to the control samples). Leaves were blended, filtered through a 100 µm nylon mesh, and stomata of epidermal peels were imaged under the 40x objective using light microscopy. Stomatal apertures in individual stomata were then measured using Image J software. WT stomata responded pronouncedly to ABA, while *ost1-3* showed insensitive stomatal closure in response to ABA (Fig. 1A).

Next, the "bubbling method" and the "whole plant method" were compared to each other in order to examine which method proved more effective for analyzing stomatal movements in response to CO_2 changes. In the bubbling method, the 5th true leaf was detached and pre-incubated in an opening buffer solution (5 mM KCl, 50 µM $CaCl_2$, 10 mM MES, pH=5.7 Tris-HCl) for 2 hours under 120 µmol m^{-2} s^{-1} light. 800 ppm [CO_2] was bubbled continuously into the treated sample's solution for 90 min (control sample was left to sit). Both WT and *ost1-3* stomata did not respond to CO_2 shifts (Fig. 1B). In the whole

plant method, whole plants were pre-incubated in 450 ppm [CO_2] for 2 hours and the 5th leaf was sampled and analyzed. For high [CO_2]-induction, plants from the 450 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 90 min and the 5th leaf was sampled and analyzed (both CO_2-controlled chambers – Percival intelles control system – were set to ~120 µmol m^{-2} s^{-1} light intensity 21°C, and ~60% relative humidity). Leaves were blended, filtered through a 100 µm nylon mesh, and stomata of epidermal peels were imaged under the 40x objective using light microscopy. Stomatal apertures in individual stomata were then measured using Image J software. WT stomata responded significantly to CO_2, while *ost1-3* showed insensitive stomatal closure in response to 800 ppm [CO_2] (Fig.1C). *P*-values illustrated that WT stomata responded poorly to CO_2 during the bubbling method compared to the whole plant method.

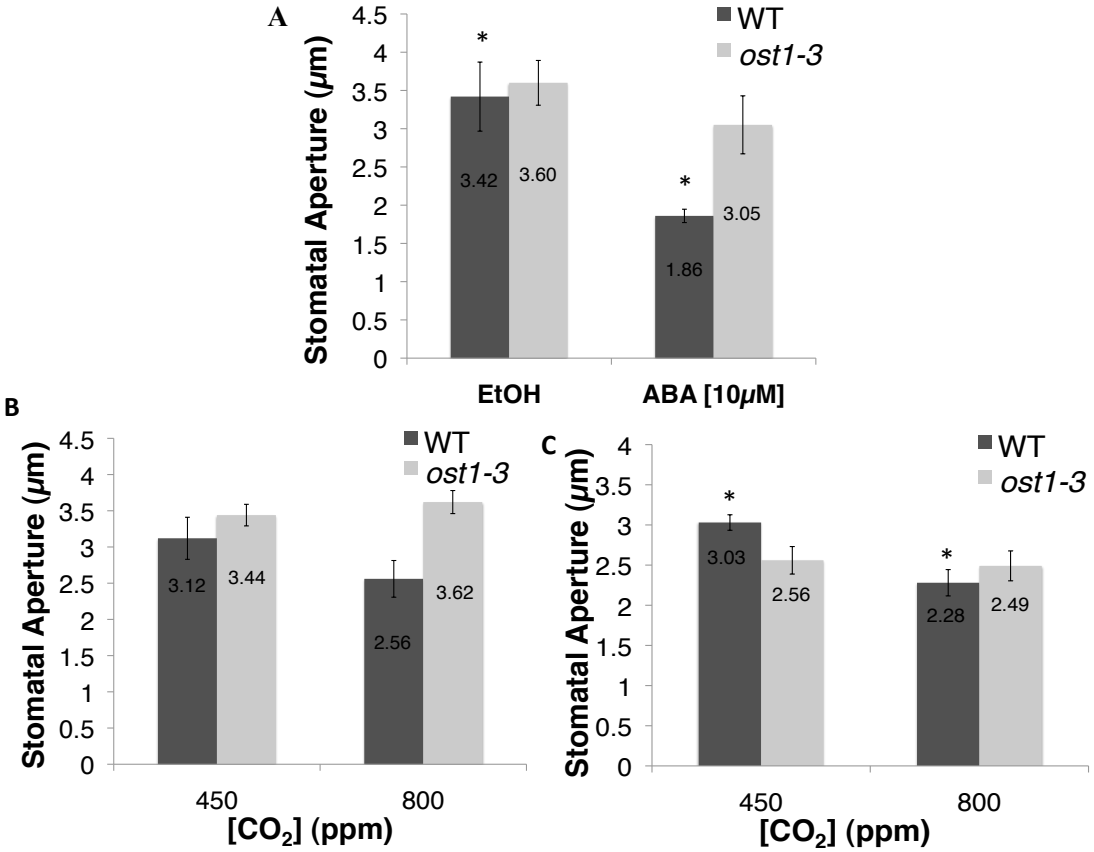


Figure 1. Stomatal movement analyses in response to ABA and CO_2 in WT (Col-0) and *ost1-3*. (A) Detached leaves were pre-incubated in opening buffer for 2 hours and 10 μM [ABA] was added to the treated samples (0.1% EtOH as solvent control). WT stomata responded to ABA (p=0.069), while *ost1-3* did not (p=0.32). n=3, total 90 stomata, pairwise Student's t-test. Data represent means ± s.e.m. (genotype and treatment blind analyses). **(B)** In the bubbling method, leaves were detached and pre-incubated in an opening buffer solution for 2 hours. 800 ppm [CO_2] was bubbled continuously to the treated samples for 90 min, while the control samples were left to sit. WT stomatal responses to CO_2 were insignificant (p=0.22). **(C)** In the whole plant method, whole plants were pre-incubated in 450 ppm [CO_2] for 2 hours and the 5th leaf was sampled and analyzed. For high [CO_2]-induction, plants from the 450 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 90 min and the 5th leaf was sampled and analyzed. WT (p=0.026) but not *ost1-3* (p=0.78) stomatal responses were significant to CO_2 shifts. n=3 plants, total 90 stomata, *p<0.07, pairwise Student's *t*-test. Data represent means ± s.e.m. (genotype blind analyses).

The process in which leaves were blended and imaged under the microscope needed to be done quickly during stomatal movement analyses in response to CO_2 changes. During one of the experiments, sample #1 required longer imaging time due to technical issues with the microscope. This sample was blended but sat out in ambient conditions waiting to be imaged, while the microscope was being fixed. WT (E1728 – Col-0 ecotype) plants were pre-incubated in 200 ppm [CO_2] for 2 hours, blended with CO_2-equilibrated water, and then imaged using light microscopy. Imaging sample #1 (italicized box) took approximately 15-20 min longer than samples #2-4. Results indicated that stomatal closure occurred rapidly when sample #1 was moved from 200 ppm [CO_2] and left out in ambient [CO_2] for 15-20 min longer than usual (Table 1).

Table 1. Stomata responded quickly to [CO_2] changes. The stomatal aperture values (μm) shown in the table below are from an experiment in which WT (E1728) plants were exposed to CO_2 shifts and their stomatal movements were analyzed. Samples were pre-incubated in 200 ppm [CO_2] for 2 hours, blended with CO_2-equilibrated water, and imaged under the light microscope. Sample #1 (italicized box) was left out in ambient CO_2 after taken out of the 200 ppm [CO_2] chamber for 15-20 min longer than samples #2-4 during the imaging process due to technical issues. ~20 stomata/sample. Data represent means ± s.e.m. (genotype blind analyses).

Samples	**Average Stomatal Aperture** (μm ± s.e.m.) WT 200 ppm [CO_2]
1	*1.654 ± 0.08, n=25 stomata*
2	3.015 ± 0.13, n=25 stomata
3	3.131 ± 0.19, n=17 stomata
4	3.034 ± 0.20, n=19 stomata

3.2. Starch metabolism and CO_2 regulation of stomatal conductance

To analyze how starch metabolism functions in CO_2 regulation of stomatal conductance, mutant plants that lacked starch were exposed to [CO_2] changes and their stomatal responses were examined and compared to WT (Col-0). *adg1* mutants cannot produce in guard cells and mesophyll cells, while *pgi1-1* mutants feature starch accumulation specifically in guard cells but not mesophyll cells (Kunz et al., 2010). Whole plants were grown for 4 weeks in a growth chamber under ambient CO_2 levels and misted one day prior to induction. Whole plants were pre-incubated in 200 ppm [CO_2] for 5 hours and the 5th leaf was sampled and analyzed. For high [CO_2]-induction, plants from the 200 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 45 min and the 5th leaf was sampled and analyzed (both CO_2-controlled chambers were set to ~130 μmol m^{-2} s^{-1} light intensity 21°C, and ~60% relative humidity). Next, the leaf epidermis was blended with deionized water, filtered using a 100 μm nylon mesh, observed under the light microscope, and stomatal aperture was analyzed using Image J software. Results indicated that high [CO_2]-induced stomatal closure was impaired in *adg1-1* and *adg1-2* mutants (Fig. 2A and C), while *pgi1-1* mutants responded to CO_2 by stomatal closure (Fig. 2B). Representative experimental results for each line are presented in Fig 2.

