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Genetic modification and mutant construction for proteins involved in the CO2 signaling
pathway of *Arabidopsis thaliana*

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

in

Biology

by

Krystal Bosmans

Committee in charge:

       Professor Julian I Schroeder, Chair
       Professor Mark Estelle
       Professor Alisa Huffaker

2022

The Master's Thesis of Krystal Bosmans is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

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ABSTRACT OF THE THESIS

Genetic modification and mutant construction for proteins involved in the CO2 signaling
pathway of *Arabidopsis thaliana*

by

Krystal Bosmans

Master of Science in Biology

University of California San Diego, 2022

Professor Julian Schroeder, Chair

As atmospheric carbon dioxide levels continue to rise, agricultural yields will be
impacted worldwide. Our laboratory obtained evidence that specific MAPKs inhibit the activity
of HT1, a negative regulator of CO_2-induced stomatal closure. The findings showed that HT1 can
phosphorylate CBC1/2 but not whether HT1 regulates CBC1/CBC2's activity. This study

analyzed stomatal conductance responses to changing CO_2 levels with a *cbc1/cbc2* double

knockout line, a dominant *ht1(A109V)* line with a permanently active HT1 protein, and a

cbc1/cbc2/ht1(A109V) triple mutant line with knocked out CBC1 and CBC2 genes and a

constantly active HT1 protein. The *cbc1/cbc2/ht1(A109V)* mutant showed stomatal conductance

values similar to *cbc1/cbc2* samples, not *ht1(A109V)*. The results suggest that the CBC1/CBC2

are epistatic to and function downstream of HT1 in the signaling pathway. Next, mutant lines of

pGC1:MPK12(K70R)-GFP/mpk12, with a K70R mutation in MPK12, and *pGC1:MPK12-*

GFP/mpk12, a rescue line, both showed stomatal conductance responses similar to Col-0.

Meanwhile, *mpk12* samples showed responses to changes in CO_2 but higher stomatal

conductance levels compared to Col-0. The data suggests that the kinase activity of MPK12 is

not required to inhibit HT1. The final experiments used a line with two mutations in mapped

HT1-mediated CBC1 phosphorylation sites, *pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2,*

showed stomatal conductance similar to the *cbc1/cbc2* samples while the rescue line

pGC1:CBC1-GFP/cbc1cbc2 with a wildtype CBC1 gene, showed stomatal responses similar to

Col-0. This suggests that the two mutated sites could be important for HT1 phosphorylation. The

information gathered here can contribute to developing agricultural lines that can withstand

rising levels of CO_2.

INTRODUCTION

Plants have been a crucial organism that impacts the living world in multiple ways such as supplying the atmosphere with oxygen through photosynthesis and acting as a food source for human and domestic animal consumption. As primary producers, it is important that plants are not exposed to stressful environmental conditions so that their growth is not negatively affected. Plants can experience stress on a daily basis from a number of abiotic sources including temperature, salinity, drought, and toxic metals in addition to biotic stresses like predation from herbivores (Gull et al., 2019). The human population has been increasing at a rapid rate since the Industrial Revolution, when the global birth rate became significantly larger. Furthermore, the global death rate declined as a result of improved technology that lead to lower mortality rates and longer life expectancy (Bongaarts, 2009). With a continuously growing population, it is important that the world can maintain a level of primary produce production that supports the global population.

Also increasing globally is the concentration of carbon dioxide (CO_2) within the atmosphere which recently has been recorded to be ~416 ppm (https://keelingcurve.ucsd.edu). Over the past 1 million years and before the industrial revolution, the atmospheric CO_2 concentration would fluctuate between 150-280 ppm but it had not surpassed 300 ppm until the Industrial Revolution. This is critical because carbon dioxide can absorb the infrared radiation (heat) of sunlight and trap it within Earth's atmosphere, this results in an overall increase in temperature (Callendar, 1938). Callendar's study also noted that there would be more concentrated radiation absorption in the lower layers of the atmosphere following an increase in atmospheric carbon dioxide. Recently, the Intergovernmental Panel on Climate Change (IPCC) stated that since the middle of the 20th century, human activities were likely responsible for the

observed increase in global temperatures (IPPC, 2014). Rising levels of carbon dioxide and increasing temperatures are just two products of climate change that are affecting our biosphere, in addition to more frequent extreme weather patterns, ocean acidification, and rising sea levels (USGCRP, 2017).

Plants are sessile organisms, meaning that they cannot move freely when their environment no longer suits them, they must either adapt to the abiotic and biotic challenges they are presented with or cease to survive (Gull et al., 2019). Water use and gas exchange are two plant activities that are crucial for survival and can be affected by changes in the environment. Plants have a cellular complex known as a stomate that resembles a pore on their leaves that responds to such changes, so they can continue to survive (Zhang et al., 2018). It is the function of stomata to maintain a level of gas exchange that is optimal for the needs of photosynthesis by keeping the aperture of the stomatal pore at an appropriate width so there is balance between water loss from transpiration and exchange of carbon dioxide and oxygen (Darwin, 1898). Each stomatal pore is surrounded by a pair of guard cells that regulate the size of the stomatal aperture to allow optimal amounts of carbon dioxide to enter the intracellular space of leaves from the atmosphere (Zhang et al., 2018). The pressure exerted on the cell wall of guard cells by the intercellular fluid is affected by changes in ion and solute osmolarity and as a result influences stomatal aperture size (Ainsworth and Rogers, 2007). Because changes in carbon dioxide concentration influence the width of stomatal apertures, it is an important subject in relation to the agriculture industry. Elevated carbon dioxide levels could lead to an increase in plant biomass depending on the conditions. An increase in plant production may positively affect crop yields because plants would have access to more carbon dioxide for use in sugar production. However, a previous study using soybeans found that high amounts of atmospheric carbon

dioxide could not counteract the negative effects that the plants experienced when they were exposed to high temperatures and drought-like conditions (Gray et al., 2016). This study also concluded that due to abiotic factors such as changes in water availability and temperature associated with climate change, it is difficult to determine overall specifically how crop yields will be affected.

