Title

Carbonic anhydrases function as mediators of CO_2-induced stomatal movements and regulators of stomatal development in Arabidopsis thaliana

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Carbonic anhydrases function as mediators of CO_2-induced stomatal movements and regulators of stomatal development in *Arabidopsis thaliana*

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

in

Biology

by

Amber Beth Ries

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Professor Stuart Brody
Professor Nigel Crawford

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The thesis of Amber Beth Ries is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Acknowledgements

Figures 1 and 2 are the same graphs that appear in: Carbonic Anhydrases Function as Upstream Regulators of CO_2-Induced Stomatal Movements in Plant Guard Cells. Hu, H; Schroeder, JI. These results are in preparation for submission.

ABSTRACT OF THE THESIS

Carbonic anhydrases function as regulators in CO_2-induced stomatal movements and development in *Arabidopsis thaliana*

by

Amber Beth Ries

Master of Science in Biology

University of California, San Diego, 2009

Professor Julian Schroeder, Chair

Stomata are small pores in the epidermes of leaves that open and close in response to a variety of environmental signals including carbon dioxide (CO_2). In wild type plants, high levels of CO_2 cause stomata to close, as well as cause a decrease in stomatal density. However, the CO_2-binding proteins that control these responses remain unknown. Through guard cell and mesophyll cell specific microarrays, two highly expressed β-carbonic

anhydrase genes, *CA1* and *CA4,* were identified. *Arabidopsis thaliana ca1ca4* double knockout mutants exhibit an insensitive stomatal closure response to 800ppm CO_2 and exhibit a higher stomatal density than wild type. To determine which gene, *CA1 and/*or *CA4,* is responsible for the CO_2 phenotype, the genomic DNA with native promoter of *CA1* and *CA4* and the cDNAs of *CA1* and *CA4* driven by a guard cell promoter were transformed into *ca1ca4* double mutant plants. In these *CA*-expressing transgenic lines, stomatal density and CO_2 response recovered to wild type phenotypes, suggesting that the *CA1* and *CA4* genes are indeed responsible for the stomatal CO_2 insensitive response as well as for the high stomatal density of the *ca1ca4* double mutant. To determine whether CA homologues from other species could complement the *ca1ca4* phenotypes, the structurally unrelated Human *CAII* controlled by a guard cell promoter was also transformed into *ca1ca4* plants. The *HmCAII* was able to restore the mutant phenotype of CO_2-induced stomatal closure as well as stomatal density. These results show that *CA1* and *CA4* function within the CO_2 signaling mechanisms that mediate CO_2 control of stomatal movements and development.

I

Introduction

Environmental effects on plant stomata

Stomata are pores in the epidermes of leaves that open and close in response to a variety of environmental factors such as light, temperature, water and carbon dioxide (CO_2). Two specialized guard cells form the stoma to control stomatal movements. Stomatal pores in the epidermes of leaves regulate water loss by transpiration as well as CO_2 influx into leaves for photosynthesis. It is known that high levels of CO_2 cause stomata to close and low levels of CO_2 cause stomata to open. According to the National Oceanic and Atmospheric Administration (NOAA), the ambient level of carbon dioxide is currently 385 parts per million (ppm) whereas in 1960, it was approximately 280ppm[1]. With the rise in continuing atmospheric CO_2, plant life and ecosystems are heavily influenced on a large scale[2]. Previous studies have shown that if the concentration of CO_2 doubles, stomatal apertures close by 20-40% in many plant species[3]. Moreover, a high level of carbon dioxide has been shown to increase continental water runoff due to a decrease in stomatal aperture and stomata density. With lower stomatal density and decreased stomatal apertures, less water evaporates through the pores, which reduces water uptake by plants and more water is left in the ground[4, 5]. Not only does transpiration decrease, but rising CO_2 levels continue to be an issue in global

warming and ecology because the photosynthetic ability of a variety of plants decreases with increased CO_2 as well[6]. Even though increased levels of CO_2 have a cascade of effects on plants, only a few genes and mutants are currently known in the literature that function in CO_2-induced stomatal movements and signal transduction [7-10]

Stomatal movements

For stomata to open, an influx of solutes into surrounding guard cells, such as potassium (K^+), chloride (Cl^-) and malate^{2-} occurs. With the influx of ions, the osmotic potential changes and forces water into the guard cells, causing them to inflate and thus openening the stomatal pore. The influx of ions is stimulated by H^+-ATPase-mediated proton efflux from the cytosol. This efflux hyperpolarizes the guard cell membrane for K^+ and activates voltage-regulated channels that mediate K^+ influx [11]. Other solutes, such as malate^{2-}, Cl^-, and nitrate (NO_3^-) contribute to anion buildup in the guard cells and increase the turgor pressure of the guard cells, offsetting the increasing positive charge of K^+. During stomatal closure, membrane depolarization occurs which creates a driving force for K^+ efflux [11, 12]. With an efflux of K^+, water and other solutes rush out of the cell to decrease the turgor pressure and therefore deflating the guard cells and causing in the stomatal pore to close. Stomatal closure is often associated with cytosolic Ca^{2+} signaling in the guard cells because Ca^{2+} down-regulates K^+ influx channels[11, 13], activates anion channels, and down regulates plasma membrane H^+-ATPases[14].

Stomatal movements are complicated and can be affected by a number of signaling compounds including abscisic acid (ABA), reactive oxygen species (ROS), nitric oxide (NO), CO_2 and Ca^{2+}. These compounds regulate stomatal apertures by transporting active ions and organic compounds including K^+, Cl^- and $malate^{2-}$ across the guard cell membrane[15]. The transport of ions in and out of guard cells will result in stomatal opening and closing. Understanding how ion transporters function and are regulated in stomatal movements is an important step toward connecting the bridge between environmental cues and stomatal movements.

Plant signal transduction

Work in plant signal transduction is complicated due to the poorly understood signaling network that elicits the correct response[16]. Previous studies have shown that several Arabidopsis mutants such as *growth controlled by abscisic acid (gca2)* and *slow anion channel-associated 1 (slac1)* are impaired in ABA signal transduction pathways as well as in their stomatal movements in response to high levels of CO_2 and in inducing an increase in cytosolic $[Ca^{2+}]$ in the guard cell[9, 10]. Studies of the guard cell signal transduction network suggest that the ABA, CO_2 and Ca^{2+} signaling pathways converge which leads to the inhibition of stomatal opening and the promotion of stomatal closure Most of the known mutants in the CO_2 signaling pathway also show impairment in ABA-induced stomatal closing, suggesting that there is a convergence point of ABA and CO_2 signaling pathways and that these

mutants are downstream of this convergence. Two mutants, *gca2* and *slac1,* have been identified that are insensitive to both ABA and CO_2 induced stomatal closure[7, 9, 10].

Mutants in stomatal movements

An ABA-insensitive mutant, *gca2,* was the first mutant shown to exhibit an impaired response to CO_2 as well as ABA[17-20]. The mutant *gca2* shows a strong insensitivity to CO_2 in cytosolic Ca^{2+}, in stomatal aperture regulation, and in CO_2 regulation of stomatal conductance changes in leaves[17]. In wild type there is a corresponding regulation of $[Ca^{2+}]$ with high and low CO_2 levels. During high $[CO_2]$, guard cells are depolarized and that depolarization dampens the cytosolic Ca^{2+} transient rate and closing stomata[7]. Disruption in *GCA2* does not alter the $[Ca^{2+}]_{cyt}$ pattern in response to changes of $[CO_2]$. Furthermore, inhibition of $[Ca^{2+}]$ of transients abrogates CO_2 induced stomatal closure[21, 22]. These observations suggest that the $[Ca^{2+}]_{cyt}$ in guard cells is an important component of the stomatal response to $[CO_2]$. These results suggest that ABA, CO_2, and Ca^{2+} signaling pathways converge near the point of *GCA2*.

Mutations in the *SLAC* gene cause impairment in S-type anion channel function in guard cells and has an insensitive stomatal movement response to a variety of stimuli including ABA and CO_2[9, 10]. The *SLAC1* gene encodes a plasma membrane protein functioning as a S-type anion channel[10]. Mutations in *SLAC1* impair S-type anion channel currents activated by cytosolic Ca^{2+} and

ABA but retain functional rapid (R-type) anion channels.[10] These data provide genetic evidence for the model that S-type anion channels are a major control mechanism for staomatal closing [23, 24]. The *slac1* mutant is insensitive to CO_2, ozone (O_3), ABA, hydrogen peroxide (H_2O_2), and NO induced stomatal closure as well as being insensitive to light/dark transitions, humidity changes, and Ca^{2+}. Both *slac1* and *gca2* show insensitivity to ABA and CO_2. In contrast, recently a mutant, *high temperature (ht1-2),* was identified. In *ht1-2*, stomatal apertures do not respond to changing levels of CO_2. However, *ht1-2* mutant plants exhibited similar ABA-induced stomatal closure in comparison to wild type. This suggested that *HT1-2* is located before the ABA and CO_2 convergence point and has a function very early in the CO_2-induced stomatal signaling pathway. HT1 encodes a protein kinase with strong mutation showing a recessive constitutive high CO_2 stomatal closing response, which was isolated by infrared thermal imaging screening for high leaf temperature[8]. Under 800 ppm CO_2 condition, stomata of wild type plants closed drastically, however, stomata were already closed and did not close further in *ht1*-2 mutant plants. In the *ht1-2* mutant, the stomatal density (number of stomata/area) and stomatal index (number of stomata/total epidermal cells) remain normal at ambient CO_2 compared to wild type[8].

Stomatal development

Stomata play a crucial role in plants yet the genes that control stomatal development and spacing are poorly understood. Stomatal development

requires three different precursor cells; the meristemoid mother cell (MMC), the meristemoid and the guard mother cell (GMC). The MMC is the first precursor cell and divides asymmetrically, to produce a meristemoid as well as a smaller sister cell. Some MMC can convert directly into GMC, while others can divide up to three times before the conversion[25]. Each time a meristemoid divides, it creates another meristemoid and a smaller sister cell[26]. The final precursor cell is the GMC that divides symmetrically to produce two guard cells[27]. This series of divisions generate a spacing pattern and a control of stomatal formation frequency determined by several environmental factors such as humidity[28], CO_2 levels[29], temperature and light[30]. Due to the many influences of the environment upon stomatal density, it can be inferred that stomatal density signals are complex and therefore there may be many factors that contribute to stomatal development.