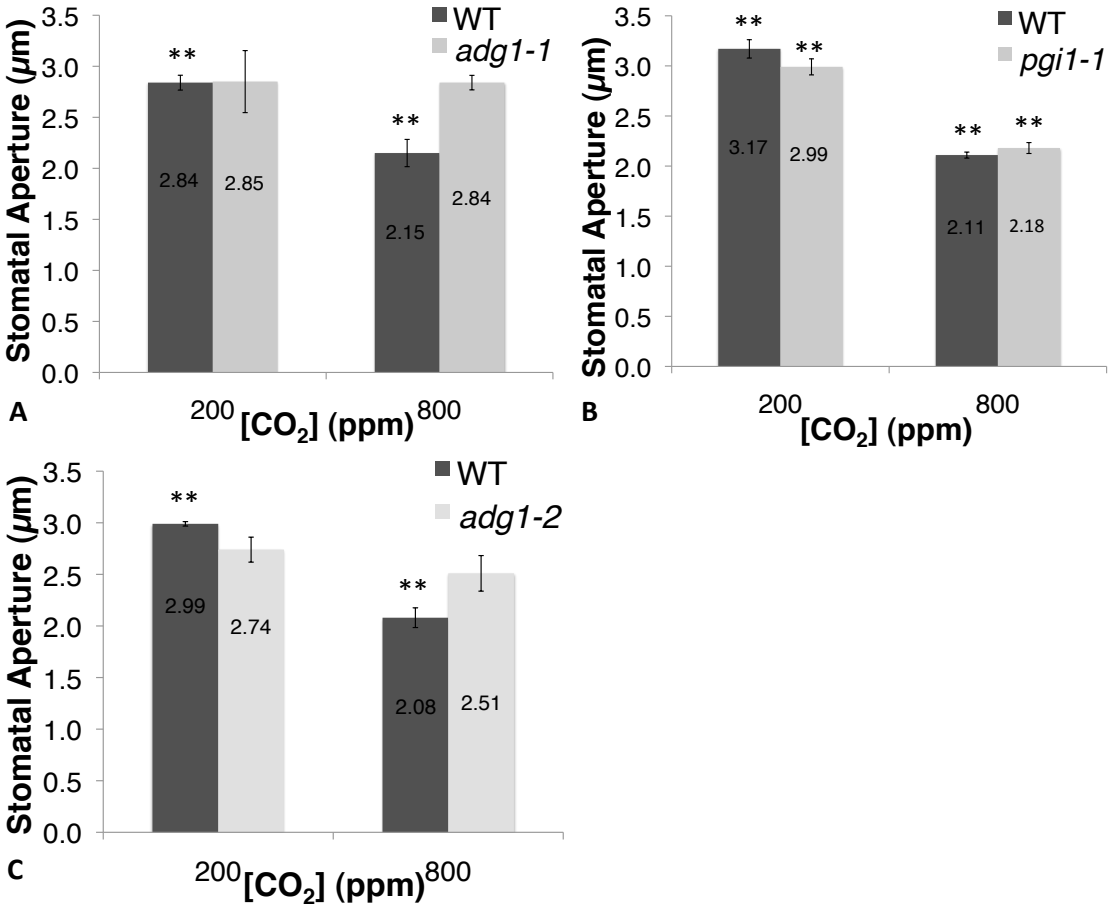


Figure 2. *adg1-1* and *adg1-2* but not *pgi1-1* showed impaired stomatal responses to CO_2 shifts in stomatal movement analyses. Stomatal apertures were measured in WT (Col-0), *adg1*, and *pgi1-1* plants in response to [CO_2] changes. Whole plants were pre-incubated in 200 ppm [CO_2] for 5 hours and the 5th leaf was sampled and analyzed. For high [CO_2]-induction, plants from the 200 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 45 min and the 5th leaf was sampled and analyzed. Leaf epidermis was blended and observed under the light microscope. **(A)** The *adg1-1* mutant that cannot produce starch in general, showed impairment to high [CO_2]-induced stomatal closure (p=0.98). **(B)** The *pgi1-1* mutant that accumulates starch specifically in guard cells, responded to CO_2 by stomatal closure. **(C)** *adg1-2* (SALK_133788), a second allele of the *adg1* mutant that also lacks starch, showed similar impaired responses as *adg1-1* (p=0.36). n=3, total 90 stomata, pairwise Student's t-test. **p<0.02. Data represent means ± s.e.m. (genotype blind analyses).

3.3. Guard cell photosynthesis and CO_2 regulation of stomatal conductance

To investigate the importance of guard cell photosynthesis during CO_2 regulation of stomatal conductance, transgenic plants (*GC-ChlaseΔN*) with severe chlorophyll reductions in guard cells were exposed to [CO_2] changes and their stomatal movements were examined and compared to WT, E1728 (Columbia-0 ecotype). Three *GC-ChlaseΔN* transgenic lines (*#4, #5, and #8*) that over-expressed chlorophyllase (Chlase) under a guard cell specific promoter showed severe chlorophyll reductions exclusively in guard cells and were used in these experiments (Dr. Tamar Azoulay-Shemer).

3.3.1. Chlorophyll auto-fluorescence measurements

Confocal microscopy analysis and Z-stack images were used to quantify guard cell chlorophyll levels in WT and transgenic plants. Epidermal peels were adhered to a microscope slide (Materials and Methods section 2.3.2. Attachment of epidermal peels to cover glass). Relative guard cell chlorophyll levels were averaged in both kidney- and thin-shaped stomata and normalized to WT (100%). Transgenic lines showed severe reduction in chlorophyll levels, up to 22.7%, 16.4%, and 14.6% of WT levels for #*4*, #*5*, and #*8*, respectively (Fig. 3A). Additionally, relative guard cell chlorophyll levels were averaged in only kidney-shaped stomata (excluding thin-shaped) and normalized to WT (100%). Transgenic lines still showed severe reduction in chlorophyll levels, up to 31.6%, 28.7%, and 17.7% of WT levels for #*4*, #*5*, and #*8*, respectively (Fig. 3B).

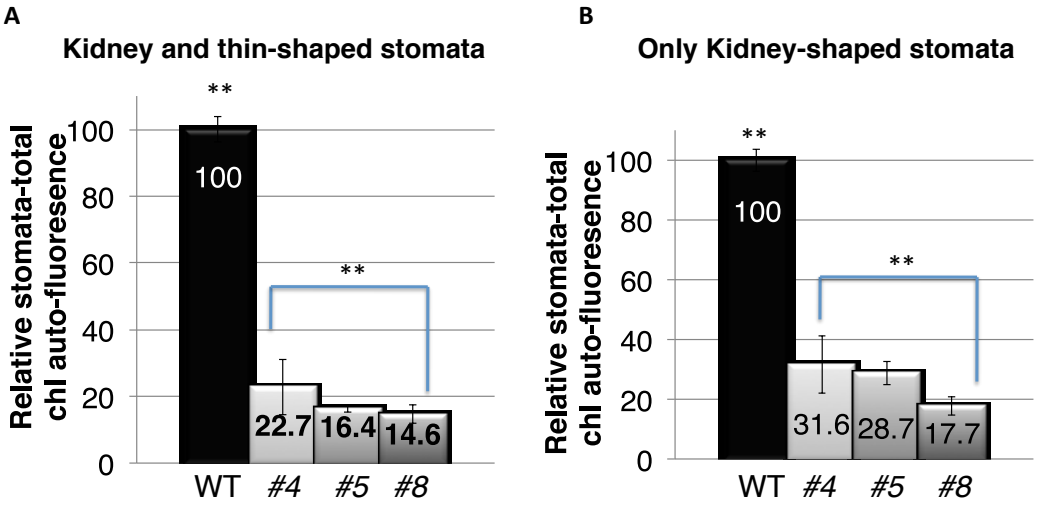


Figure 3. *GC-ChlaseΔN* stomata suffered from severe chlorophyll reduction. Confocal microscopy analyses (Z-stack images) were used to analyze the relative (to WT=100%) average chlorophyll auto-fluorescence in **(A)** both kidney-shaped and thin-shaped stomata of *GC-ChlaseΔN* transgenic lines and **(B)** only kidney-shaped stomata (excluding thin-shaped stomata) of the transgenic lines. WT (E1728) n=8 plants, lines *#4*, *#5*, *#8* n=5 plants, ~24 stomata/sample, pairwise Student's *t*-test. ***p*<0.01, data represents means ± s.e.m. Acknowledgments to Dr. Tamar Azoulay Shemer for assistance with data analysis and plotting.

3.3.2. More than 90% of the *GC-ChlaseΔN* guard cells showed reduced chlorophyll levels

The chlorophyll auto-fluorescence data in individual guard cells was further analyzed by Dr. Tamar Azoulay-Shemer and statistically analyzed and plotted by Dr. Aaron Stephan, who programmed a R-script. The data is represented as a scatter-plot. Each dot corresponds to the individual chlorophyll fluorescence of a guard cell relative to WT (normalized to 100). Black dots and grey dots represent kidney-shaped stomata and thin-shaped stomata, respectively. Horizontal lines depict the mean and standard deviation for each plant. A statistical analysis of all the data revealed that for all three

transgenic lines analyzed, more than 90% of guard cells displayed reduced chlorophyll levels beyond one-standard deviation of the WT mean (Fig. 4).