In environments with low concentrations of carbon dioxide, the stomatal aperture widens to allow more carbon dioxide enter while in environments with high concentrations of carbon dioxide, the stomatal aperture narrows and allows less carbon dioxide to enter even if the light conditions are optimal for photosynthesis (Zhang et al., 2018). As a result of long-term exposure to higher than ambient atmospheric carbon dioxide levels, plant stomata become narrower, the overall stomatal density on leaves is reduced, the intracellular temperatures of leaves increase, and water-use efficiency is affected (Hetherington and Woodward, 2003). If stomata remain closed it is possible that the plant could die from overheating or from lack of access to the carbon dioxide needed to build sugars for growth and development. Multiple studies have predicted or observed a decrease in the crop yield of grains when they are exposed to higher temperatures or drought, an increasingly common occurrence as atmospheric carbon dioxide levels continue to rise (Parry, 2007; Hatfield, 2016; Shim et al., 2017).

With the looming threat of climate change, specifically rising carbon dioxide levels in our generation, there is a lot interest in understanding the signaling pathways that induce stomatal closure. The two main pathways being studied include one triggered by carbon dioxide levels and another triggered by production of the plant stress hormone abscisic acid (ABA) in response to drought conditions. This work described herein will be focusing on the signaling pathway that is induced by changes in carbon dioxide levels. This pathway starts with the entry of CO_2 into the

intracellular space of the leaf through the opening of the stomatal pore (Zhang et al., 2018). The CO_2 is then reversibly converted into bicarbonate (HCO_3^-) by multiple beta-carbonic anhydrases (β-CA) (Hu et al., 2010). There are three major known gene families of carbonic anhydrases, alpha, beta, and gamma, all of which have homologs in our plant model system, *Arabidopsis thaliana* (Hewett-Emmett and Tashian, 1996). Specifically, the βCA1 and βCA4 carbonic anydrases expressed in guard cells convert the entering carbon dioxide into protons and bicarbonate, the latter of which stimulates the downstream signaling cascade that results in changes to the width of stomatal apertures (Hu et al., 2010, Xue et al., 2011).

The next protein involved in the carbon dioxide signaling pathway is a type of mitogen-activated protein kinase (MAPK) and in *A. thaliana,* the MPK12 and MPK4 genes/proteins mediate the stomatal CO_2 response (Jakobson et al., 2016, Tõldsepp et al., 2018). Previous studies with *MPK4-silenced Nicotiana tabacum* have shown stomata to have regular movements when exposed to ABA treatments but lacked stomatal closing in environments with high CO_2 levels (Gomi et al., 2005, Hettenhausen et al., 2012, Marten et al., 2008). These observations lead to an interest to investigate whether the MPK4 protein had a similar role in *A. thaliana*. A double mutant where MPK4 was suppressed in guard cells while MPK12 was disrupted via a T-DNA insertion showed similar results, where ABA-induced stomatal closure was functioning, while stomatal responses to elevated carbon dioxide were impaired (Tõldsepp et al., 2018). Additionally, *in vitro* experiments from this same study concluded that kinase activity from MPK4/MPK12 was not directly activated by bicarbonate levels suggesting that there is another component of the signaling pathway upstream of the MAP kinases (Tõldsepp et al., 2018). MPK12 then goes on to inhibit the protein kinase high leaf temperature1 (HT1) by unknown

mechanisms which may then affect directly or indirectly two convergence of blue light and CO_2 (CBC1/2) kinases (Hõrak et al., 2016; Hiyama et al., 2017).

MPK12 has previously been shown to interact with HT1 by inhibiting its activity (Jakobson et al., 2016). Jakobsen also found that HT1 has the ability to activate itself via autophosphorylation but is inhibited by active MPK12 *in vitro*. These observations were further confirmed in SDS page gel experiments that included wildtype HT1, kinase dead HT1, and multiple MPK12 proteins with varying levels of activity (Jakobson et al., 2016).

Experiments using mutant *A. thaliana* lines in two of seven known HT1 alleles (*ht1-2* and *ht1-3*) showed genetically dominant disruption of stomatal signaling induced by elevated CO_2 levels but similar stomatal aperture widths compared to wildtype following treatment with ABA indicating that it was only carbon dioxide-induced stomatal signaling that was impacted (Hashimoto-Sugimoto et al., 2016). An A109V mutation in HT1 resulted *in vitro* in a reduced ability of MPK12 to inhibit the autophosphorylation activity of HT1 so that it remains active, although there have not yet been any *in vivo* studies (Hõrak et al., 2016). HT1 was shown *in vitro* to phosphorylate both wildtype CBC1/CBC2 and kinase dead CBC1/CBC2 proteins that lost their ability to autophosphorylate themselves or phosphorylate other proteins (Hiyama et al., 2017). However, the effect of this *in vitro* phosphorylation remains unknown. This same study also observed that CBC1/CBC2 did not phosphorylate HT1 despite the protein kinase activity of CBC1 and CBC2. The fact that HT1 can activate CBC1/CBC2 has led to hypotheses that CBC1/CBC2 could function in the same signaling pathway as HT1 and CBC1/CBC2 impact stomatal closure.

Downstream in the signaling pathway of CBC1/CBC2 is the S-type anion channel-associated 1 (SLAC1) protein that controls the efflux of ions that leave the guard cells, thereby

reducing the turgor pressure leading to a reduction in the stomatal aperture size. Some ions that have been suggested to be responsible for these fluctuations in the carbon dioxide signaling pathway include calcium (Ca^{2+}) and potassium (K^+) (Webb et al., 1996, Schroeder et al., 1984).