Different genes regulate cell division through transitions along the pathway leading to the cells' terminal fates. The number of asymmetric divisions of the MMC determines the number of stomata that form[31]. This type of patterning involves spatial cues from existing stomata that determine the location of newly forming stomata[32]. It has also been shown that not only do the stomata themselves give spatial cues but so do the stomatal precursors, such as the MMC, meristemoid and the GMC. Therefore multiple signals and cues are responsible for the fate of the newly forming stomata. By studying different mutants in the stomatal development pathways, more is understood about the function of certain genes and the role they play in stomatal formation

and stomatal density.

Mutants in stomatal development

Mutants have been identified for each precursor cell division, causing identifiable phenotypes depending on the function of the mutated gene. Many of these mutants inherit poorly functioning stomata because the mutations occur in pathways that cause formation, rather than mutations involved in stomatal spacing. These mutants include *speechless (spch), mute (mute)* and *fama (fma)* [3334]. The mutant *spch* is identified by an epidermis made of only pavement cells and this observed phenotype is due to a disruption of the first asymmetric cell division of the stomata formation. In *mute*, cells are arrested in the meristemoid stage and the pavement cells form a rosette pattern around the meristemoid cell. While in *fama,* repetitive divisions of the GMCs produce a series of undeveloped guard cells and form "fake mouths" [33]. All these three mutants have lost the function of stomatal formation; however, mutants can arise causing different types of spacing phenotypes.

There are several known mutants that do not follow the wild type spacing and distribution patterns. These mutants have phenotypes depending on the function of the gene in the stomatal development pathway. The mutant *too many mouths (tmm)*, has a phenotype that produces clusters of stomata, caused by improper spacing of stomata. The *TMM* gene negatively regulates asymmetric division in neighboring cells[35]. Another mutant, *stomata density and distribution (sdd1),* shows a 2-fold increase in stomatal density and an

increase in stomatal index throughout the entire plant. The *SDD1* gene is a negative regulator of entry into the stomatal pathways inhibiting stomata from forming[36]. Many of the stomatal density and stomatal index mutants have visible phenotypes at 365ppm CO_2; however there are phenotypes that are only seen during a change in environmental signals, such as at 500ppm CO_2 as shown in this M.S. thesis.

Carbon dioxide affects stomatal development

Carbon dioxide levels cycle yearly between seasons (+/-10ppms) and have been increasing for over the last century[1]. Since the industrial revolution, carbon dioxide levels have increased by almost 40% and with the increased atmospheric CO_2, a change in stomatal density has occurred as well. This change has been documented throughout time as shown in fossil over the past 400 million years imprints[37]. It has been accepted that long-term exposure to elevated CO_2 has occurred in parallel with a decrease in stomatal apertures, as well as a decrease in leaf stomatal density and stomatal index. For CO_2 control of stomatal density only one gene, *HIC,* has been identified that affects this CO_2 response[38]. The *HIC* gene encodes a fatty acid elongase and is hypothesized to affect a repressor in guard cell walls by altering wall permeability through long chain lipids[38]. In wild-type plants, stomatal numbers increase with low CO_2 concentrations and decrease with high levels of CO_2. In *hic* mutant plants, stomata numbers increase under high levels of CO_2 and stomatal spacing rules remain unaffected[29, 38]. Because *hic* mutants retain

normal stomatal distribution, it suggests that this gene may be involved in a separate regulatory pathway.

Carbonic anhydrases

Carbonic Anhydrases (CAs) function in many biochemical processes involving carboxylation or decarboxylation reactions. CAs catalyze the reversible reaction of $CO_2 + H_2O \leftarrow \rightarrow HCO_3^- + H^+$ and play roles in photosynthesis, respiration, pH regulation, inorganic carbon transport, ion transport, and water and electrolyte balance[39]. CAs were first discovered in bovine erythrocytes in 1933[40] and many isoenzymes have since been discovered in most mammalian tissues and cell types as well as being abundant in plants and algae[41]. CAs can be divided into four classes (αCA, βCA, γCA and δCA) based on amino acid sequence similarity and have been shown to evolve independently of each other [42]. In animals, the main CA class belongs to the αCA class, while in other eukaryotes all α, β, and γCAs are found. The δCAs have only been found in the marine diatom *Thalassiosira weissflogii*[43]. The αCA class contains human isoforms, periplamic enzymes from the unicellular green alga *Chlamydomonas reinhardtii*, and homologues in plants. In plants and algae, α, β, and γCA classes exist and the β class is the most studied[44]. The βCA family appears to be targeted to chloroplasts and mitochondria of algae and monocotyledonous and dicotyledonous plants[42].

In the Arabidopsis genome, there are eight αCAs, six βCAs, and four γCAs genes. Since these enzymes catalyze the reversible reaction of CO_2 +

$H_2O \leftarrow \rightarrow HCO_3^- + H^+$, it might be predicted that these genes function in CO_2 response pathways, mediating stomatal movements and stomatal development. It was hypothesized that CO_2 sensors maybe exist in a gene family, which have multiple gene functions, like many of the other stomatal movement control mechanisms. The *ca1ca4* double mutant was identified in the Schroeder laboratory that has an insensitive response to CO_2-induced stomatal movements, and retains a wild-type ABA response. The stomatal density was also affected in *ca1ca4* double mutant plants. The current research shows that the carbonic anhydrases regulate both CO_2-induced stomatal movements and development.

II

Materials and Methods

Plant growth

Seeds were sterilized for 15 minutes with 0.5% SDS and 75% ethanol. Seeds were then washed twice with 100% ethanol and allowed to air-dry in the hood. Dry seeds were then sprinkled on ½ MS plates and incubated for 48 hours in 4°C to stimulate germination. Plates were then placed in a growth room with a 16 hour light, 8 hour dark cycle. The light in the growth room is white fluorescent bulbs at 70$\mu E/m^2$ on the average. At approximately a 10 day-old stage, the seedlings were transferred to autoclaved Sunshine Mix Professional Blend soil. To the autoclaved soil, 25 grams of Cleary and 2.2 grams of Marathon per 2.5 kg of soil were added as fungicide and insecticide respectively. Plants were watered 3 times a week. For stomatal movement experiments, plants were grown under high humidity to insure healthy leaves and are tested at approximately 3-4 weeks of age.

Epidermal leaf peels

Epidermal leaf peels of *Arabidopsis thaliana* were prepared by gluing leaves, the lower epidermal side down, to a small, round, microscope cover slide with Medical Adhesive glue. Glue is applied with a razor blade to streak the glue across the slide. The top portion of the leaf is gently removed with a

razor blade leaving the epidermis layer remaining on the slide. The slide is then placed in incubation buffer in a Petri dish to expose the epidermal cells. Leaves the of same age and size, were chosen to ensure appropriate results.

Leaf incubation

Leaf epidermes were obtained as stated above and were then incubated in a Petri dish in a growth chamber with the bioassay buffer containing 10mM KCl, 50μM $CaCl_2$, and 10mM MES. The buffer was adjusted to a pH of 6.15 with Tris. Depending on the experimental conditions, leaf epidermes were incubated in the buffer from 30 minutes to three hours.

Stomatal aperture measurements

Epidermal samples were taken out from the buffer and adhered to a glass slide with a hole drilled in the middle, creating a shallow well (1.22 cm^2, 200 µl capacity). One drop of buffer solution was placed on top of the sample to prevent the leaf from drying out as well as to keep the same condition as incubation. Just prior to measurements, at the end of the experiment, 0.0007% (wt/vol) of fluorescein diacetate (dissolved in acetone) was added to distinguish the living cells from the dead cells. The length of the entire stomata as well as the width of the stomatal pore (stomatal aperture) were measured. The measurement was taken from the program *Image J* and is measured in pixels on the computer; therefore a conversion is required (50μm /2.945 pixels) to convert pixels into micrometers (μm) for this particular

microscope/computer configuration. Error bars were calculated by taking the standard deviation of the stomatal counts from 20 different pictures, and that number was divided by the square root of the number of pictures (20).

Carbon dioxide treatment

During the incubation period, buffer was placed in 50mL Falcon tubes and bubbled with different concentrations of CO_2 through Teflon tubing, 800ppm, ambient air (~365ppm) and 0ppm CO_2. To achieve each of these concentrations of CO_2, different methods were used. To obtain 800ppm CO_2 levels, a tank of compressed gas is attached to the Teflon tubing and is fed into a growth chamber and pumped into the Falcon tubes with incubation buffer for bubbling 1 hour. Ambient air is pumped into tubing and into the growth chamber as well via an aquarium fish pump. 0ppm CO_2 is obtained by filtering the air through soda lime to remove CO_2 from the air. Once bubbled, samples are placed in Petri dishes that have holes in the lids, large enough to feed the tubing through, along with wax that borders the hole in order to decrease escaped gas as well as to hold the tube in place while the air bubbles through. The dish, once assembled, is wrapped with parafilm. The dish with tubing and sample will remain in the growth chamber until time of measurement.

ABA treatment

When ABA experiments are carried out, leaf epidermes are prepared. Samples are pre-incubated in opening buffer (10mM KCl, 50μM CaCl2, 10mM MES, pH of 6.15 with Tris) for three hours. Stocks were created for 1mM and 10mM of ABA stocks were prepared and therefore 10μl of each was added to 10mL of stomata bioassay buffer to get 1μM and 10μM respectively. After 30 or 60 minutes treatment in the various concentrations, stomata apertures were measured and analyzed. These experiments were double blinded, for the sample as well as for the concentration of ABA.

Stomatal closure in response to malate and bicarbonate

For stomata closure experiments in response to other compounds, leaf epidermes are prepared the same way as CO_2 and ABA experiments. Samples are pre-incubated in bioassay buffer (10mM KCl, 50μM CaCl2, 10mM MES, pH of 6.15 with Tris) for three hours in Petri dishes and then incubated in the buffer with different concentration of malate and bicarbonate. For malate, 20mM malate or 20mM malate plus 800ppm CO_2 was added and samples were treated for 30 minutes. For bicarbonate a concentration of 2.7mM of bicarbonate was chosen which is equilibrated to the same as 800ppm CO_2 in solution. Samples were treated for thirty minutes and 40 stomata were measured for each condition. These experiments were double blinded, for the sample as well as for the concentration of malate or bicarbonate.