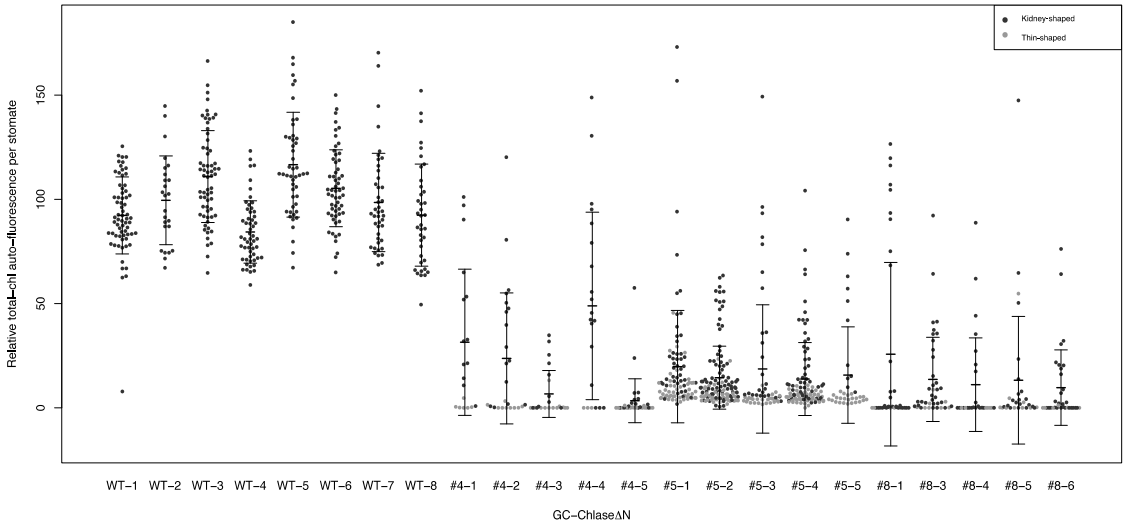


Figure 4. More than 90% of the *GC-ChlaseΔN* guard cells showed reduced chlorophyll levels. Total chlorophyll auto-florescence of individual guard cells was analyzed from confocal microscopy using Z-stack images. Each dot in the scatter plot corresponds to the chlorophyll fluorescence of an individual guard cell relative WT (normalized to 100). Black dots and grey dots represent kidney-shaped stomata and thin-shaped stomata, respectively. Horizontal lines depict the mean and standard deviation for each plant. Acknowledgments to Dr. Tamar Azoulay-Shemer and Dr. Aaron Stephan for assistance with data analysis and plotting.

3.3.3. Kidney-shaped but not thin-shaped stomata of *GC-ChlaseΔN* lines responded to CO_2 shifts

Stomatal movement analyses in response to CO_2 shifts were performed in WT (E1728 – Columbia-0 ecotype) and *GC-Chlase*Δ*N* transgenic plants to study how guard cell photosynthesis functions in regulation of stomatal conductance. Whole plants were grown for 4 weeks in a growth chamber under ambient CO_2 levels. Whole plants were pre-incubated in 200 ppm [CO_2] for 2 hours and the 5th leaf was sampled and analyzed.

For high [CO_2]-induction, plants from the 200 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 45 min and the 5th leaf was sampled and analyzed (both CO_2-controlled chambers were set to ~140 µmol m^{-2} s^{-1} light intensity, 21°C, and ~60% humidity). Next, the leaf epidermis was blended quickly with CO_2-equilibrated water (200/800ppm CO_2, correspondingly), filtered using a 100 µm nylon mesh, observed under the light microscope, and stomatal aperture was analyzed using Image J software.

Stomatal movement analyses revealed that kidney-shaped stomata of the *GC-ChlaseΔN* lines responded to high [CO_2] by stomatal closure (Fig. 5), while thin-shaped stomata remained constitutively closed under both low and high CO_2 levels (Fig. 5). Additionally, kidney-shaped stomata of the transgenic lines did not open as pronouncedly as WT following 200 ppm [CO_2]-induction.

All stomatal movement analyses in response to CO_2 changes were genotype blind during the experiment. However, the genotype was known as the thin-shaped phenotypes of the transgenic lines were easily detected when the data was being analyzed. But the treatment was made unknown as the file names were changed and later identified after the data was analyzed. All experiments included n=4 plants, except the data shown in Fig. 5C, in which n=3 only for WT plants.

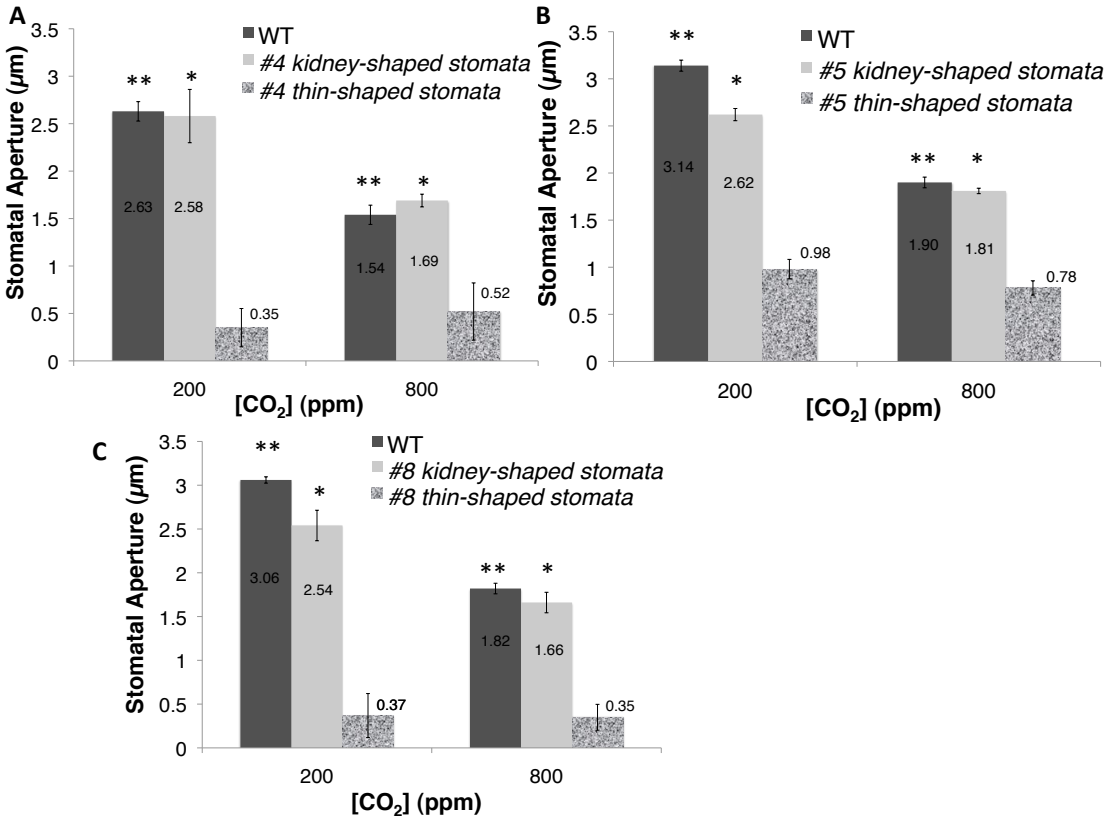


Figure 5. Kidney-shaped stomata of *GC-ChlaseΔN* plants responded to high [CO_2]-induction, while thin-shaped stomata remained continuously closed. Stomatal apertures were measured in WT (E1728) and the three *GC-ChlaseΔN* transgenic lines (*#4*, *#5*, *#8*) in response to [CO_2] changes. Whole plants were pre-incubated in 200 ppm [CO_2] for 2 hours and the 5th leaf was sampled and analyzed. For high [CO_2]-induction, plants from the 200 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 45 min and the 5th leaf was sampled and analyzed. Leaf epidermis was blended with CO_2-equilibrated water (200/800ppm CO_2, respectively) and observed under the light microscope. Kidney-shaped stomata of *GC-ChlaseΔN* responded to high [CO_2]-induction by stomatal closure, while thin-shaped stomata remained constitutively closed under both low and high CO_2 levels (p>0.1). n=4 plants, total 120 stomata. **p<0.001, *p<0.05, pairwise Student's t-test. Data represent means ± s.e.m. (treatment blind analyses).

4. DISCUSSION

4.1. Developing an innovative method for stomatal movement analyses in response to CO_2 changes

Stomatal movement analyses in response to CO_2 shifts have been an integral part in understanding how plants regulate stomatal conductance. The most prevalent analysis is the bubbling method that has been utilized by many labs; however, this method has a few limitations. Detaching a leaf may cause mechanical wounding and alter its viability and condition, which may result in stomata behaving differently than when leaves are intact. It is well known that wounding induces different pathways that can influence stomatal aperture, a few of which include ABA (Leon, 2001) and H_2O_2 (Guan & Scandalios, 2000; Pei et al., 2000) upregulation. Therefore, an innovative stomatal movement analysis, in which whole plants were induced in CO_2-controlled chambers, was more favorable when choosing the method of analysis. In addition, inducing whole plants in these chambers is much more simple and elegant than bubbling CO_2 into a solution through a tube. The CO_2 levels in this new method are controlled and monitored in a sealed chamber, while the CO_2 levels inside the solution cannot be monitored. It was also important to make sure that the detached leaf had good contact with the opening buffer solution during the bubbling method. However, it appeared that the CO_2 that was being bubbled moved the leaves and sometimes reduced their contact with the solution, which may have affected stomatal responses. Moreover, this innovative method included adding CO_2-equilibrated water and using this water when blending the epidermis prior to imaging under the microscope. The water was left in a flask and placed in the CO_2-controlled chamber 24 hours prior to the experiment so the corresponding [CO_2] would have enough time to diffuse into the flask (gas takes a longer time to equilibrate in

liquid). The epidermis was then blended with this [CO_2]-equilibrated water as opposed to water that had ambient CO_2 levels. Numerous stomatal movement analyses in response to CO_2 shifts were conducted (Data not shown). Results suggested that fast imaging was crucial for significant stomatal responses to CO_2 during these experiments. Stomata reacted quickly to [CO_2] changes (Table 1). Therefore, it was imperative that preparing the slide and imaging it under the microscope be done as fast as possible during stomatal movement analyses in order to obtain accurate data.