In guard cells, an influx of K^+ via inward K^+ channels is coupled with H^+ extrusion that creates a current that contributes to the opening of stomata (Schroeder et al., 1984, Assmann et al., 1985, Schroeder et al., 1987). Changes in ion concentrations like these result in an osmotic influx of water into the guard cells that facilitates the opening of stomata via the resulting turgor pressure increase. A previous study found that in mutant *SLAC1* guard cells, the rate of stomatal opening was greatly reduced but not completely disrupted (Laanemets et al., 2013). CBC1/CBC2 are hypothesized to inhibit SLAC1 ion channels directly or indirectly by unknown mechanisms (Hiyama et al., 2017). SLAC1 influences changes in stomatal aperture but because there are so many signaling pathways that converge on SLAC1 it is unknown whether or how the two components interact (Hiyama et al., 2017).

There has been much research done on carbon dioxide-induced stomatal closure that is supported by genetic evidence, but the knowledge of what specific proteins interact and regulate targets in the signal transduction pathway is far from complete. While previous studies have shown that HT1 and CBC1/CBC2 interact *in vitro*, it is currently unknown if they indeed are in the same pathway and if CBC1/CBC2 functions downstream of HT1. One goal of this study was to determine through *in vivo* experiments whether the CBC1/CBC2 proteins act downstream of HT1 in the carbon dioxide-induced signal transduction pathway. Therefore, a *cbc1/cbc2/ht1(A109V)* triple mutant transgenic *A. thaliana* line was developed for use in stomatal conductance experiments to determine if CBC1/CBC2 are epistatic to HT1, which can be interpreted as CBC1 and CBC2 most likely acting downstream of HT1 in the carbon dioxide

signaling transduction pathway. Our hypothesis is that the mutated *ht1(A109V)* protein keeps

stomata open through activation of CBC1/CBC2 and that its phenotype will be dependent on the

existence of CBC1/CBC2 in the pathway. If the triple mutant exhibits a phenotype similar to the

cbc1/cbc2 double mutant instead of the *ht1(A109V)* phenotype, it would suggest that

CBC1/CBC2 do function downstream of HT1.

Another goal of this study was to explore the importance of kinase activity in MPK12

and possible HT1 phosphorylation sites on CBC1. There have been preliminary experiments *in*

vitro that suggest that the kinase activity of MPK12 might not be not required for its ability to

mediate CO_2 signal transduction, but there have been no experiments that explore whether this is

consistent *in vivo*. There has also been unpublished new research from our laboratory exploring

possible phosphorylation sites for HT1 on the CBC1 protein. Mass spectrometry experiments

were performed following *in vitro* phosphorylation assays that focused on CBC1 with and

without the presence of bicarbonate. Comparing the two data sets, eight potential

phosphorylation sites were identified as possible mutation targets that could affect HT1's ability

to phosphorylate CBC1 for *in vitro* experiments, and our set of experiments builds on that data.

To explore both of these topics, *Agrobacterium* vectors were used to make *pGC1:MPK12-*

GFP/mpk12, *pGC1:MPK12(K70R)-GFP/mpk12*, *pGC1:CBC1-GFP/cbc1cbc2*, and

pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2 mutant *A. thaliana* lines that were screened and

selected for use in stomatal conductance experiments. The results gathered were compared to the

in vivo responses gathered from Col-0 (wildtype control accession), *mpk12* mutant plants, and

cbc1/cbc2 double mutant plants. The use of these genetic mutants would determine whether the

data that has been observed in in multiple *in vitro* phosphorylation studies have consistent results

in vivo. Overall, the information gathered from these experiments could be used for developing

mutant lines in agricultural products that can tolerate the increasing atmospheric levels of carbon

dioxide we are experiencing as a result of climate change.