Stomatal density measurements

Leaf epidermes were made from medium sized leaves (the same size and type from each sample) from four different plants of each genotype and 5 pictures were taken of each leaf on the top and on the bottom side for a total of 20 microscope images were taken per side. For those twenty pictures, stomata and pavement cells were counted on each image. Stomatal density number is calculated by averaging the stomata number per picture area and multiplying it by a conversion factor. The Nikon lens used was a 20x magnification, therefore, around 20 pictures is approximately equal $1mm^2$. To convert one picture to be the same as $1mm^2$, the stomata pictures are multiplied by 5.636636. For those calculated on the 40x lens, stomatal numbers were multiplied by 23.045.

Plant growth at high (500ppm) and low (150ppm) in CO_2 growth chamber

Plant lines were also grown in 500ppm CO_2 as well as 150ppm CO_2 controlled chamber with 70% humidity and 16 hours light, 8 hours dark. The light in the growth room is white fluorescent bulbs at $70\mu E/m^2$ on the average. To achieve 500ppm CO_2, CO_2 was pumped into the chamber and the levels were analyzed with a CO_2 sensor to keep the chamber at ~500ppm CO_2. For the levels of ~150ppm, air was pumped through soda lime, which removes the CO_2 from the air. .

Crossing plants

Under a binocular microscope with a pair of fine forceps, all siliques, open flowers and budding flowers were removed from the existing stem leaving only those that had not blossomed. Also the meristem of the bud packet was removed in order to prevent any further flower formation. The outside sepals were removed, as well as the petal and the stamens, therefore leaving only the carpels. The following day, the carpel will have extended and can then be pollinated by pollen from the stamen of another genotype plant. The carpels pollinated were marked with tape and watched over the next few days. Those that became siliques were successfully crossed. Seeds were harvested, planted, and genotyping of the plants were carried out.

DNA extraction

DNA extraction buffer was made that contained 200mM Tris, 250mM NaCl, 25mM EDTA and 0.5%SDS. Leaves were ground with a pestle and 400ul of DNA extraction buffer was added. Samples were centrifuged at 11,000rpm for 8 minutes. The supernatant was transferred to a new tube (350μl) and 350μl of isopropanol was added and mixed. Samples are then centrifuged again at 12,000rpm for 10 minutes. The supernant is discarded and samples were washed with 500μl of 75% ethanol and centrifuged again at 12,000rpm for 5 minutes. Supernant is discarded and DNA is dried at room condition. Once dry, 50μl of TE buffer is added to the sample.

Isolation of *ca3* and *ca5* homozygous lines

DNA was extracted from Salk lines for *ca3* (SALK_144106C) and *ca5* (SALK_044970). Samples were genotyped for homozygous lines by PCR. PCR was run at 32 cycles of 30s at 95°C, 30s at 55°C, 1m30s at 72°C. Reactions were carried out for both the presence of a T-Band as well as the absence of a genotype band. For primers used refer to Table 1.

Crossing and screening for *ca1ca2ca4ca6* and *ca1ca2ca3ca4* plants

Plant lines *ca1ca2ca4* and *ca1ca4ca6* were crossed in both directions in order to screen for a *ca1ca2ca4ca6* mutant. The T-DNA and genotype bands were screened by PCR (mentioned above) to isolate homozygous lines for *ca1ca2ca4ca6.* Plant lines *ca1ca2ca4* and *ca3* were crossed and lines were selected for the presence of both *ca3* and *ca4*. Due to the location of the mutants, there needs to be a crossover between *ca3* and *ca4* because they are both on chromosome 1.

Table 1: Primers used for βCAs

CA1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAGGTACTTAACCCTAAT
CA1R	GGGGACCACTTTGTACAAGAAAGCTGGGTACGAAGCTACAAATCTACA
CA2F	CACATCCCTACCTCTGAAAGTCTC
CA2R	ACGAAGCCTTCCTTAATTCGTT
CA3gF	CTTGATTCCAACAAGTTAGATGCC
CA3gR	TATGTCGACAGAGTCGTACG
CA3R	GATTCACGAGTTAGTCCATCTCAC
CA4F	CACCGAGCTAGGAAGAGCCTTACCAAG
CA4R	GATCAATGGCTTCAATGCCAAGAAG
CA5gF	CCCTTAATCCAAATCCTCCATC
CA5gR	TTGGAGCTTGAGCATCTGCCAG
CA5R	CACACCGGCTATGACCAATGACT
CA6F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCGTTTACACTAGGT
CA6R	GGGGACCACTTTGTACAAGAAAGCTGGGTATTTCGTATGTACAAAGATACA
LBa1	ATTTTGCCGATTTCGGAAC

Plant Material

Table 2: Plant material for the six expresses βCAs found in guard cells. All Arabidopsis lines used were of the Columbia (Col-0) background:

Gene	Mutant	
CA1	*ca1*	SALK_106570
CA2	*ca2*	SALK_145785
CA3	*ca3*	SALK_144106C
CA4	*ca4*	WiscDsLox50D11
CA5	*ca5*	SALK_044970
CA6	*ca6*	SALK_044658
HT1	*ht1-2*	Koh Iba lab

III

Results

High CO_2 (800ppm)-induced stomatal closure is impaired in *ca1ca4* mutant plants

Previous gas exchange analyses in intact leaves showed that CO_2-induced stomatal conductance was greatly impaired in *ca1ca4* mutant plants in response to changes of CO_2. To analyse whether the impaired CO_2 response in intact leaves is linked to CO_2-induced stomatal movements, the CO_2-induced stomatal movements in leaf epidermes were also analyzed.

Leaf epidermes from wild type (Columbia backgroud, col) and mutant plants were pre-incubated in bioassay buffer containing 10mM MES, 10mM KCl, 50μM $CaCl_2$ and a pH of 6.15 with TRIS. After one hour, the samples were then moved to incubation buffer pre-treated with 365ppm or 800ppm CO_2 for 30 minutes. Forty stomata per experiment were measured and the data demonstrated that high CO_2-induced stomatal was also greatly impaired in *ca1ca4* plants, while at ambient CO_2, no difference was observed compared to wild type plants (Figure 1). These data suggests that CAs play a role in CO_2-induced stomatal movement.

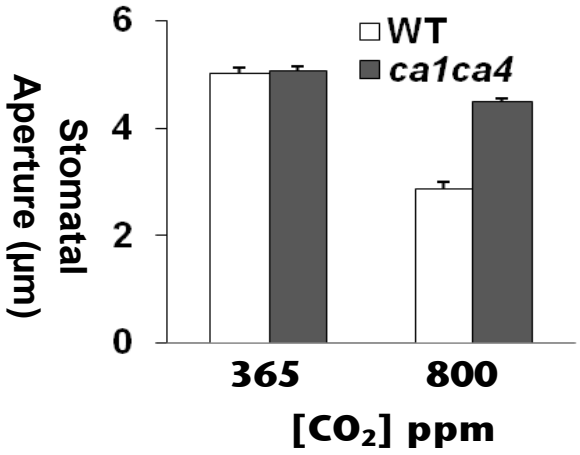

Figure 1: High CO_2-induced stomatal closure in WT and *ca1ca4* plants. The graph shows that *ca1ca4* exhibits a CO_2 insensitive response to increased levels of CO_2 compared to wild-type. Three experiments (n=3), each experiment 40 stomata for a total of 120 stomata per sample were measured. Error bars depict means ± s.e.m.

***ca1ca4* retains functional ABA-induced stomatal closure**

Stomata in the *ca1ca4* mutant show a weak response to high levels of CO_2. Most known CO_2-induced stomatal movement mutants are also ABA insensitive mutants, and therefore *ca1ca4* plants were also tested for ABA-induced closure. To test if ABA-induced stomatal closure is also affected in *ca1ca4* mutant plants, leaf epidermes from wild type and *ca1ca4* plants were treated with 1μM or 10μM ABA. After 1 hour exposure, the stomatal apertures were measured and no difference was observed between the col and double mutant plants either at 1μM or 10μM ABA, unlike other CO_2-insenstive mutants previously reported. These data showed that CA functions before the CO_2 and ABA convergence point, further supporting that CA1 and/or CA4 are responsible in early CO_2 signaling. (Figure 2)

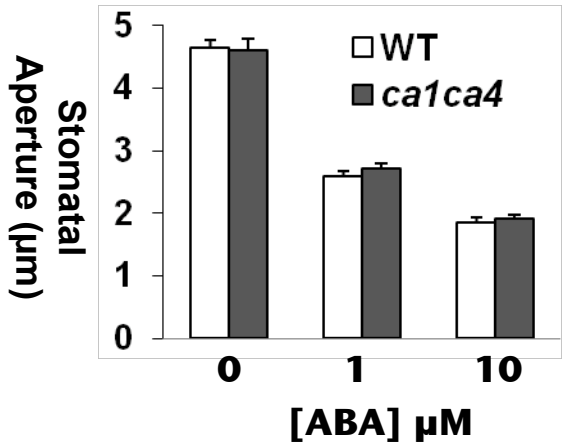

Figure 2: ABA-induced stomatal closure remains functional in *ca1ca4* mutant plants. WT and *ca1ca4* both respond to ABA shown by stomatal closure after one hour exposure to ABA. This experiment was conducted in three trials (n=3) measuring 40 stomata per sample and condition for a total of 120 stomata measured. Error bars depict means ± s.e.m, for n=3

Malate induced stomatal closing

ABA is well known to cause stomatal closure. Other compounds, such as malate, have been shown to induce stomatal closure. Malate induces stomatal closure by increasing the apoplastic malate concentration and opening anion channels in guard cells that allow for malate to be released, therefore closing stomata. Malate has been shown to be a factor in CO_2-induced stomatal closing with similar effects as high CO_2. Stomatal movement bioassays were performed to analyze the malate-induced closure pattern in *ca1ca4* and col plants. After treatment with 20mM malate and 20mM malate with 800ppm CO_2 for 30 minutes, statistically there was a significant difference in stomatal apertures observed between col and *ca1ca4* (Figure 3). The *ca1ca4* double mutant stomata are less responsive to malate, just as they are to high levels of CO_2 shown in Figure 1.