Comparison between the bubbling method (Fig. 1B) and the whole plant method (Fig. 1C) revealed that the whole plant method induced stronger stomatal responses to CO_2 in WT plants. WT stomata closed more pronouncedly following high CO_2 induction.

4.2. Starch metabolism and CO_2 regulation of stomatal conductance

In order to study guard cell starch metabolism and its involvement in CO_2 regulation of stomatal conductance, stomatal movement analyses in response to CO_2 shifts were performed on *adg1* and *pgi1-1* mutants. The two allelic mutants of ADGase (Kunz et al., 2010), *adg1-1* and *adg1-2*, that cannot produce starch in both guard cells and mesophyll cells, showed impairment to high [CO_2]-induced stomatal closure (Fig. 2A and C), while *pgi1-1* mutants that feature starch accumulation specifically in guard cells but not mesophyll cells, responded to high [CO_2] by stomatal closure. One hypothetical explanation for these results is based on the effects malate has as an osmolyte. Since the starch biosynthesis pathway is blocked in guard cells of *adg1* mutants (Kunz et al., 2010), then malate to starch conversion is inhibited; thus, malate accumulates in the guard cell, increasing osmolarity, resulting in guard cell turgor increase. Without malate being able to convert to starch following high CO_2-induction, osmolarity levels will remain high in the guard cell and the stomata will be inhibited to close. This assumption can be supported by stomatal movement analyses in response to CO_2 shifts, showing that *adg1* stomatal apertures were approximately the same after both 200 and 800 ppm [CO_2]-inductions (Fig. 2A and C). Additionally, few studies may support this hypothesis. One study suggested that malate accumulation is correlated with stomatal aperture (Allaway, 1973; Cyanea & Faba, 1973; Pearson & Milthorpe, 1974; Vavasseur & Raghavendra, 2005), while another study explained how there existed an importer, AtABCB14, that transported malate from the apoplast into the guard cell (Lee et al., 2008), which can increase guard cell turgor.

On the other hand, a different explanation can suggest that starch deficiency in guard cells and mesophyll cells of the *adg1* mutant cannot provide a source of starch to breakdown into malate. It was previously proposed that R-type anion channels are activated by an increase in apoplastic malate concentrations (Hedrich & Marten, 1993; Hedrich et al. 1994). Moreover, Cl^- and malate efflux through these R-type anion channels have been shown to play an important role in stomatal closure (Schmidt & Schroeder, 1994). Additional data displayed that increased [CO_2] resulted in increased apoplastic malate concentrations by 50-100%, and an increase in apoplastic malate concentration resulted in stomatal closure (Rainer Hedrich et al., 1994). However, it is still unknown whether apoplastic malate is provided for solely by the mesophyll cell or by guard cells; or both. Since there is no starch to breakdown into malate in both guard cells and mesophyll cells, then *adg1* mutants cannot provide a source of apoplastic malate. Without apoplastic malate, R-type anion channels cannot be activated, thus stomatal closure is inhibited; which may be one explanation for the impaired high [CO_2]-induced stomatal closure response observed in *adg1* mutants. However, since the *pgi1-1* mutants responded to high [CO_2]-induction by stomatal closure, this may suggest that the deficiency of starch in guard cells but not mesophyll cells is causing the impaired stomatal closure phenotype of *adg1* mutants. Perhaps, starch accumulation specifically in guard cells in *pgi1-1* mutants, can provide a source for malate, which can be lost from the guard cell upon efflux into the apoplast and activate anion channels necessary for high [CO_2]-induced stomatal closure. Moreover, additional studies may further support this hypothesis. Hedrich and Marten (1993) demonstrated how external malate concentrations affected the properties of a specific anion channel located in the guard cells, GCAC1

(Guard Cell Anion Channel 1). Following activation of this channel, anion efflux through GCAC1 would decrease guard cell turgor and result in stomatal closure. They also showed that the binding site for malate to GCAC1 is located on the extracellular side of the channel. In fact, cytoplasmic malate proved to be ineffective in activating GCAC1 (Hedrich & Marten, 1993). These studies proposed apoplastic malate as a potential activator of GCAC1 and regulator of stomatal aperture. Therefore, a lack of apoplastic malate and hence, inability to activate GCAC1 may explain why *adg1* mutants showed impairment to high [CO_2]-induced stomatal closure.

So far little research has been done on starch metabolism and stomatal conductance. The only research in this field includes analyses of stomatal conductance in response to red and blue light (Talbott & Zeiger, 1993) but not CO_2 shifts. One study investigated another mutant that lacked starch, *pgm* (Lasceve, Leymarie, & Vavasseur, 1997), and showed that slow stomatal opening occurred during dark conditions in WT but not *pgm* mutants. In addition, this mutant responded more slowly to blue light. Another publication (Nunes-Nesi et al., 2011) analyzed mitochondrial succinate-dehydrogenase and fumarase anti-sense lines, which showed a correlation between malate and fumerate concentrations in the apoplast and stomatal conductance. The mitochondrial-succinate dehydrogenase anit-sense lines contained low levels of malate and fumarate, and therefore had a larger stomatal aperture due to a higher starch concentration and carbon assimilation rate. Conversely, the mitochondrial fumarase antisense lines showed high malate and fumarate and low starch concentrations. Thus, carbon assimilation rates and stomatal conductances were markedly reduced. They displayed that stomata of WT and transgenic lines closed when incubated with malate or fumarate (apoplastic

concentration), which provides additional evidence that apoplastic malate is responsible for stomata closure.

In the future, quantification of metabolites by measuring the concentrations of starch, malate, and other metabolites in this pathway in guard cells and comparing them to whole plant concentrations would give better insight to further elucidate the role of starch metabolism in CO_2 regulation of stomatal conductance. Additionally, engineering a transgenic plant by using an *adg1* mutant that expresses ADGase specifically in mesophyll cells but not guard cells, and performing stomatal movement analyses in response to CO_2 shifts in this transgenic line, would lead to a better understanding of how mesophyll starch metabolism functions in regulation of stomatal conductance.

4.3. Guard cell photosynthesis regulation of CO_2 stomatal conductance

Data have shown that guard cells in isolated epidermal peels that do not contain any mesophyll cells, responded to CO_2 (Frechilla, 2002). This proposes that the epidermis may contain some kind of CO_2 sensor. Additional studies suggested that guard cells could possibly contain their own photosynthetic activity, which control stomatal apertures (Shimazaki, Gotow, & Kondo, 1982; Gotow, Taylor, & Zeiger, 1988). In contrast, other experts believe that the organic compounds produced by mesophyll cell photosynthesis can regulate stomatal conductance (Roelfsema et al., 2006). Nevertheless, stomatal movement analyses in response to CO_2 shifts were performed in *GC-ChlaseΔN* (Dr. Azoulay-Shemer) transgenic plants in order to elucidate the role of guard cell photosynthesis in CO_2 regulation of stomatal conductance. Stomatal conductance responses to CO_2 changes in *GC-ChlaseΔN* showed that kidney-shaped but not thin-shaped stomata responded to high [CO_2]-induction by stomatal closure (Fig. 5). All three *GC-ChlaseΔN* transgenic lines showed kidney-shaped stomata with severely reduced chlorophyll levels and thin-shaped stomata with approximately zero chlorophyll (Fig. 3 and 4). Thin-shaped stomata displayed GFP expression when observed via confocal microscopy (Fig. S1. B), indicating that they are still viable. However, these guard cells are constantly closed, lose their turgor, and collapse, suggesting that a certain threshold of guard cell chlorophyll is required for basic stomatal functioning. Furthermore, scatter plot data (Fig. 4) showed that 35-67% of kidney-shaped stomata contained less than 10%, and 29-47% contained ≤ 2%, of WT guard cell chlorophyll levels, suggesting that stomata probably collapse only under extreme reduced levels of chlorophyll. Additionally, thin-

shaped stomata cannot assimilate carbon to produce enough osmolytes and generate adequate turgor, and hence collapse.

Statistical analysis of individual guard cells (Dr. Tamar Azoulay-Shemer and Dr. Aaron Stephan) showed that more than 90% of *GC-ChlaseΔN* guard cells had severely reduced chlorophyll levels (Fig 4). Further quantification revealed that the average chlorophyll auto-fluorescence in kidney-shaped stomata of the *GC-ChlaseΔN* lines was significantly reduced, by ~68-74% of WT levels (Fig. 3).

On the other hand, kidney-shaped stomata closed following high [CO_2]-induction, but did not open to the same extent as WT following 200 ppm [CO_2]-induction (Fig. 5). Since these stomata suffered from reduced energy sources such as ATP and NADPH (Willmer & Fricker, 1996), originating from their decreased levels of photosynthesis in guard cells, then these guard cells may not be producing enough osmolytes required for stomatal opening as compared to WT. However, kidney-shaped stomata of these transgenic lines may be generating turgor from other sources in order to counter their severe reduction in guard cell chlorophyll levels. A previous publication proposed that osmolytes such as malate are transported from the apoplast into the guard cell via an importer, AtABCB14 (Lee et al., 2008), inducing an increase in turgor. Additionally, guard cell starch levels in these transgenic lines are severely reduced (Analysis by Dr. Azoulay-Shemer). Previous studies have suggested that starch reserves in *Vicia faba* guard cell chloroplasts may be vital in generating enough turgor for stomatal opening (Outlaw & Manchester, 1979). However, this study was conducted in *Vicia faba* and not in *Arabidopsis* plants.