RESULTS

1.1 Genotyping and isolation of the cbc1/cbc2/ht1(A109V) triple mutant.

In order to test our hypothesis *in vivo*, a *cbc1/cbc2/ht1(A109V)* triple mutant line had to be constructed. This was achieved by crossing a T-DNA *cbc1/cbc2* double mutant line with an *ht1(A109V)* line. Polymerase chain reaction (PCR) experiments were pursued in the T1 generation to test if the crossing was successful by confirming that the CBC1 and CBC2 genes were heterozygous for each T-DNA insertion with a wildtype allele that originated from the *ht1(A109V)* parent plant. In following generations, samples were first tested to determine whether a sample was homozygous for each of the CBC1 and CBC2 T-DNA insertions before the HT1 gene was amplified via PCR (Fig. 1a-b). DNA samples were then purified and sent in for sequencing to confirm that the A109V point mutation was present and homozygous (Fig. 1c). A successful point mutation would show a nucleotide change of C→T in sequencing with only a single peak, not two peaks overlapping which would indicate that the mutation is heterozygous (Fig. 1d). Samples that met these criteria were selected for use in experiments that would test their stomatal responses to imposed shifts in the atmospheric concertation of carbon dioxide. This *cbc1/cbc2/ht1(A109V)* triple mutant line was compared to WT, *cbc1/cbc2*, and *ht1(A109V)* plants that were all selected for use as controls.
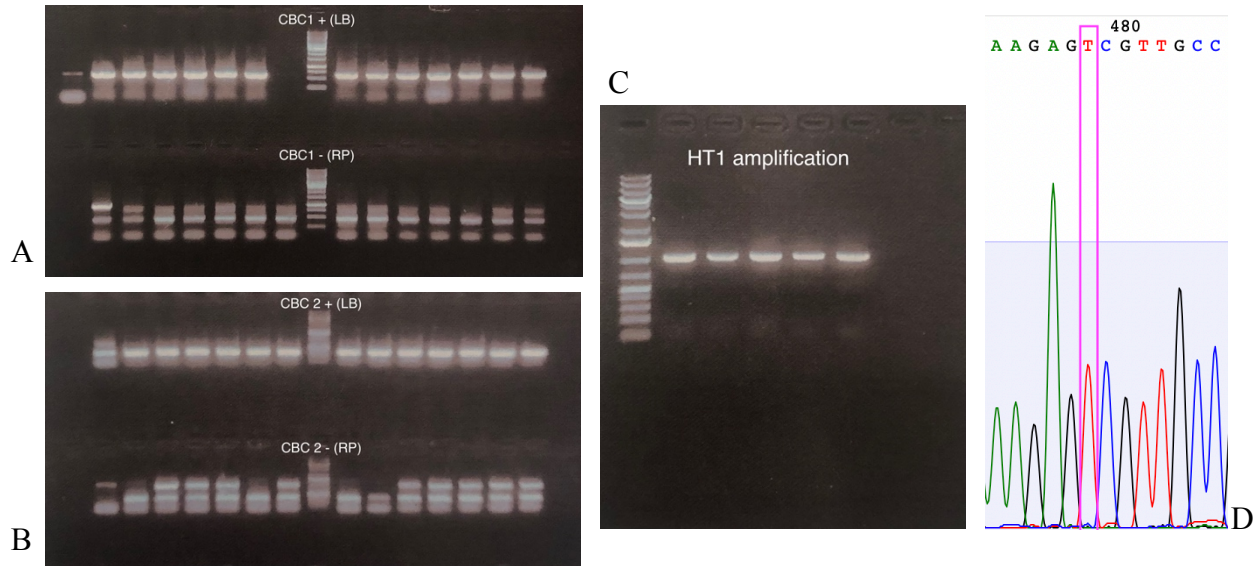
Figure 1. Polymerase chain reaction and HT1 genetic sequencing used to identify *cbc1/cbc2/ht1(A109V)* triple mutant samples. a-b, PCR results testing for the presence of T-DNA insertions and homozygosity in each CBC gene (A: *cbc1* and B: *cbc2*). One primer pair was used to test for this insertion, with the results shown in the top row of each gel. In the top row, the presence of a band not seen in the WT sample indicates the presence of the insertion as the LB primer attaches to the left side border of the insertion. Another primer pair was testing for homozygosity of the insertion with the results shown in the bottom row. In the bottom row, having the same bands as the WT sample indicates the lack of insertion because if there were, the distance between the two primers would be too long for the WT band to form. **c,** PCR gel showing results of amplification of the HT1 gene from the genomic DNA of candidates that were sent in for genetic sequencing **d,** Genetic sequencing results showing a successful homozygous point mutation of *ht1(A109V)*.

Table 1. PCR primers used for construction of *cbc1/cbc2/ht1(A109V)*.

	Primer Sequence	Tm(°C)
SALK.LBb1.3	5'-ATTTTGCCGATTTCGGAAC	52
SAIL_LB1	5'-TAGCATCTGAATTTCATAACCAATCTC	58
SALK_005187_LP	5'-ACACCGAAAGAAAGCGAAGAG	55
SALK_005187_RP	5'-TTCGGAAAGAAAGTTTCGATTC	52
SAIL_740_G01_LP	5'-TTTATGCCAAACAGCAACCTC	54
SAIL_740_G01_RP	5'-TCTTTTCCCATTTTCCTCTGC	53
HT1 (A109V check F55)	5'-AACGACGGTTAGATTCTCG	55
HT1 (A109V check R58)	5'-CAAAAGCAAATTGTTGGACTTTAG	58

**Table 2.** Length of expected DNA fragment using primers from Table 1.

	Expected DNA Length (bp)
CBC1 insertion	~650
WT CBC2	1103
CBC2 insertion	~800
WT CBC2	1140
HT1 amplification	1003

1.2 Whole leaf gas exchange experiments of the cbc1/cbc2/ht1(A109V) triple mutant.

The goal of the experiment was to compare the CO_2 concentration-induced changes in stomatal conductance among the genotypes described above. Stomatal conductance is a measurement of the amount of water vapor that is exiting a leaf through its stomata, of the three different mutants to each other and wildtype (WT). The stomatal conductance phenotype quantitatively reflects the degree of stomatal opening in a plant, higher values indicate a wider stomatal aperture while lower values indicate more narrow apertures. For the experiments, a LiCOR 6400 portable photosynthesis machine automatically changed the concentration of carbon dioxide within the chamber that contained the sample being tested and collected data on changes in stomatal conductance. A program where the concentration of carbon dioxide was shifted from ambient ~400 ppm to high >800 ppm to low <180 ppm was chosen. The WT plants showed normal basal stomatal conductance values of ~0.15 mol H_2O $m^{-2}s^{-1}$ when exposed to ambient levels of CO_2 (Fig. 2). In a high CO_2 environment, stomatal conductance values of approximately ~0.10 mol H_2O $m^{-2}s^{-1}$ and lower indicate stomatal closure. In environments with low levels of CO_2, values of ~0.18 mol H_2O $m^{-2}s^{-1}$ and higher indicate more open stomata; both of these responses were observed in the WT samples (Fig. 2). The *cbc1/cbc2/ht1(A109V)* triple mutant has both CBC genes knocked out in addition to a mutated HT1 protein that is not deactivated in elevated CO_2. The triple mutant showed stomatal conductance values similar to the *cbc1/cbc2* double knockout mutant where the stomata remained narrower than WT even when CO_2 levels fluctuated. These results are the opposite of the *ht1(A109V)* single mutant samples that maintained more open stomatal values as a result of the mutated HT1 protein being constantly active. The data from the experiments showed that during carbon dioxide level