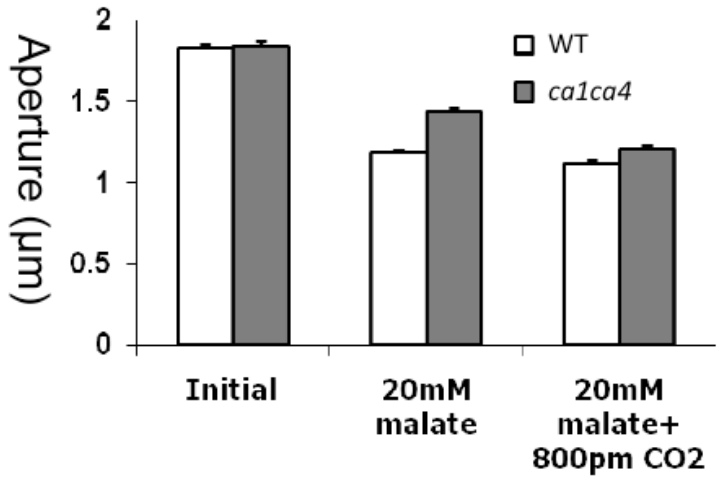

Figure 3: Stomatal closure in response to malate. This experiment was three trials (n=3) measuring 40 stomata per sample and condition for a total of 120 stomata measured. Error bars depict means ± s.e.m.

Bicarbonate has the similar effect on stomatal closure as CO_2

Malate was shown to induce stomatal closure. Bicarbonate (HCO_3^-) has also been recognized to decrease stomatal apertures. Bicarbonate is produced from the reaction catalyzed by carbonic anyhdrases in plant cells, $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. Therefore adding more bicarbonate is indirectly the same as adding more CO_2 to the solution because this equation is in equilibrium. It was calculated that 800ppm is comparable to a solution with a concentration of 2.7mM of bicarbonate after equilibrium at pH 7.1. Interestingly, exposure to bicarbonate has the very similar effect as high CO_2 both in col and *ca1ca4* plants, the stomatal course was impaired in *ca1ca4* plants compared to col plants at the same condition (Figure 4). This shows that perhaps bicarbonate can transduce the CO_2 signal and may be responsible for stomatal closure induced by high CO_2.

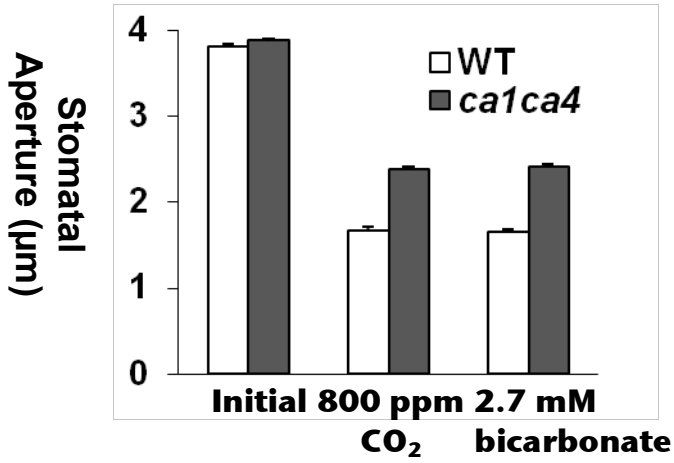

Figure 4: Stomatal movement analyses in col and *ca1ca4* plants applied with bicarbonate and CO_2. Choosing a concentration of 2.7mM of bicarbonate was the same as using 800ppm CO_2 in equilibrium. This experiment was three trials (n=3) measuring 40 stomata per sample and condition for a total of 120 stomata measured. Error bars depict means ± s.e.m.

Analyses of stomatal densities in *ca1, ca4, ca1ca4, ca1ca2ca4,* and col.

Previous studies have shown that stomatal density increases in *ca1ca4* mutant plants, indicating these two CAs regulate stomatal development, as well as stomatal movements. To analyze whether this stomatal development is mediated by CAs, stomata density in leaves both adaxial (Figure 5) and abaxial (Figure6) were measured in *ca1, ca4, ca1ca4, ca1ca2ca4* and col plants. These plants were grown in high CO_2 (500ppm) and low CO_2 (150ppm) growth chambers. Col shows expected results, where stomata numbers are high at 150ppm and low at 500ppm on abaxial and adaxial leaf surfaces. Both single mutants show a small phenotype. However, the *ca1ca4* shows opposite as col, where stomata numbers decrease at 150ppm and increase at 500ppm CO_2. The *ca1ca2ca4* shows a large increase in stomata number at 500ppm CO_2. These results show that CAs play a role in CO_2-induced stomatal development on both abaxial and adaxial sides of leaves.

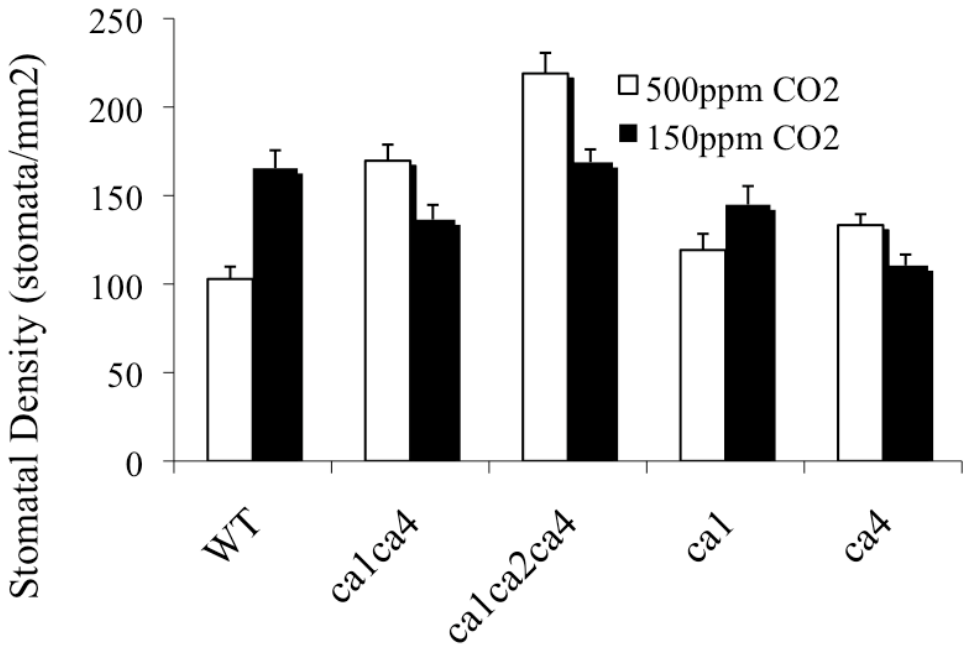

Figure 5: Stomatal densities of abaxial side of WT, *ca1ca4, ca1ca2ca4, ca1,* and *ca4* four leaves growing at 150 ppm and 500 pmm. Four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

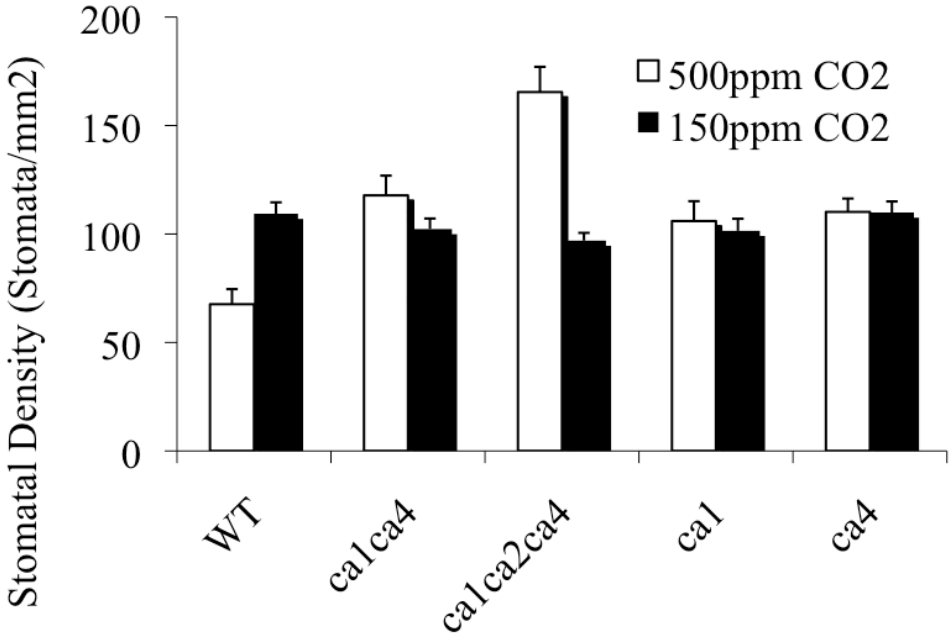

Figure 6: Stomatal densities of abaxial side of col, *ca1ca4, ca1ca2ca4, ca1,* and *ca4* four leaves growing at 150 ppm and 500 pmm. Four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of 1mm^2.

Stomatal density of *ca1ca4, ht1-2, ca1ca4ht1-2* and col

Knowing that *ca1ca4* and *ht1*-2 are the only two known CO_2 mutants with functional ABA response, stomatal density was tested in col, *ca1ca4, ht1-2* and *ca1ca4ht1-2* grown at 150ppm CO_2 and 500ppm CO_2. At low CO_2, col has a higher stomata number that at high CO_2, whereas *ca1ca4* mutant plants show a reverse phenotype, with higher numbers of stomata with 500ppm CO_2. *ht1-2* previously has shown a wild type density pattern at ambient CO_2 condition, and the results in Figures 7 and 8 also show wild type phenotype in stomatal density. The triple mutant, *ca1ca4ht1-2,* show variable results, demonstrating that many aspects of stomatal density are still unknown.