Nevertheless, results displayed that kidney-shaped stomata in *GC-ChlaseΔN* lines responded to CO_2 shifts. However, it is still not completely clear if the kidney-shaped stomata that do not contain any chlorophyll in these transgenic lines can respond to CO_2 shifts. Two previous studies have shown that photosynthesis in guard cells is not required for normal stomatal responses to CO_2 (Hu et al., 2010; Roelfsema et al., 2006). In both studies, plants were treated with nonflurazon, inhibiting caretenoid synthesis, leading to albino leaves with guard cells that lacked functional chloroplasts. Yet, these albino leaf patches showed normal stomatal responses to CO_2. Moreover, another publication showed that stomata of the Orchid genus, *Paphiopedilum,* which lacked chloroplasts entirely, still maintained functional stomata and responded to CO_2 (Nelson & Mayo, 1975). Yet, these studies used orchid species, which differ from *Arabidopsis thaliana* species, and the use of chemicals such as nonflurazon could have other physiological side effects on the plant. These issues need to be taken into account when considering the mechanisms that regulate stomatal conductance.

Because results suggested that normal chlorophyll levels might not be necessary in guard cells for CO_2 regulation of stomatal conductance, this implies that mesophyll cells play a central role in regulation of stomatal conductance. Additional analysis, by Dr. Azoulay-Shemer showed that the whole plant chlorophyll levels of all *GC-ChlaseΔN* lines were not affected, indicating that mesophyll chlorophyll levels were intact. Different studies have suggested that mesophyll photosynthetic activity directly monitors stomatal aperture in response to CO_2 changes (Assmann, 1999; Morison, 1998; Mott, 1990, Hedrich et al., 1994). However, my experiments focused on photosynthesis specifically in guard cells and how they regulate stomatal conductance. Therefore, more

research is necessary in order to understand the involvement of mesophyll photosynthesis in CO_2 regulation of stomatal conductance.

Smaller stomatal apertures in the transgenic lines could have been attributed to developmental effects. A previous publication has shown a negative correlation between stomatal density and stomatal aperture as a compensation mechanism (Büssis, von Groll, Fisahn, & Altmann, 2006). However, stomatal index and density analyses by Dr. Azoulay-Shemer (Data not shown) had confirmed that stomatal development in these transgenic lines was normal. Nevertheless, the extent to which guard cells and mesophyll cells function in CO_2 regulation of stomatal conductance still remains unclear. Therefore, future analysis requires tracking the response of the kidney-shaped stomata in *GC-Chlase*Δ*N* that do not contain any chlorophyll in these transgenic lines in order to determine whether or not chlorophyll in guard cells is required for normal CO_2 regulation of stomatal conductance. The role of mesophyll photosynthesis in regulation of stomatal conductance is also another topic that requires further investigation.

5. APPENDIX

5.1. Guard Cell Regulation and Auxin Signaling

Auxin is a plant hormone and is mainly known for its role in plant growth (Bennett & Scheres, 2010). IAA5 is a transcription factor (TF) that negatively regulates auxin signaling (Nakamura et al., 2003). *pIAA5(PROMOTER)-iaa5d2r-GUS #1-3* is a transgenic line that expresses a mutated version of the Iaa5 TF, which binds to the promoter and down-regulates auxin signaling (Kang & Dengler, 2002; Lee *et al*., 2006; Okushima *et al*., 2007; Schlereth *et al*., 2010). Consequently, these plants are phenotypically smaller and hyposensitive to drought (Dr. Eilon Shani – unpublished results). A triple mutant, *iaa 5,6,19*, and the transgenic line, *pIAA5-iaa5d2r-GUS #1-3*, were analyzed in order to independently study the role of IAA in ABA-regulation of stomatal movements.

Stomatal movement analyses in response to ABA were performed on these plants and compared to WT (Columbia-0 ecotype). Whole plants were grown in a growth chamber for 3 weeks under ambient CO_2. The method employed was the same as described in Results Section 3.1. Improving methods for stomatal movement analyses in response to CO_2 chagnes, with the exception that the detached leaves were incubated in opening buffer for 3 hours under 170 μmol m^{-2} s^{-1} light and ABA-induction time was increased to 90 min. Results indicated that WT stomata did not respond to ABA. Therefore, it was not possible to compare the triple mutant and transgenic line to WT stomatal responses. However, since the triple mutant and transgenic line both showed a greater stomatal closure response to ABA than WT, this suggested that they are hypersensitive to ABA (Fig. A1).

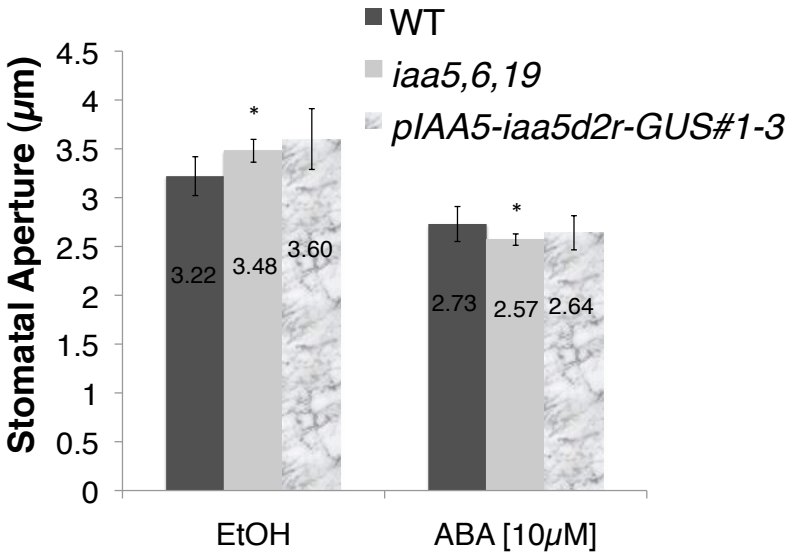
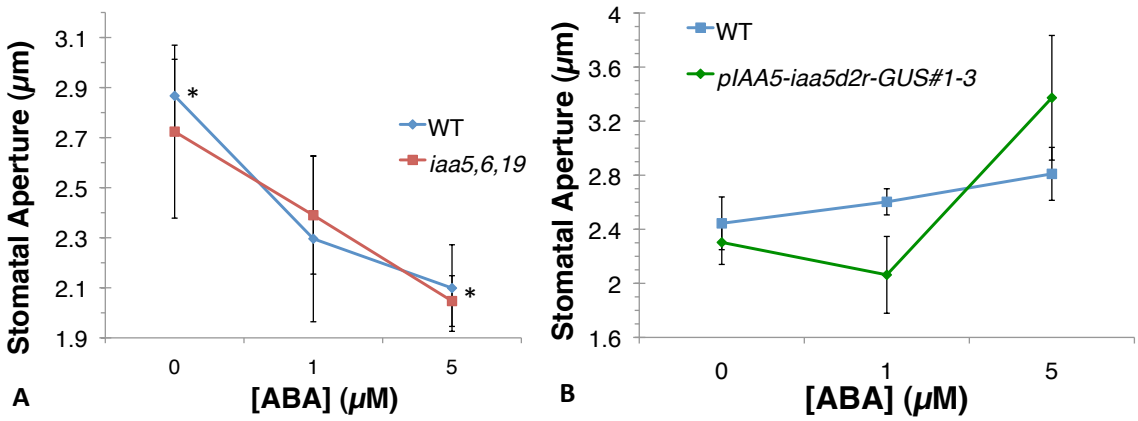


Figure A1. Stomatal movement analysis in response to ABA in *iaa5,6,19*, *pIAA5-iaa5d2r-GUS #1-3*, and WT (Col-0). Plants were pre-incubated in opening buffer solution for 3 hours. 10 μM [ABA] was added to the treated samples for 90 min (0.1% EtOH to solvent control). Epidermis was blended and imaged under light microscope. WT stomatal responses to ABA were insignificant (p=0.15), while *iaa5,6,19* (*p=0.007) and *pIAA5-iaa5d2r-GUS #1-3* (p=0.07) were more pronounced. n=3 plants, total 90 stomata, pairwise Student's t-test. Data represent means ± s.e.m. (genotype and treatment blind analyses).

In order to test whether these mutant and transgenic lines were hypersensitive to ABA, *iaa5,6,19* and *pIAA5-iaa5d2r-GUS #1-3* were exposed to different concentrations of ABA and their stomatal responses were compared to WT (Col-0). Whole plants were grown in a growth chamber for 4 weeks under ambient CO_2. Detached leaves were pre-incubated in opening buffer (pH=5.7) and 130 μmol m^{-2} s^{-1} light for 2 hours. 0, 1, or 5 [ABA] (μM) were added to the samples for 1 hour. Concentrations of ABA were adjusted by diluting with the corresponding amount of EtOH. Epidermis was blended and analyzed under light microscope. 0 μM [ABA] (EtOH) acted as the baseline control.