fluctuations, the *cbc1/cbc2/ht1(A109V)* triple mutant responded similarly to the *cbc1/cbc2* double mutant as opposed to the *ht1(A109V)* single mutant.
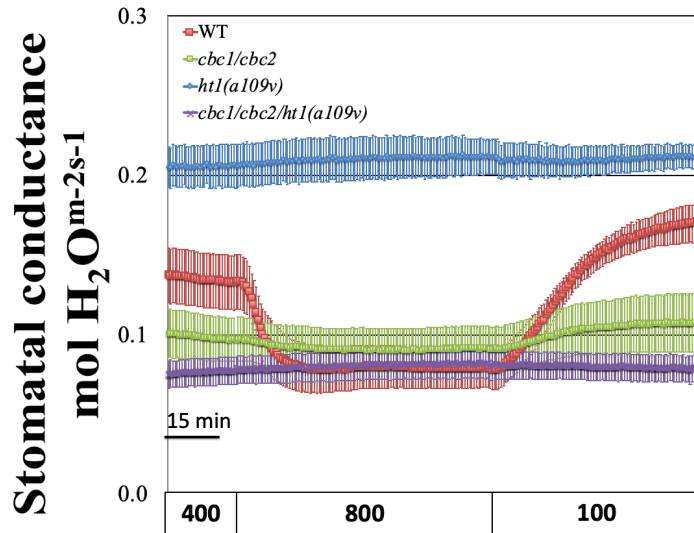
Figure 2. Stomatal conductance comparisons of Col-0, *cbc1/cbc2*, *ht1(A109V)*, and *cbc1/cbc2/ht1(A109V)*. The stomatal conductance of samples of *Arabidopsis thaliana* were measured at 400 ppm (parts per million) of CO_2 for 30 minutes, 800 ppm of CO_2 for 60 minutes, and 150 ppm of CO_2 for 50 minutes with a LiCOR 6400 portable photosynthesis system. The average value is represented for genotypes where more than one sample was tested. WT (n=4), *cbc1/cbc2* (n=4), and *ht1(A109V)* (n=4) lines were all used as controls to compare to the *cbc1/cbc2/ht1(A109V)* triple mutant line (n=4).

1.3 Construction of MPK12 and CBC1 GFP vectors.

To determine if the 256 and 280 amino acid sites were crucial for the function of CBC1, we decided to transform background *cbc1/cbc2* double mutant plants with two vectors. One vector would have a wildtype CBC1 protein and one vector would express a CBC1 protein with point mutations in two of our hypothesized phosphorylation sites (Fig. 3a). A similar approach was taken to test if kinase activity was required for MPK12 function where a wildtype MPK12 allele and a MPK12 with a K70R point mutation were transformed into *mpk12* background plants via *Agrobacterium* (Fig. 3b). This K70R mutation was in a location where a mutation could impact the kinase activity of MPK12 and therefore its ability to inhibit HT1. A promoter specific to guard cells called *pGC1*, developed in the Schroeder lab, was selected as results have shown strong guard cell expression and therefore was selected to be the promoter for these constructs (Yang et al., 2008). Green fluorescent protein was added to the C-terminal as a tag so that candidates with the vector can be easy to identify using microscopy (Fig. 3a-b). Phusion U Polymerase was used to amplify the genes of interest before USER cloning and Phusion Hot Start II Polymerase was used for amplification before SLiCE cloning, the primers used for both of these cloning reactions are listed in Table 3. Expected lengths of fragments are listed in Table 4 and were used to determine if the cloning was successful before these fragments were transformed into vectors. Additionally, after each cloning step, plasmid purification was done on colonies so that sequencing, done by Retrogen Inc, could confirm that the point mutations were present. The USER cloning allowed for the genes of interest to be placed into a vector with a GFP tag downstream of the gene and the SLiCE cloning was used to place the gene of interest - GFP fragment into a vector with the *pGC1* promoter. The final constructs were vectors that had *pGC1* as its promoter, green fluorescent protein (GFP) downstream of the gene, and contained