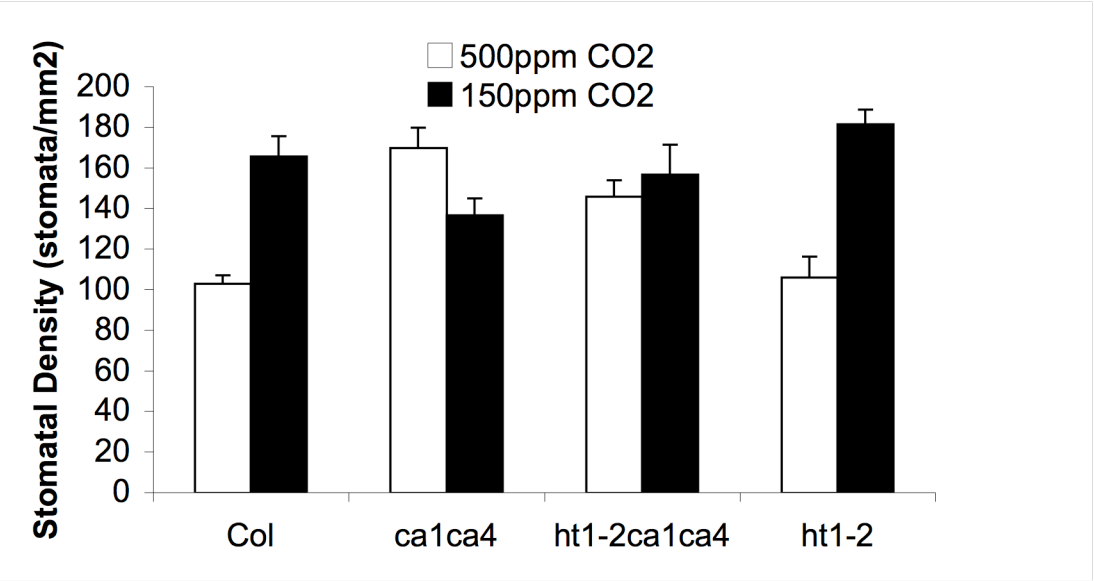

Figure 7: Stomatal densities of abaxial side of col, *ca1ca4, ht1-2ca1ca4,* and *ht1-2* four leaves growing at 150 ppm and 500 pmm. Four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

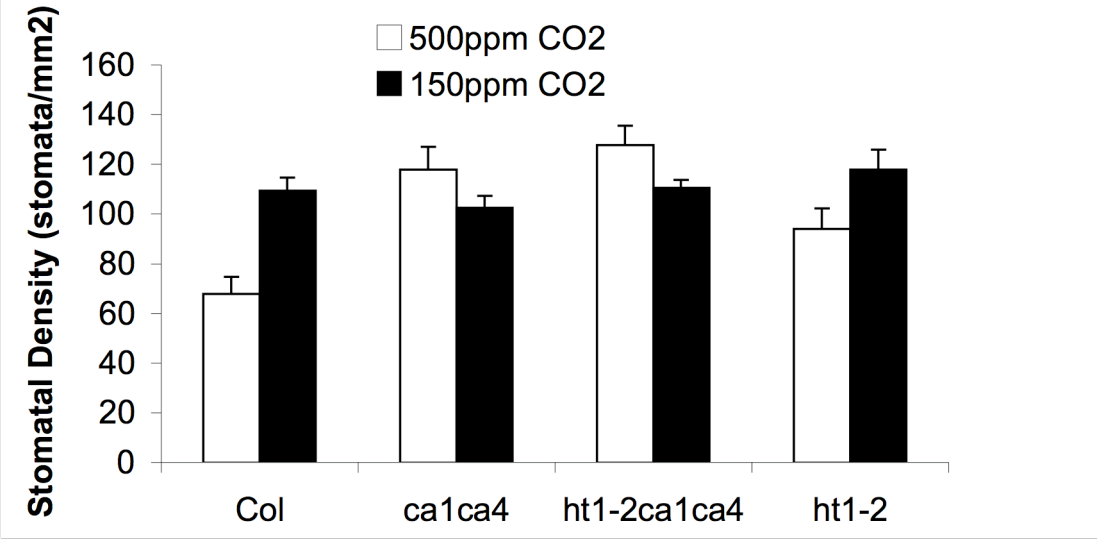

Figure 8: Stomatal densities of adaxial side of col, *ca1ca4, ht1-2ca1ca4,* and *ht1-2* four leaves growing at 150 ppm and 500 pmm. Four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

Stomatal movements and stomatal densities in *ca1ca4* plants transformed with *CA1* and *CA4* genomic DNA

To analyze which gene, CA1 or *CA4*, is more responsible for the CO_2 phenotype, the genomic DNA of wild-type was expressed in the *ca1ca4* double mutant to check for complementation (CA1g(1) and CA4g(2)). Stomatal density was calculated for ambient CO_2 and 500ppm CO_2 and genomic DNA from *CA1* and *CA4* genes recover the wild-type stomatal density phenotype for those grown under 500ppm CO_2 as well as normal conditions (Figure 9). These results show that both *CA1* and *CA4* cDNA and genomic DNA are able to show complementation.

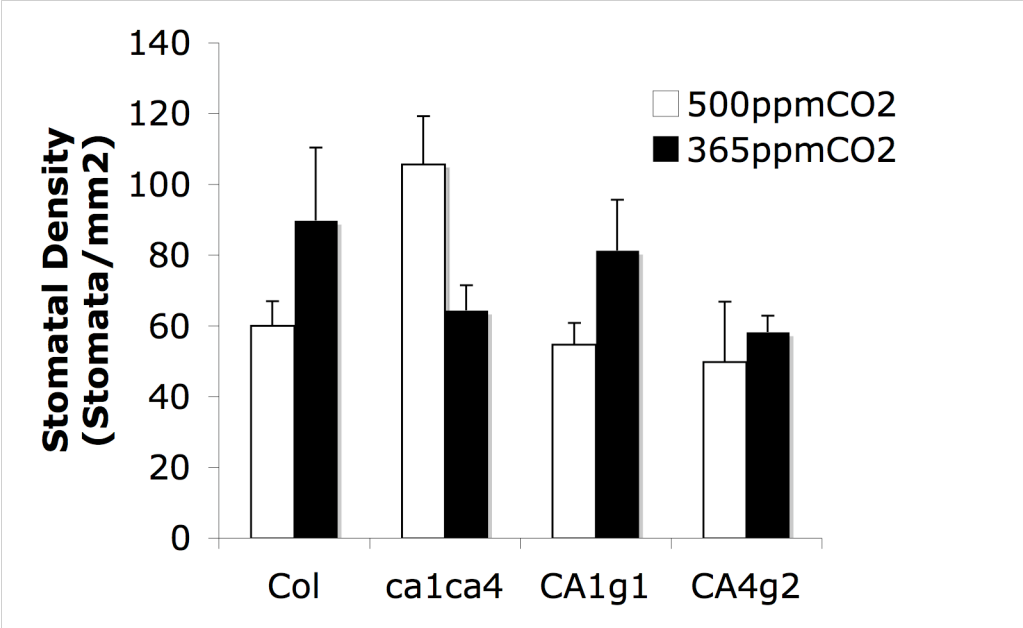

Figure 9: Stomatal density of the abaxial side of the leaf. For each line, col, *ca1ca4,* CA1g1 and CA4g2, four leaves from four different plants were used from each line and 5 pictures per leaf were taken. Plants were grown at both 500ppm CO_2 and 365ppm CO_2. Each sample is an average of 20 pictures and correlated to an area of 1mm^2.

Stomatal movements and stomatal densities in *ca1ca4* plants transformed with *CA1* and *CA4* cDNA

In order to analyze which gene, *CA1* or *CA4,* is more responsible in the CO_2 phenotype the, cDNA of *CA1* or *CA4* from WT controlled by a guard cell promoter was transformed into *ca1ca4* double mutant. Lines of CA1d-2, CA1d-6, CA1d18, CA4d-1 and CA4d-2 were analyzed as *CA1* or *CA4* expressing transgenic *ca1ca4* plants. The stomatal densities of these lines were analyzed at 500ppm CO_2 growth conditions. CA1d-2, CA1d-6 and CA1d-18 all show WT phenotype for stomatal density (Figure 10). CA4d-1 and CA4d-2 also show WT phenotype for stomatal density (Figure 11). As shown in figure 10 and 11, it was found that expression of *CA1* or *CA4* in double mutant recover the stomatal development phenotype.

To analyze if CA1d-2 and CA1d-18 would complement CO_2-induced stomatal movement, CA1d-2, CA1d-18, col and *ca1ca4* were treated with 800ppm CO_2. Both CA1d-2 and CA1d-18 show WT phenotype to CO_2-induced stomatal closure (Figure 12).

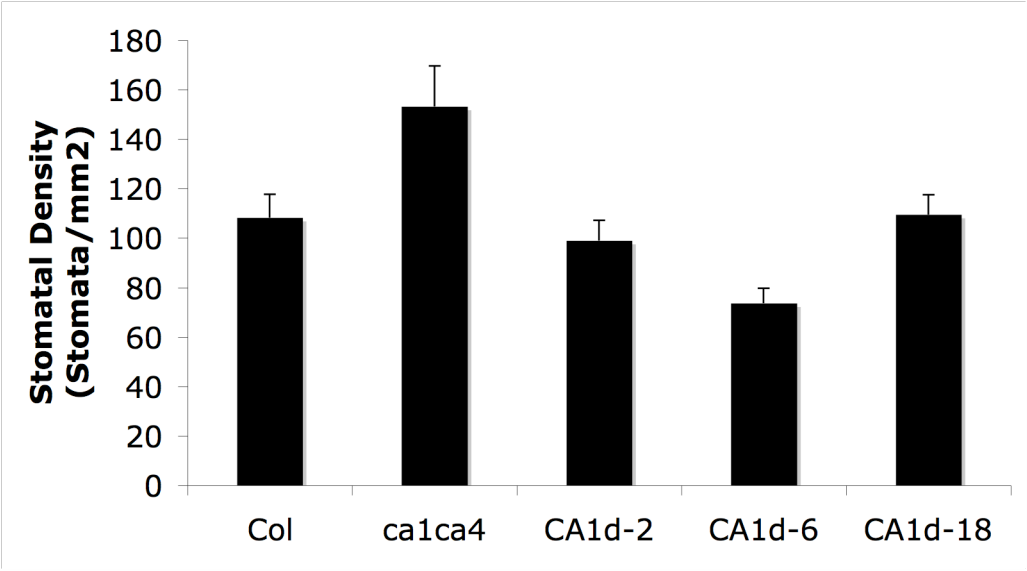

Figure 10: Stomatal density in abaxial side of the leaf. For each line, col, *ca1ca4,* CA1d-2, CA1d-6, CA1d-18, four leaves from four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