Results indicated that WT responded significantly to 5 µM [ABA] but not to 1 µM [ABA], while *iaa5,6,19* responded similarly as WT, however, with a lower *p*-value (Fig. A2.A). In the second experiment, WT and *pIAA5-iaa5d2r-GUS #1-3* stomata did not show any responses to ABA (Fig. A2.B).



p-values, n=3

mM	Col-0	*iaa5,6,19*
0-1	0.222	0.475
0-5	0.029	0.182
1-5	0.635	0.281

p-values, n=3

mM	Col-0	*pIAA5-iaa5d2r-GUS#1-3*
0-1	0.519	0.513
0-5	0.256	0.135
1-5	0.415	0.086

Figure A2. *iaa5,6,19* and *pIAA5-iaa5d2r-GUS #1-3* were exposed to different concentrations of ABA Detached leaves were pre-incubated in opening buffer and 130 µmol m^{-2} s^{-1} light for 3 hours. 0, 1, or 5 [ABA] (µM) were added to the samples for 1 hour. **(A)** WT (Col-0) responded significantly only to 5 µM [ABA] (**p*=0.029) but not to 1 µM [ABA], while *iaa5,6,19* showed a similar but weaker response. **(B)** WT (Col-0) and *iaa5d2r-GUS #1-3* did not show responses to ABA. All *p*-values are listed under the figure. n=3 plants, total 90 stomata, pairwise Student's *t*-test. Data represent means ± s.e.m. (genotype and treatment blind analyses).

Results from Fig. A1 and Fig. A2 are contradictory so no definitive conclusions can be made. Nevertheless, this was all the data collected from this project. Further investigation is necessary in order to make conclusions about these plants and their role in guard cell regulation and auxin-signaling.

5.2. PCR and Genotyping

5.2.1. C-NAD-MDH2 (Cytosolic-NAD-Dependent Malate Dehydrogenase 2)

Different studies have proposed that extracellular malate, an osmolyte and activator of R-type anion channels, is involved in regulation of stomatal conductance (Rainer Hedrich et al., 1994). Malate dehydrogenase (MDH) is a reversible enzyme that reduces oxaloacetate to malate by using NADH as energy (Tomaz et al., 2010). There are 3 MDH located in the cytosol. However, only the NAD-MDH2 was highly expressed (approximately 70-fold more $(8.396)^2$) in guard cells than mesophyll cells (analysis from Schroeder Lab – Affymetrix data). Therefore, the C-NAD-MDH2 knockout line was used to observe its affects on CO_2-regulation of stomatal conductance. A gabi-kat line number 136F09 was ordered and genotyped to confirm this mutant line (http://www.gabikat.de/db/showseq.php?line=136F09&gene=At5g43330).

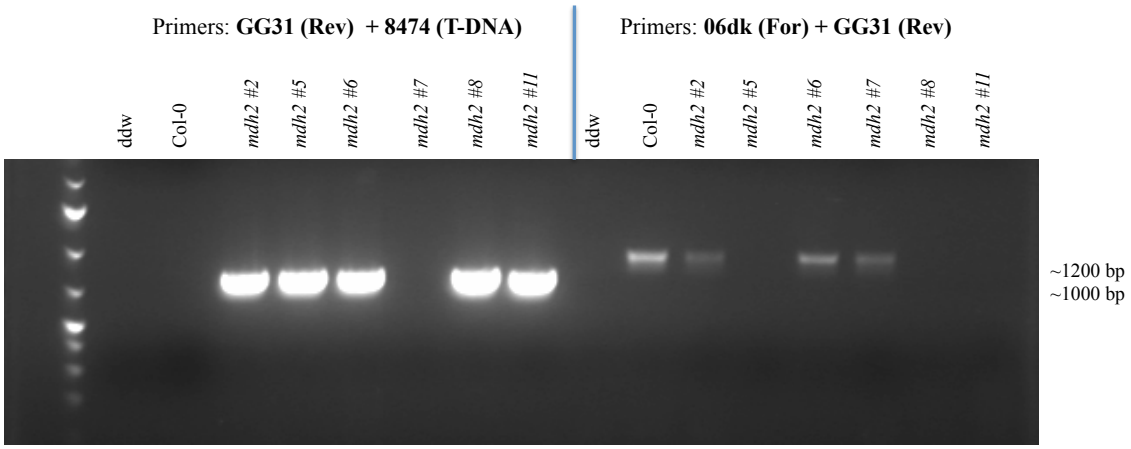


Figure A3. *mdh2* genotyping. DNA gel of ddw (- control), Col-0 (+ control), and *mdh2* plants *#2, #5, #6, #7, #8, #11* using primers 06dk (For), GG31 (Rev), and 8474 (T-DNA). *mdh2* lines *#5, #8, and #11* were confirmed as homozygous to the mutation. The PCR was performed under the following conditions: 94°C for 2 min; 37 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 90 s; and 72°C for 5 min. Primer sequences are listed in Table A2.

To investigate how CO_2 levels regulate stomatal conductance, a stomatal movement analysis in response to CO_2 shifts was conducted on *mdh2 #8* and compared to WT (Col-0). The method employed in this analysis was the same as described in Results section 3.2. Starch metabolism and CO_2 regulation of stomatal conductance, with the exception that plants were induced in 150 ppm [CO_2] instead of 200 ppm [CO_2] for low [CO_2]-induction. Results suggested that high [CO_2]-induced stomatal closure was impaired in *mdh2 #8* (Fig. A4).

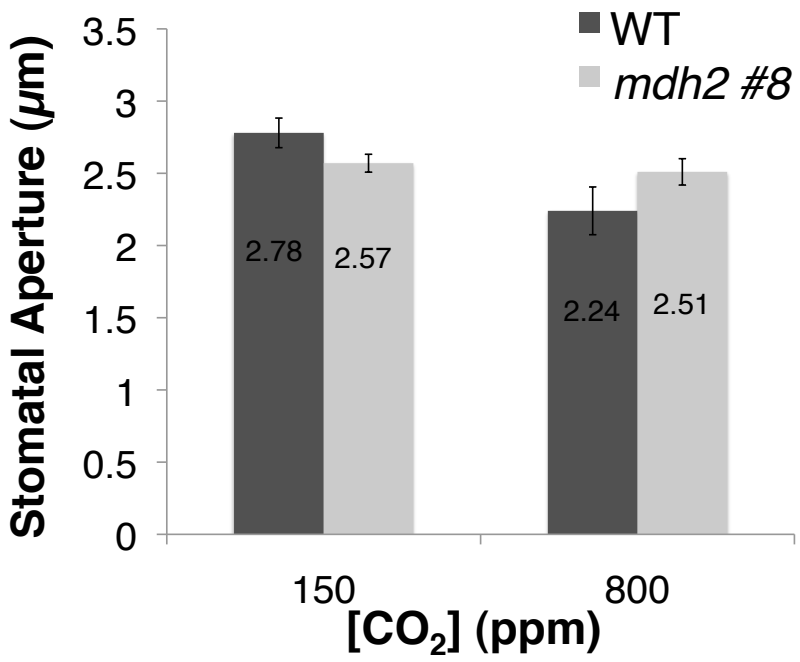


Figure A4. *mdh2 #8* high [CO_2]-induced stomatal closure may be impaired. Stomatal apertures were measured in WT (Col-0) and *mdh2 #8* plants in response to [CO_2] changes. Whole plants were pre-incubated in 150 ppm [CO_2] for 5 hours and the 5th leaf was sampled and analyzed. For high [CO_2]-induction, plants from the 150 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 45 min and the 5th leaf was sampled and analyzed. Leaf epidermis was blended and observed under the light microscope. WT (p=0.06) stomata responded to CO_2 shifts (p=0.06), while *mdh2 #8* did not (p=0.6). n=3, total 90 stomata, pairwise Student's t-test. Data represent means ± s.e.m. (genotype blind analyses).

No conclusions can be made because Dr. Azoualy-Shemer's gas exchange results did not correlate with this stomatal movement analysis. This analysis was done once and repetitions are necessary. Gas exchange analysis will be performed after knocking out all 3 cytoplasmic MDH and seeing if this triple mutant will have any stomatal responses to CO_2 shifts.

5.2.2. NPC4 (Non-Specific Phospholipase C4)

Diacyglycerol (DAG) is a lipid messenger molecule that has been suggested to impact plant responses to ABA (Peters et al., 2010). *NPC4* knockout plants displayed decreased ABA sensitivity in stomatal movements and tolerance to hyperosmotic stress. Therefore, the *NPC4* knockout was ordered and genotyped to confirm the mutation in order to observe its stomatal responses to CO_2.

http://www.arabidopsis.org/servlets/TairObject?id=4010766651&type=germplasm

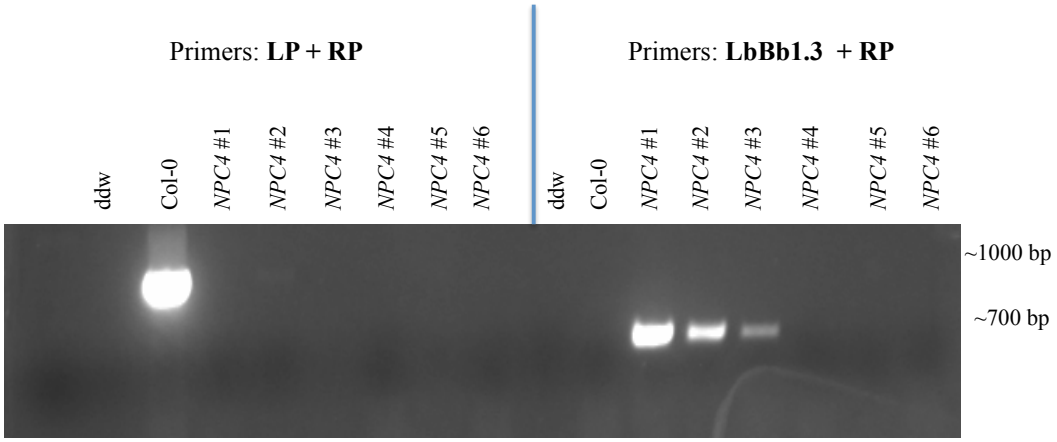


Figure A5. *NPC4* genotyping. DNA gel of ddw (- control), Col-0 (+ control), and *NPC4* plants *#1-6* using primers LP, RP, and LbBb1.3 (T-DNA). *NPC4* lines *#1-3* were confirmed as homozygous to the mutation. The PCR was performed under the following conditions: 94°C for 2 min; 32 cycles of 94°C for 20 s, 59°C for 40 s, 72°C for 70 s; and 72°C for 10 min. Primer sequences are listed in Table A2.