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the desired WT or mutant CBC1 or MPK12 gene (Fig. 3a-b). Once the final constructs were confirmed via genetic sequencing they were transformed into their respective background plants of *Arabidopsis thaliana* using the *Agrobacterium* transformation method. The vectors that contained an MPK12 gene were transformed into *mpk12* plants while the vectors that contained a CBC1 gene were transformed into *cbc1/cbc2* plants. This was to ensure that the proteins in the plants had the sequence of the gene from the vectors since before the transformation the plants did not have a functioning protein at all. Thousands of seeds were collected and screened on hygromycin plates and samples that germinated were transferred to separate pots and grown in the growth room using a cycle of 16 hours of day with 8 hours of night. When plants were 2-3 weeks old, a sample was taken to determine if the plant did contain the vector with the gene of interest via microscopy. Because there was a GFP tag attached to the end of this gene it made selection of T1 and T2 candidates easy as fluorescence of guard cells were checked using a confocal microscope and compared to wildtype guard cell autofluorescence (Fig. 4a-b).
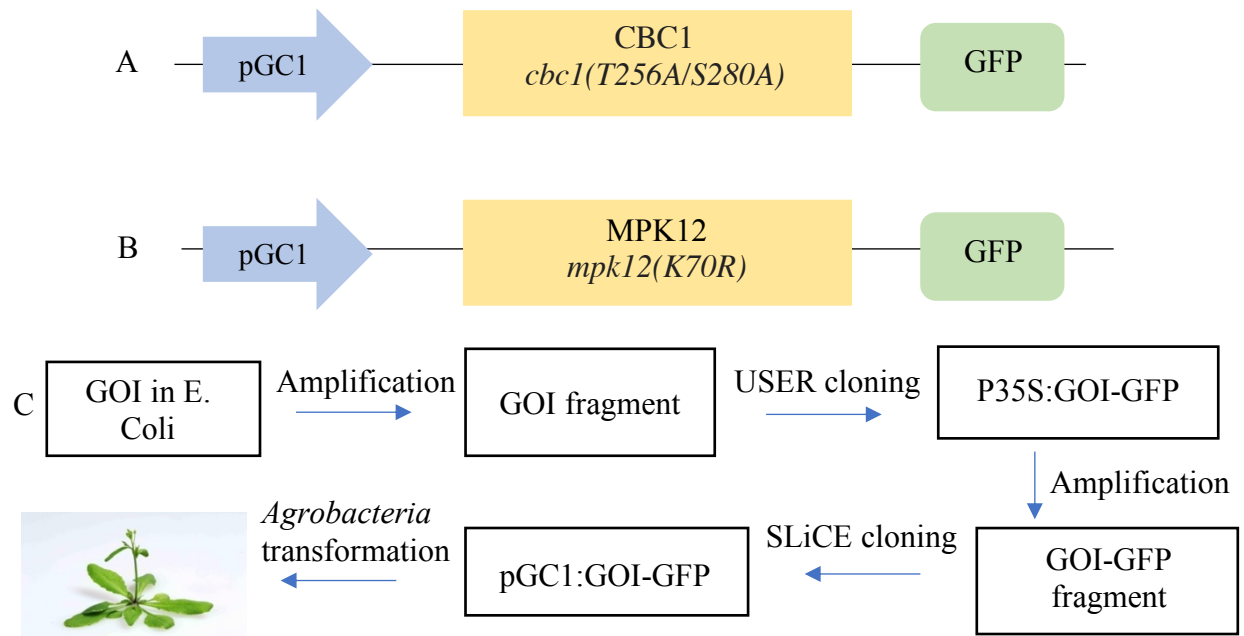
Figure 3. Construction plan for MPK12 and CBC1 GFP vectors. a-b, A strong guard cell promoter (*pGC1*) was placed upstream of the gene of interest. Downstream of the gene of interest was green fluorescence protein (*GFP*) that can be used as a marker to determine via confocal microscopy whether samples contain the mutant protein. **c,** a step by step flowchart of how the genes of interest were cloned for the construction of the vectors that were transformed into *A. thaliana* mutant background plants that were mutants for the transforming vector's gene of origin.
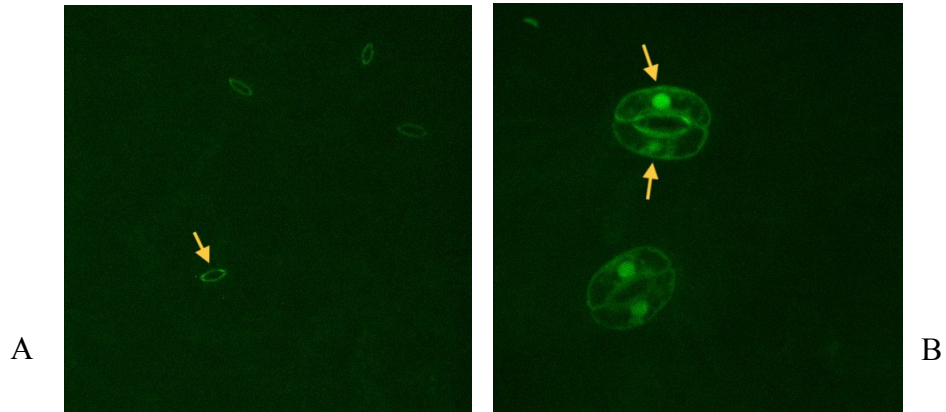
Figure 4. Confocal results of guard cell GFP fluorescence in a *pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2* candidate. These images were taken with a confocal microscope at a magnification of 100x using a GFP filter. **a,** example of stomatal autofluorescence observed in WT samples. **b,** a candidate from one of the *pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2* lines displaying high fluorescence within the guard cells.

Table 3. Primers used for cloning of GFP vectors using USER and SLiCE cloning systems.

	Primer Sequence	Tm(°C)
CBC1_USER_F55	5'-ggcttaauATGAAGGAGAAGGCGGAG	55
CBC1_-stop_USER_R60	5'-ggtttaauTGGGCCTCGGTGTCGG	60
MPK12.user.F54	5'-ggcttaauATGTCTGGAGAATCAAGCTCTG	54
MPK12.user.R54	5'-ggtttaauGTGGTCAGGATTGAATTTGACAG	54
CBC1_InF_pGC1_F55	5'-aaattctagaggatccatgAAGGAGA	55
MPK12_InF_pGC1_F54	5'-aaattctagaggatccATGTCTGGAGAATCAAGCTC	54
GFP_InF_Nos_R52	5'-cggtacccgggatccTTATTTGTATAGTTCATCCATGC	52

Table 4. Length of expected DNA fragment using primers from Table 3.

	Expected DNA Length (bp)
CBC1 User Cloning	~1200
MPK12 User Cloning	~1200
CBC1 pGC1/Nos	~1900
MPK12 pGC1/Nos	~1900

Table 5. Clones, plasmids, and vectors used from or added to the Julian Schroeder's lab stocks.

Ref #	Stock Type	Clone Name	General Description	Vector	Strain	Antibiotic
3949	DNA Box #41	pGEX-6P1-user	USER compatible E. coli overexpression vector	pGEX-6P1-user	N/A	Carbenicillin
4551	DNA Box #58	pGEX-4T-1-MPK12	wide-type MPK12	N/A	N/A	N/A
4554	DNA Box #58	pGEX-4T-1-MPK12-K70R	Kinase-inactive MPK12(K70R)	N/A	N/A	N/A
5502	Glycerol Stock Box #70	pGEX6P-CBC1	CBC1 protein expression	pGEX6P-1user	BL21	Ampcillin
5953	DNA Box #75	GST-CBC1-T256/S280A	E. Coli Expression vector	pGEX-6P-1	N/A	Carbenicillin
5969	DNA Box #75	pGC1:CBC1-GFP	Stable Expression vector	pCAMBIA1300	N/A	Kanamycin/ Hygromycin
5970	DNA Box #75	pGC1:CBC1(T256/ S280A)-GFP	Stable Expression vector	pCAMBIA1300	N/A	Kanamycin/ Hygromycin
5971	DNA Box #75	pGC1:MPK12-GFP	Stable Expression vector	pCAMBIA1300	N/A	Kanamycin/ Hygromycin
5972	DNA Box #75	pGC1:MPK12(K70 R)-GFP	Stable Expression vector	pCAMBIA1300	N/A	Kanamycin/ Hygromycin
5922	DNA Box #75	35S:user-GFP	Protoplast Transient Expression vector	pUC18	N/A	Carbenicillin

1.4 Whole leaf gas exchange experiments of pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2 and pGC1:CBC1-GFP/cbc1cbc2 mutant lines.