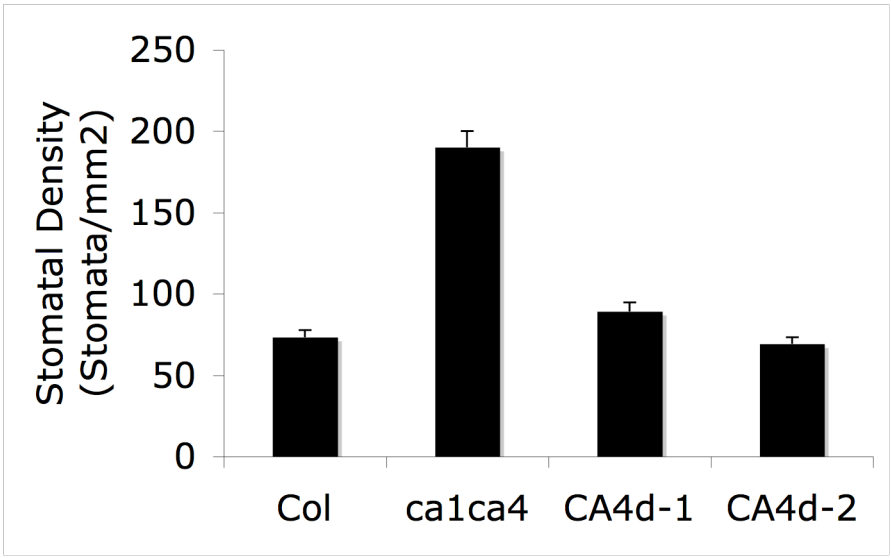

Figure 11: Stomatal density in abaxial side of the leaf. For each line, col, *ca1ca4,* CA4d-1 and CA4d-2, four leaves from four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

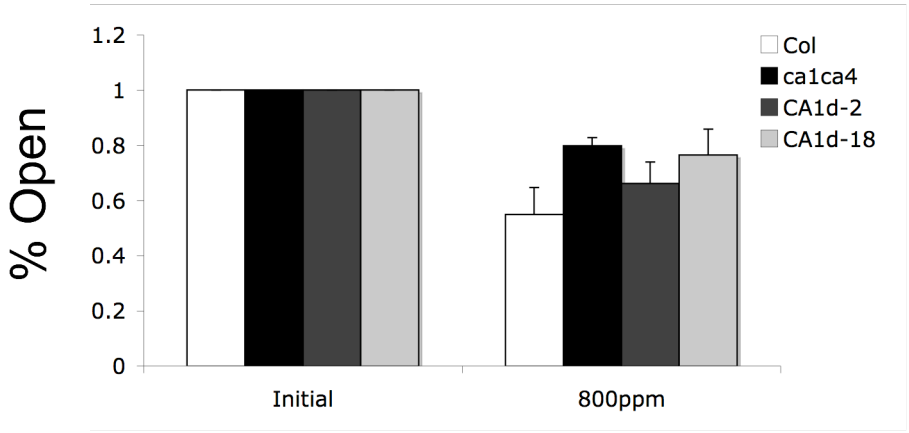

Figure 12: WT, *ca1ca4*, CA1d-2 and CA1d-18 were tested with 800ppm CO_2. Samples were incubated for 1 hour in 10mM MES, 10mM KCl, 50μM $CaCl_2$ and a pH of 6.15 with TRIS. Results show three experiments (n=3) with 40 stomata being measured each trial for a total of 120 stomata per sample. Numbers are adjusted so that initial is 100% open and the treatment is the percent open compared to initial. Error bars depict means ± s.e.m.

Stomatal aperture response with 365ppm and 800ppm CO_2, in col (WT) and *CA*-overexpressing plants

Genomic DNA as well as cDNA shows complementation. To test the effects of a dosage increase of these genes, *CA1* and *CA4* cDNA was transformed into wild type. The samples incubated in the buffer for one hour and then moved to the pre-equilibrated test condition of 800ppm CO_2 for 30 minutes. In Figure 13 it is seen that all four ox lines clearly have wild-type CO_2 phenotypes when treated with 800ppm CO_2. This shows that a dosage increase of CA does not affect CO_2-induced stomatal movement.

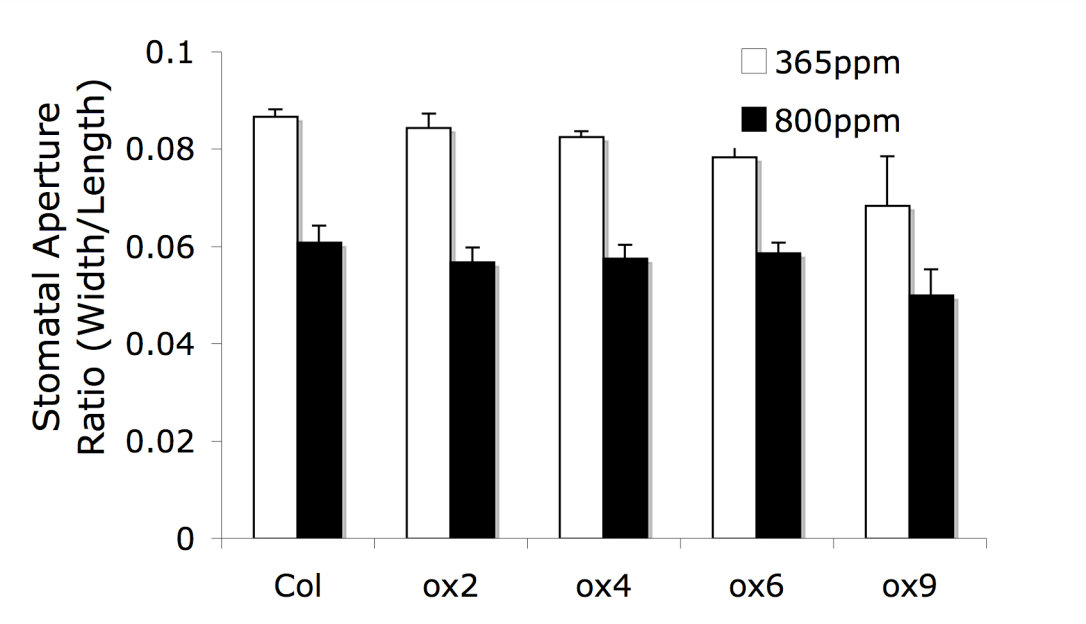

Figure 13: WT and four different over expression lines were tested with both 365ppm and 800ppm CO_2 responses. Results show three experiments (n=3) with 40 stomata being measured each trial for a total of 120 stomata per sample. The graph shows that all four over expression lines are able to restore the wild-type phenotype for CO_2. Error bars depict means ± s.e.m.

Stomatal density of *ca1ca4, col,* ox2, ox6 and ox9

Knowing that the *ca1ca4* double mutant plants have a phenotype for stomatal density, stomatal densities of overexpressing plants were tested with 150ppm as well as 500ppm. In Figure 14 and 15, col, *ca1ca4,* ox2, ox6 and ox9 show different stomata density at the various concentrations. At low CO_2, col has a higher stomata number at high CO_2, whereas *ca1ca4* mutant plants show a reverse phenotype, with higher numbers of stomata at 500ppm CO_2. For the abaxial side of the leaf, the over-expression maintain close to wild-type numbers under 500ppm CO_2 growth conditions, but under 150ppm CO_2 growth conditions, over-expression lines have a lower number than wild-type (Figure 14). For the adaxial side, all over-expression lines maintain wild-type phenotypes under 500ppm and 100ppm CO_2 (Figure 15). The variety seen in stomatal density could be contributed to various levels of CA expression in these lines.

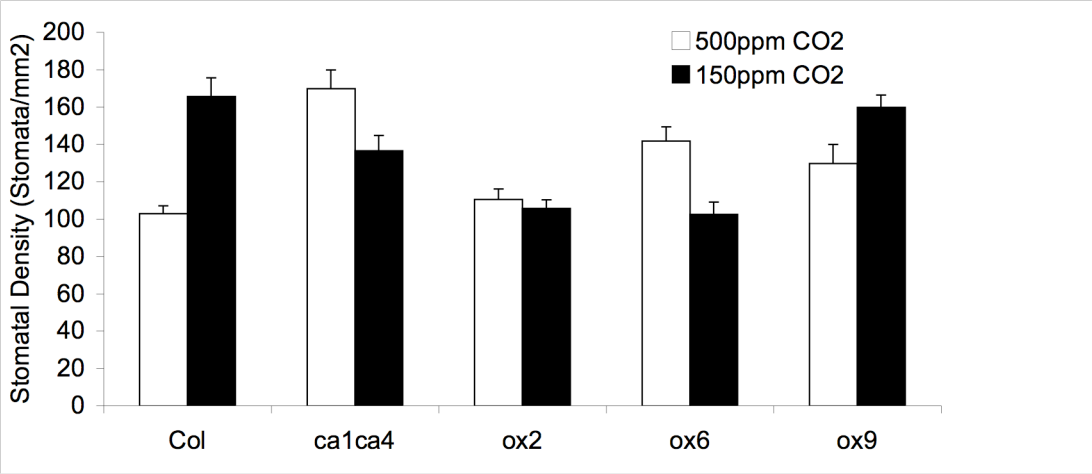

Figure 14: Stomata density of the abaxial side of the leaf. For each line, col, *ca1ca4,* ox2, ox6 and ox9, four leaves from four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