Gas exchange and stomatal movement analyses in response to CO_2 shifts (Dr. Azoualy-Shemer) will be performed on *NPC4* homozygous plants (*#1-3*) to see how CO_2 levels regulate stomatal conductance in *NPC4* plants.

5.2.3. Phospholipase D (PLD) alpha and delta double mutant

Phospholipase D was previously published to be involved in responses to abiotic stress and ABA signaling (Lin, Tai, Peng, & Tzen, 2002). ABA-induced stomatal closure

was suppressed in *pldα+δ* double mutants. Therefore, these mutant lines were ordered and genotyped.

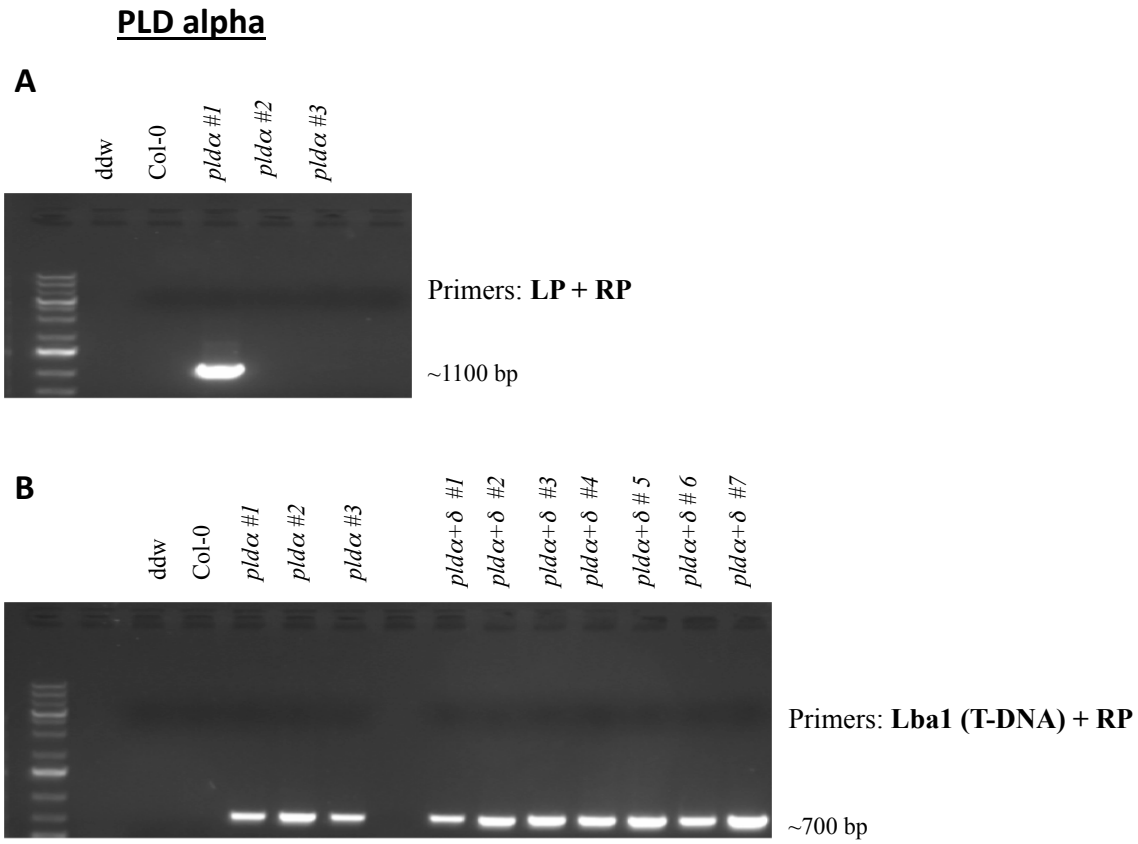


Figure A6. *pldα* genotyping. (A) DNA gel of ddw (- control), Col-0 (+ control), and *pldα* plants *#1-3* using LP and RP primers. **(B)** DNA gel of ddw (- control), Col-0 (+ control), *pldα #1-3* and *pldα+δ #1-7* using Lba1 (T-DNA) and RP primers. All *pldα+δ* plants are positive for the alpha t-DNA insertion. The PCR was performed under the following conditions: **(A)** 94°C for 2 min; 30 cycles of 94°C for 20 s, 59°C for 40 s, 72°C for 70 s; and 72°C for 10 min. **(B)** 94°C for 2 min; 30 cycles of 94°C for 20 s, 59°C for 40 s, 72°C for 50 s; and 72°C for 10 min Primer sequences are listed in Table A2.

PLD delta

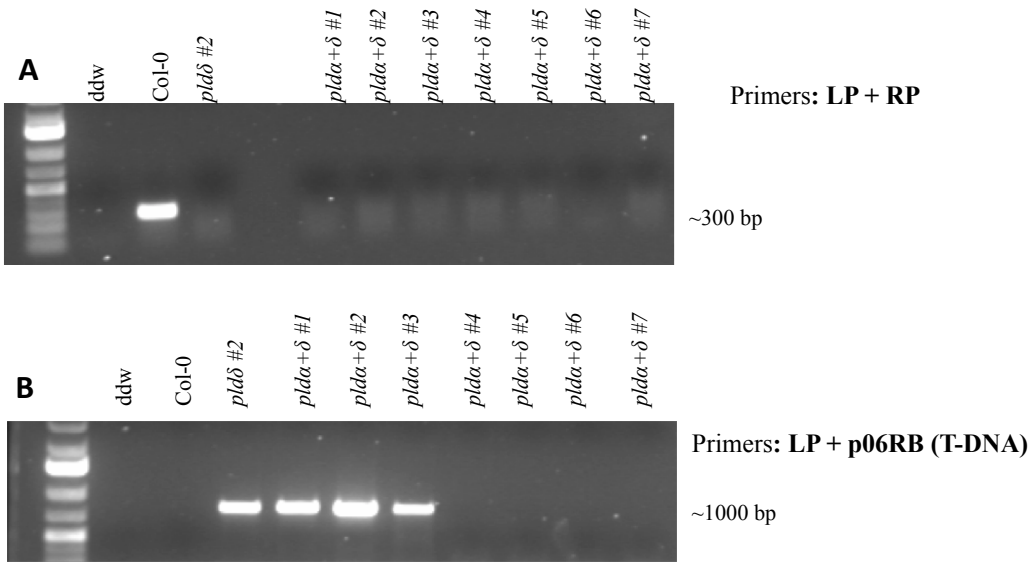


Figure A7. *pldδ* genotyping. (A) DNA gel of ddw (- control), Col-0 (+ control), and *pldδ* plants *#2*, and *pldα+δ* plants *#1-7* using LP and RP primers. **(B)** DNA gel of ddw (- control), Col-0 (+ control), and *pldδ* plants *#2*, and *pldα+δ* plants *#1-7* using LP and p06RB (T-DNA) primers. *pldα+δ* plants *#1-3* may be homozygous for the double mutation. The PCR was performed under the following conditions: **(A)** 94°C for 2 min; 30 cycles of 94°C for 20 s, 51°C for 40 s, 72°C for 60 s; and 72°C for 10 min. **(B)** 94°C for 2 min; 30 cycles of 94°C for 20 s, 58°C for 40 s, 72°C for 60 s; and 72°C for 10 min. Primer sequences are listed in Table A2.

Results indicated that all PLD double mutant plants were homozygous for the alpha subunit mutation (Fig. A6). However, not all were positive for the delta mutation, suggesting that some are heterozygote for the delta subunit mutation (Fig. A7). *pldα+δ* plants (*#1-3*) were grown and seeds were collected. These seeds will be genotyped again to confirm the double mutation of these plants. *pldα+δ* double mutants will be further analyzed by gas exchange analysis (Dr. Azoulay-Shemer).

Table A1. T-DNA names and AGI numbers to corresponding mutant lines.

Gene	Mutant	T-DNA Name	AGI #	Insertion
C-NAD-MDH2	*mdh2*	GABI_136F09	At5g43330	Chr. 5: 17390895
NPC4	*npc4*	SALK_046713	At3g03530	Chr. 3: 843300
PLD Alpha	*pldα*	SALK_053785	At3g15370	Chr. 3: 5331890
PLD Delta	*pldδ*	KAZUSA T-DNA tag line	At4g35790	Chr. 4: 16955518

Table A2. Sequences of primers used for genotyping.