As expected, the WT stomata became narrower when carbon dioxide levels increased and more open when the CO_2 levels decreased (Fig. 5). The *cbc1/cbc2* double mutant does not have functioning CBC1 or CBC2 proteins and these samples showed low levels of stomatal conductance ~0.085 mol H_2O m^{-2}s^{-1} and displayed no clear response to an increase in CO_2 levels and a small increase in stomatal conductance (to ~0.10 mol H_2O m^{-2}s^{-1}) when the CO_2 concentration changed to 100 ppm (Fig. 6). Samples from the *pGC1:CBC1-GFP/cbc1cbc2* have a WT CBC1 protein but no functioning CBC2 protein. These samples had responses to carbon dioxide shifts that we more comparable to the WT samples with a basal stomatal conductance that was slightly elevated (~0.16 mol H_2O m^{-2}s^{-1}) (Fig. 5). The other mutant line, *pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2*, has a nonfunctional CBC2 protein and a CBC1 protein with two point mutations at potential phosphorylation sites. These samples exhibited low stomatal conductance levels that did not change dramatically when CO_2 levels increased to 800 ppm and only increased slightly when CO_2 levels decreased, similar to the *cbc1/cbc2* mutant results (Fig. 5). The results from the experiments show that the WT and *pGC1:CBC1-GFP/cbc1cbc2* plants both had similar responses to changes in CO_2 concentrations by having narrower stomata when carbon dioxide levels are high and more open stomata when carbon dioxide levels are low. Conversely, both the *cbc1/cbc2* and *pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2* samples showed overall trends of low stomatal conductance levels that did not indicate acclimation to fluctuations in carbon dioxide levels.
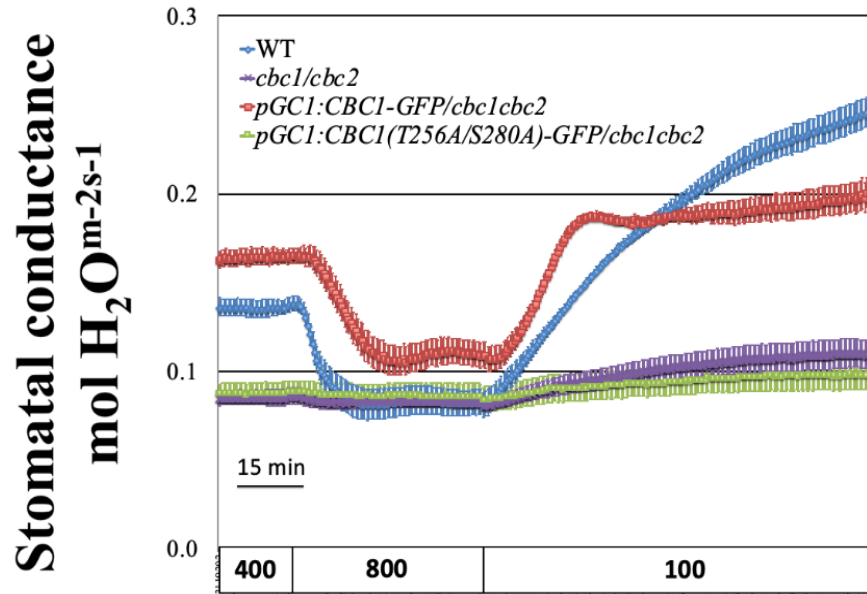
Figure 5. Stomatal conductance comparisons of WT, *cbc1/cbc2*, *pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2* **and** *pGC1:CBC1-GFP/cbc1cbc2*. The stomatal conductance of samples of *Arabidopsis thaliana* were measured at 400 ppm of CO_2 for 30 minutes, 800 ppm of CO_2 for 45 minutes, and 150 ppm of CO_2 for 90 minutes with a LiCOR 6400-XT portable photosynthesis system. The average value is represented for genotypes where more than one sample was tested. WT (n=4) and *mpk12* (n=4) lines were used as controls and compared to the *pGC1:CBC1-GFP/cbc1cbc2* (n=4) and *pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2* (n=4) mutant lines.

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1.5 Whole leaf gas exchange experiments of pGC1:MPK12-GFP/mpk12 and

   pGC1:MPK12(K70R)-GFP/mpk12 mutant lines.

The goal for this set of experiments was to determine whether kinase activity was

required for MPK12 function in the carbon dioxide signaling pathway by exposing different

genotype samples to changing atmospheric carbon dioxide concentrations and observing their

stomatal responses. The results for the WT control samples showed stomatal closure in high

carbon dioxide concentrations and more open stomata under low carbon dioxide concentrations

as expected (Fig. 6). The *mpk12* control samples do not have a functioning MPK12 protein and

showed an elevated stomatal resting state compared to WT (~0.24 vs. ~0.13 mol H_2O m^2s^{-1}) and

remained elevated during the shifts in atmospheric CO_2 levels which is consistent with other

academic literature (Fig. 6). The *pGC1:MPK12-GFP/mpk12* samples are *mpk12* plants that have

a WT MPK12 allele and so should exhibit a rescue phenotype that looks similar to the WT