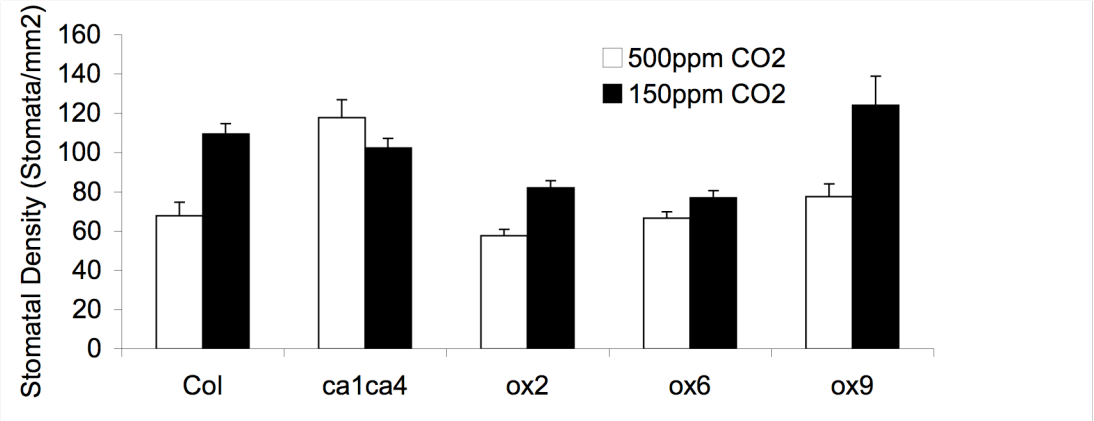

Figure 15: Stomatal density of the adaxial side of the leaf. For each line, col, *ca1ca4,* ox2, ox6 and ox9 four leaves from four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

***ca1ca4* mutant plants transformed with Human CAII recover wild-type phenotypes for stomatal density and CO_2 response.**

Col and *ca1ca4* have different stomatal density at different levels of CO_2. Previous data have clearly shown that CAs play a role in CO_2-induced stomatal movement and development. Introduction of CAs genomic DNA with their native promoters or cDNA controlled by a guard cells into *ca1ca4* plants are able to restore wild type phenotype. To analyze whether CAs from other species also can complement this stomatal CO_2 response, Human CAII was transformed into *ca1ca4* plants. Three different transformed lines, HmII-3, HmII-6 and HmII-8, were used to analyze the stomatal response (Figure 16). The data demonstrated that all these three lines recovered to wild-type plant phenotype, indicating that carbonic anhydrases activity play a role in stomatal movements at high levels of CO_2.

Stomatal densities analyses were then performed to analyze the high stomatal density of *ca1ca4* also could be restored by *HmIICA* . The data were shown in Figure 17 and 18, but it seems that CO_2-induced stomatal development are more complicated.

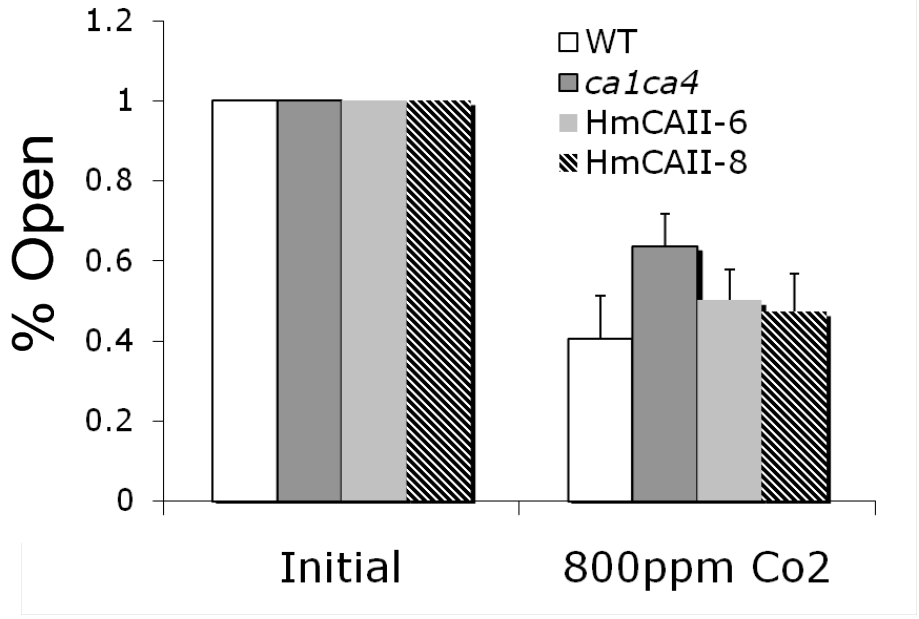

Figure 16: Stomatal response of high CO_2 in human *CAII* expressing transgenic *ca1ca4* plants, *ca1ca4* and col plants**.** Results show three experiments (n=3) with 40 stomata being measured each trial for a total of 120 stomata per sample. Numbers are adjusted so that initial is 100% open and the treatment is the percent open compared to initial. Error bars depict means ± s.e.m.

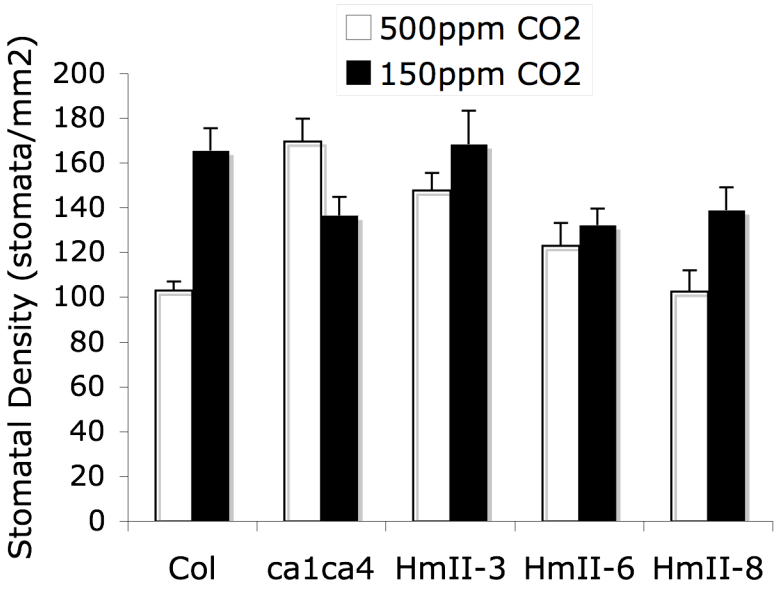

Figure 17: Stomatal density of the abaxial side of the leaf. For each line, col, *ca1ca4,* HmII-3, HmII-6, and HmII-8 four leaves from four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

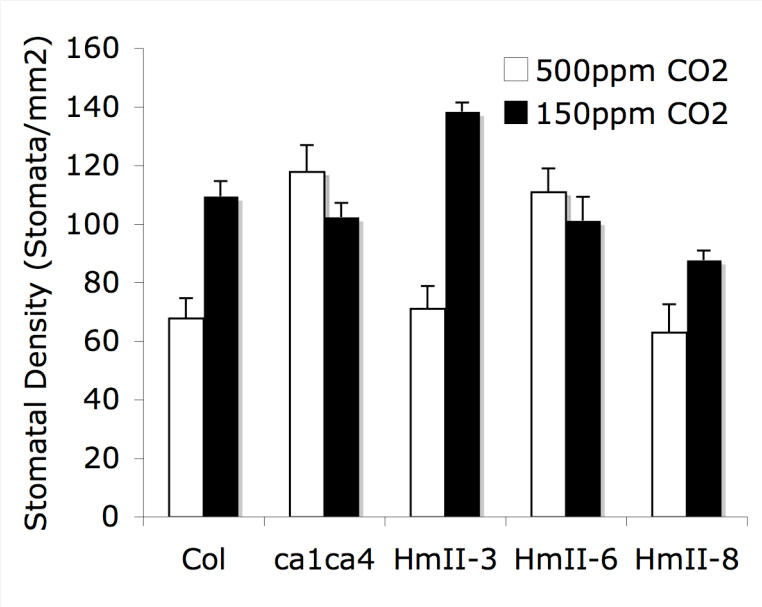

Figure 18: Stomatal density of the adaxial side of the leaf. For each line of col, *ca1ca4,* HmII-3, HmII-6 and HmII-8 four leaves from four different plants were used from each line and 5 pictures per leaf were taken. Each sample is an average of 20 pictures and correlated to an area of $1mm^2$.

ABA-induced stomatal closing in Human *CAII* expressing *ca1ca4* plants

Two HmII lines were chosen to check the effects of ABA response in stomatal closure with comparison to WT and *ca1ca4*. Stomatal movement experiments were carried out by making epidermal strips for col, *ca1ca4*, HmII-6 and Hm11-8 lines and incubated in opening buffer (5mM MES, 10mM KCl, 50μM $CaCl_2$, pH 6.15) for 3 hours. Lines were then treated with 10μm ABA for 30 minutes. Stomatal aperture (μm) were measured and the data were adjusted to represent the percent the stomata were open in comparison to the initial condition. From the data we can analyze that the Human *CAII* expressing plants exhibited hypersensitivity ABA phenotype. (Figure 19)

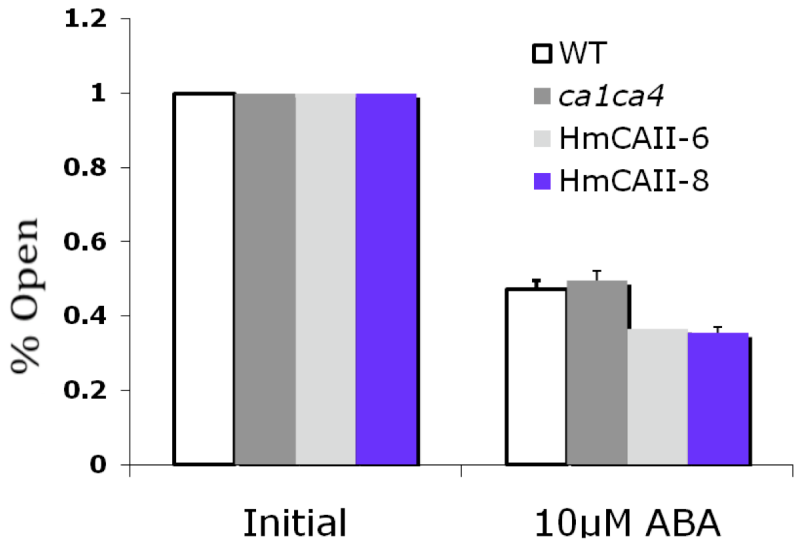

Figure 19: WT, *ca1ca4,* HmII-6, and Hm11-8 plants were treated 10µM ABA. Epidermal strips were incubated in 5mM MES, 10mM KCl, 50uM $CaCl_2$, pH 6.15 for three hours. Samples were then treated with ABA for one hour. All lines respond to ABA. This experiment was three trials (n=3) measuring 40 stomata per sample and condition for a total of 120 stomata measured. Error bars depict means ± s.e.m.