Gene	Mutant	Primers used (5'→ 3')
C-NAD-MDH2	*mdh2*	**GG31 (R)**: AGTGTGTTTCTGTTATTGTGCAGG **06dk (F)**: TTGCTAACATACATGAGCATCACA **T-DNA (8474)**: ATAATAACGCTGCGGACATCTACATTTT
NPC4	*npc4*	**LP**: AATTCCACCCACACACAAGAG **RP**: CTACGAGGCATTGAGATCGAG **T-DNA (LbBb1.3)**: ATTTTGCCGATTTCGGAAC
PLD Alpha	*pldα*	**LP**: ATTAAGTGCAGGGCATTGATG **RP**: CAAGGCTGCAAAGTTTCTCTG **T-DNA**: **(LBa1)**: TGGTTCACGTAGTGGGCCATCG
PLD Delta	*pldδ*	**LP**: CAGATCCATAGCTACCGATG **RP**: TTATGGAAGATAGACCAACC **T-DNA**: **(p06RB)**: TTCCCTTAATTCTCCGCTCATGATC

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7. SUPPLEMENTAL INFORMATION

Characteristics of *GC-ChlaseΔN* transgenic plants

Characteristics of *GC-ChlaseΔN* transgenic lines were analyzed and compared to WT (E1728 – Columbia-0 ecotype). This enhancer trap line was used as E1728 and all *GC-ChlaseΔN* plants expressed GFP constitutively in their guard cells so that they were easy to identify under the confocal microscope. Pictures of 28-day-old plants from the T3 generation revealed that *GC-ChlaseΔN* transgenic plants displayed normal morphology as WT (Fig. S1.A). Confocal microscopy analysis showed that guard cell chlorophyll auto-fluorescence levels (displayed as red emission) were dramatically reduced in *GC-ChlaseΔN* plants (Fig. S1.B). Additionally, all *GC-ChlaseΔN* transgenic plants were found to have two differently shaped stomata; kidney-shaped (Fig. S1.C) and thin-shaped (Fig. S1.D), as detected using light microscopy. Note that the control line (E1728) did not contain any thin-shaped guard cells.

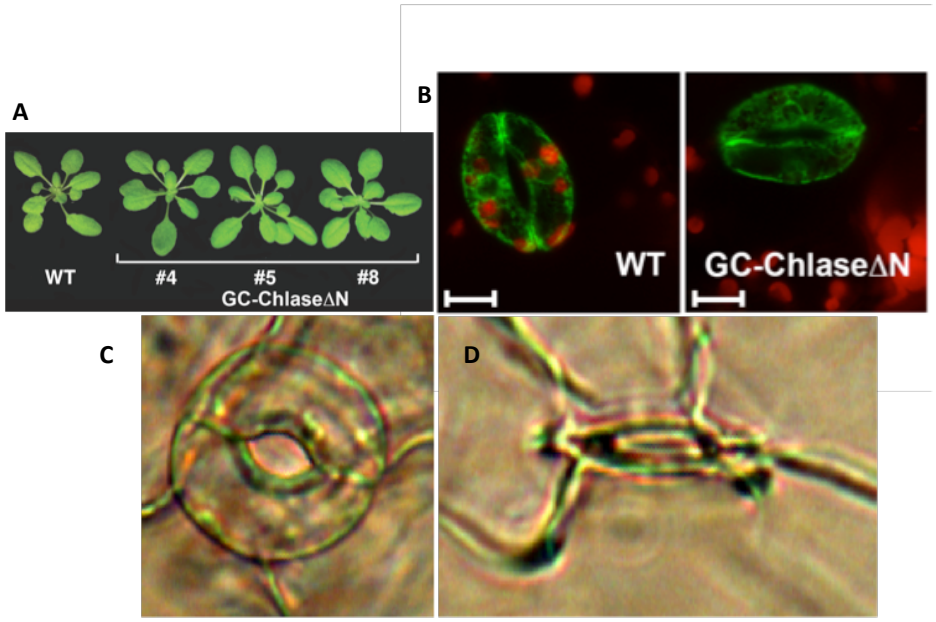

Figure S1. Characterization of *GC-ChlaseΔN* transgenic lines. (A) Images of 4-week-old WT and transgenic plants from the T3 generation demonstrated normal morphology **(B)** Confocal microscopy images of WT and *GC-ChlaseΔN* stomata showed GFP expression and severe chlorophyll reduction in guard cells of the transgenic lines. Light microscopy images of a **(C)** kidney-shaped guard cell (*#8*) and **(D)** thin-shaped guard cell (*#8*). Acknowledgments to Dr. Azoulay-Shemer (A) and (B).

Experimental repeats of stomatal movement analyses in response to CO_2 changes in *adg1-1* and *pgi1-1* mutants showed consistent results

Stomatal movement analyses in response to CO_2 changes were repeated in *adg1-1* and *pgi1-1* mutants and compared to WT (Col-0) responses. The methods employed and results were the same as described in Results section 3.2. Starch metabolism and CO_2 regulation of stomatal conductance (Fig. 2). WT and *pgi1-1* mutants responded to high CO_2-induced stomatal closure (Fig. S2.C and D), while *adg1-1* mutants showed impairment to high CO_2-induced stomatal closure (Fig. S2.A and B).

Additionally, stomatal movement analyses in response to CO_2 changes were repeated in *adg1-1* and *pgi1-1* mutants and compared to WT with longer CO_2-induction times in order to determine whether kinetics changed stomatal responses previously observed in Fig. 2 and Fig. S2.A and B. All methods are the same as described in Fig. 2 (Results section 3.2. Starch metabolism and CO_2 regulation of stomatal conductance), with the exception that both 200 ppm and 800 ppm [CO_2]-induction times were 24 hours each instead of 5 hours and 45 min, respectively. Relatively, WT showed the strongest response to CO_2-induced stomatal closure (p=0.0007), *pgi1-1* showed some response (p=0.007), and *adg1-1* showed the weakest response (p=0.05) (Fig. S2.E and F).

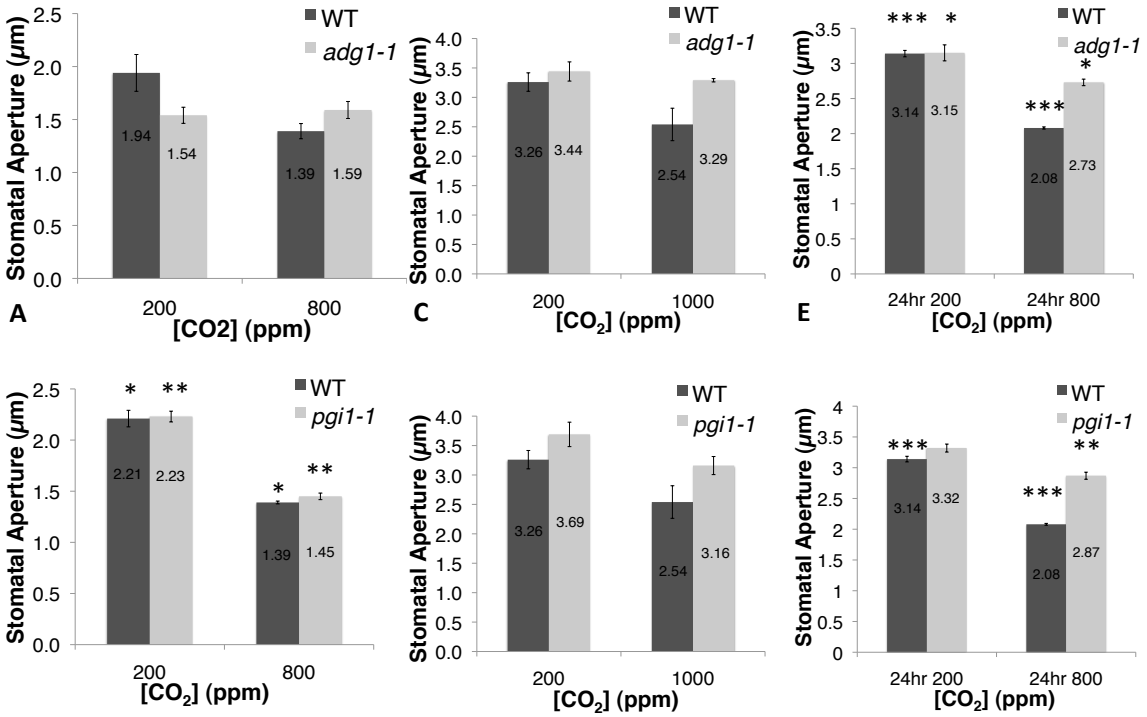

Figure S2. Experimental repetitions confirmed *adg1-1* but not *pgi1-1* showed impaired stomatal closure following high [CO_2]-induction. Stomatal apertures were measured in WT (Col-0), *adg1-1*, and *pgi1-1* plants in response to [CO_2] changes. Whole plants were pre-incubated in 200 ppm [CO_2] for 5 hours and the 5th leaf was sampled and analyzed. For high [CO_2]-induction, plants from the 200 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 45 min and the 5th leaf was sampled and analyzed. Leaf epidermis was blended and observed under the light microscope. **(A)** and **(C)** *adg1-1* showed impairment to high [CO_2]-induced stomatal closure, while **(B)** and **(D)** *pgi1-1* mutants responded to CO_2-induced stomatal closure. **(E)** and **(F)** Whole plants were pre-incubated in 200 ppm [CO_2] for 24 hours and the 5th leaf was sampled and analyzed. For high [CO_2]-induction, plants from the 200 ppm [CO_2]-controlled chamber were transferred to the 800 ppm [CO_2]-controlled chamber for an additional 24 hours and the 5th leaf was sampled and analyzed. Leaf epidermis was blended and observed under the light microscope. **(E)** *adg1-1* showed impaired responses to CO_2 shifts as compared to WT, while **(F)** *pgi1-1* showed slight impairment in responses to CO_2 shifts as WT. n=3, total 90 stomata, pairwise Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001. Data represent means ± s.e.m. (genotype blind analyses).