IV

Discussion

Stomata are pores in the epidermis of leaves that open and close in response to a variety of environmental factors, such as light, temperature, water and carbon dioxide**.** High levels of CO_2 cause stomata to close, whereas low levels of CO_2 cause stomata to open. It is hypothesized that carbonic anhydrases (CAs) may function in CO_2 sensing because these enzymes are known to catalyze the reversible reaction of $CO_2 + H_2O \leftarrow \rightarrow HCO_3^- + H^+$. Most mutants in the CO_2 signaling pathway also show ABA insensitive stomatal closure. Research has shown that ABA, CO_2 and Ca^{2+} signaling pathways converge at the point of *GCA2* or downstream[7, 16]. The only mutant known until now to have a CO_2 stomatal movement phenotype with functional ABA response is *ht1-2*[8]. Recently, the *ca1ca4* double mutant has been discovered in the Schroeder laboratory that has a CO_2-induced stomatal movement insensitive phenotype and maintains functional ABA response. The *ca1ca4* mutant also has an increase in stomatal density in comparison to wild type when grown at ambient CO_2. These data indicate that these two carbonic anhydrases regulate both CO_2 controlled stomatal movements and CO_2 controlled stomatal development and *CA1* and *CA4* function before the ABA and CO_2 convergence point in plant signaling.

In previous studies, four single mutants (*ca1, ca2,* ca4 and *ca6*) demonstrated normal CO_2 sensitivity in mature leaves by gas exchange analysis. Double mutants *ca1ca4, ca4ca6, ca1ca6* were therefore created as well as *ca1ca4ca6* and *ca1ca2ca6* triple mutants. Of these mutants, the *ca1ca4* and *ca1ca4ca6* mutant showed the strongest CO_2 impaired response, and had the same phenotype. Therefore, studies with the *ca1ca4* double mutants underwent further experiments to demonstrate that the *CA1 and CA4* genes play a role in the CO_2 signaling pathway. In order to discover where in the CO_2 signaling pathway the *ca1ca4* mutant resided, a variety of tests on the *ca1ca4* mutant were conducted.

The *ca1ca4* double mutant was tested in comparison to wild type for CO_2-induced stomatal closure. The results in this thesis show that *ca1ca4* stomata have an insensitive stomatal response to high CO_2 concentrations (Figure 1). Most known CO_2 response mutants also show an ABA insensitivity phenotype, indicating that they are involved in the ABA signaling pathway, the *ca1ca4* mutant was then tested for ABA response (Figure 2). Either at low ABA condition or high ABA condition, *ca1ca4* double mutant showed similar stomatal ABA response as wild type plants, further indicating that CAs function early in the CO_2 signaling pathway and that function occurs before the CO_2 and ABA convergence point.

Due to the fact that guard cells respond to a number of environmental stimuli, the *ca1ca4* double mutant was then tested with known stomatal closing signals, such as malate and bicarbonate. Malate has been shown to

be a factor in CO_2-induced stomatal closing. Malate induces stomatal closure by increasing the apoplastic malate concentration and opening anion channels in guard cells that allow for malate to be released, therefore closing stomata. Malate has been shown to be a factor in CO_2-induced stomatal closing with similar effects as high CO_2. When the *ca1ca4* double mutant was tested alongside wild type with either 20mM malate or 20mM + 800ppm CO_2, col and *ca1ca4* both close with malate and with malate and 800ppm CO_2 (Figure 3), but *ca1ca4* plants were less sensitive to malate. Malate is regulated on several levels, and the results are a combination of malate-induced closure in addition to CO_2-induced closure.

Bicarbonate has been shown to be an external source of CO_2 due to the fact that bicarbonate is in the reaction catalyzed by CA: $CO_2 + H_20 \leftrightarrow HCO_3^- + H^+$. Therefore, a higher concentration of bicarbonate would be equal to high CO_2 concentrations. It was calculated that 2.7mM bicarbonate would be the same as bubbling 800ppm CO_2 into the solution at pH=7.1. When col and *ca1ca4* stomata were exposed to 800ppm CO_2 or 2.7mM bicarbonate, results (Figure 4) show that *ca1ca4* and col respond identically to 2.7mM bicarbonate as they do to 800ppm CO_2. Because col and *ca1ca4* respond similarly to CO_2 as to bicarbonate, bicarbonate could be responsible in the CO_2-induced stomatal closure. CAs not only are responsible for the production of bicarbonate, but also have a role in stomatal development.

The *ca1ca4* double mutant has a much higher stomatal density than col, at concentration of 500ppm CO_2. When stomatal density was calculated

for col, *ca1ca4, ca1ca2ca4, ca1* and *ca4*, both *ca1ca4* and *ca1ca2ca4* have a much larger stomatal density at the concentration of 500ppm CO_2 on the abaxial side (Figure 5) and the adaxial side of the leaves (Figure 6). These results indicate that CAs must play a role in the development of stomata as well as CO_2-induced stomatal movement.

The only mutant known that functions before the ABA and CO_2 convergence point is *ht1-2.* Knowing that the *ca1ca4* phenotype for stomatal density is high, the stomatal densities of *ht1-2, ca1ca4ht1-2, ca1ca4,* and col were also determined (Figure 7 and 8). Results show that the *ht1-2* maintains a wild-type stomatal density pattern, while the triple mutant *ca1ca4 ht1-2* has the *ca1ca4* phenotype. Research in stomatal density is complicated and many environmental signals are involved in addition to CO_2. Many results of stomatal density vary from leaf to leaf and plant to plant which can explain why some plants stomatal density is unclear. However, results with ca1ca4 show that CAs function in stomatal development under high and low CO_2 levels.

The *ca1ca4* double mutant is mutated in the carbonic anhydrase genes that are highly expressed in guard cells. In order to analyze which gene is responsible for the CO_2 response, Genomic DNA of *CA1* and *CA4* was expressed in the double mutant plants. Stomatal densities were analyzed in these randomly selected transgenic lines (CA1g(1) and CA4g(2)and it was found that both *CA1* and *CA4* genes recover the stomatal density phenotype of double mutant plants (Figure 9). These data demonstrated that both the *CA1* and *CA4* genes are responsible for the CO_2-inducded stomatal closure

insensitivity response as well as for the high stomatal density phenotype. To analyze whether expression of *CA1* and *CA4* only in guard cells also can complement the stomatal CO_2 responses, the cDNA of *CA1* and *CA4* from WT, controlled by a guard cell promoter, was transformed into *ca1ca4* double mutant (CA1d-2, CA1d-6, CA1d18, CA4d-1, CA4d-2 lines were randomly chosen for analyses). Stomatal density analyses of these lines suggests that both *CA1* and *CA4* genes can restore to the wild type density (Figures 10 and 11). Stomatal CO_2 response in these transgenic lines was also analyzed. With exposure to 800ppm CO_2 for 30 min, CA1d -2 and CA1d-18 both close the stomata to similar degrees as col, showing that complementation of *CA1* or *CA4* in guard cells can complement the insensitive stomatal response of *ca1ca4* under high CO_2 (Figure 12). To understand more of the CAs function in CO_2 signaling pathway, *CA1* and *CA4* were also transformed into wild-type guard cells. The over-expression lines were tested for stomatal density and for CO_2-induced stomatal closure response. Interestingly, for CO_2-induced closure, all lines showed the wild type phenotype (Figure 13). This could be because there is saturation in the CO_2 response. Observations for stomatal density show that under 500ppm CO_2 conditions, on both abaxial and adaxial sides, stomatal density has a wild-type phenotype (Figure 14 and 15). However, under 150ppm CO_2 conditions, there is a large variety between the different lines, which could correlate to different levels of expression in these lines, leading to different stomatal density counts. These results show that

CO_2-controlled stomatal development is complicated and perhaps the amount of functional CA is responsible in the development of stomata.

To analyze if other CA homologues from other species could complement the *ca1ca4* phenotypes of CO_2-induced stomatal movement as well as stomatal density, human *CAII* was transformed into the *ca1ca4* double mutant plants. The *HmCAII* was able to restore the mutant phenotype for CO_2 induced stomatal closure as well as stomatal density (Figures 16-18). Because the *HmCAII* was able to restore the mutant phenotype further demonstrates that the CA activity is a main mechanisms for controlling both CO_2-induced stomatal movements and stomatal development. In conclusion, the *ca1ca4* phenotype of stomatal density and the insensitive CO_2-induced stomatal movement phenotype can be recovered by either cDNA or genomic DNA if *CA1* and *CA4* from wild-type as well as from Human *CAII*.

The previous experiments were carried out with *CA1* and *CA4* from the βCA class, of which both highly expressed in guard cells. Currently in progress are two different sets of crosses to isolate the following quadruple mutants, *ca1ca2ca4ca6* and *ca1ca2ca3ca4.* An isolated homozygous Salk line, *ca3,* was crossed with *ca1ca2ca4* to screen for a *ca1ca2ca3ca4*. However, *ca3* and *ca4* are on the same chromosome, so a crossover needs to occur. Also, *ca1ca2ca4* was crossed with *ca1ca4ca6* to obtain a *ca1ca2ca4ca6* mutant. Screening for these two mutant lines is in progress. With these quadruple mutants, we hope to find out more of the function of CAs in plant stomata and eventually obtain a *ca1ca2ca3ca4ca5ca6* knockout for a

complete shutdown of the βCA family if such a mutant is at all viable. This research shows that the CA genes *CA1* and *CA4* function in CO_2 regulated stomatal movements and development. Further research on these genes will provide insights into CO_2 signaling and stomatal development pathways.

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