results. In this experiment, these plants had lower levels of stomatal conductance when exposed

to higher levels of CO_2 and higher stomatal conductance when CO_2 levels decreased, an overall

response that was similar to our WT controls (Fig. 6). The *pGC1:MPK12(K70R)-GFP/mpk12*

plants were *mpk12* plants that were transformed with an MPK12 protein that has a K70R amino

acid mutation that prevents kinase activity since the ATP-binding site was mutated. The plant

samples that expressed kinase inactive MPK12, showed stomatal responses that were similar to

both WT and *pGC1:MPK12-GFP/mpk12* as the stomatal conductance increased with low levels

of CO_2 and decreased in response to higher CO_2 concentrations (Fig. 6). The data from this

experiment showed that the samples from the *pGC1:MPK12-GFP/mpk12* and

pGC1:MPK12(K70R)-GFP/mpk12 mutant lines had similar responses to the WT samples in

relation to their basal stomatal conductance levels and stomatal responses to changes in

atmospheric carbon dioxide levels.
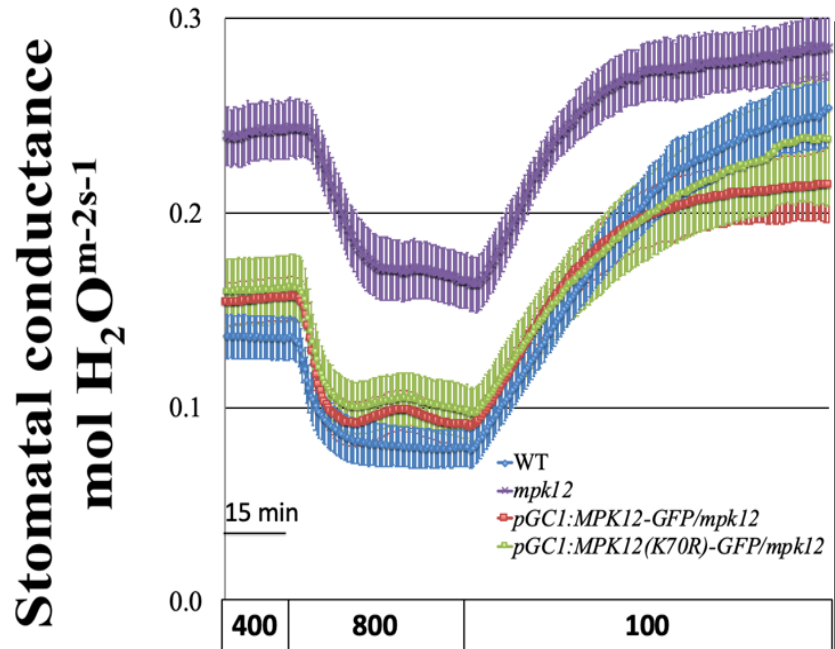
Figure 6. Stomatal conductance comparisons of WT, *mpk12*, *pGC1:MPK12-GFP/mpk12* and *pGC1:MPK12(K70R)-GFP/mpk12*. The stomatal conductance of samples of *Arabidopsis thaliana* were measured at 400 ppm of CO_2 for 30 minutes, 800 ppm of CO_2 for 45 minutes, and 150 ppm of CO_2 for 90 minutes with a LiCOR 6400-XT portable photosynthesis system. The average value is represented for genotypes, where more than one sample was tested. WT (n=6) and *mpk12* (n=5) lines were used as controls and compared to the *pGC1:MPK12-GFP/mpk12* (n=6) and *pGC1:MPK12(K70R)-GFP/mpk12* (n=6) mutant lines.

DISCUSSION

cbc1/cbc2/ht1(A109V)

Previous research has shown that in *Arabidopsis thaliana* and in other C3 and C4 land plants, fluctuations in atmospheric levels of carbon dioxide affect the stomatal conductance levels due to changes in the width of the stomatal aperture. Recent research has suggested that the early CO_2 signaling pathway is not directly mediated by the signaling pathway triggered by the plant stress hormone abscisic acid (Hsu et al., 2018). Additionally, HT1, a protein kinase present within the carbon dioxide signaling pathway was shown to have the ability to phosphorylate both wildtype and kinase dead CBC1/CBC2 proteins *in vitro* (Hiyama et al., 2017). However, the effect or *in vivo* relevance of this CBC1 phosphorylation has remained unknown. Separate *in vitro* experiments indicated that phosphorylation of CBC1 by HT1 was inhibited by MAP kinase proteins MPK4 and MPK12 when bicarbonate was present (Y. Takahashi, Unpublished). Taking these results into account, a hypothesis that CBC1/CBC2 are epistatic to HT1 in the carbon dioxide signaling pathway was developed and one goal of this study was to test this theory *in vivo* using genetic mutants.

The goal for this set of experiments was to determine if the CBC1/CBC2 proteins operated in the carbon dioxide-induced signal transduction pathway, specifically downstream of HT1, by comparing the stomatal conductance phenotypes of mutants that had different activity levels of HT1, CBC1, and CBC2. Current hypotheses for models of this pathway are based on previous yeast two-hybrid and phosphorylation assay experiments that suggested that the proteins MPK4/MPK12 are able to interact and inhibit HT1 activity (Jakobson et al., 2016). The genetically dominant HT1 point mutation A109V was chosen because previous experiments

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have shown that this mutation allows for stomata to remain open and stomatal conductance levels are elevated (Hõrak et al., 2016) (; Y. Takahashi et al., unpublished).

Our experimental data showed that despite the *cbc1/cbc2/ht1(A109V)* triple mutant being exposed to varying levels of atmospheric carbon dioxide, the stomatal conductance remained low and had responses similar to the *cbc1/cbc2* samples (Fig. 2). In contrast, the *ht1(A109V)* samples retained elevated stomatal conductance levels when compared to Col-0 (WT control) samples and showed minimal fluctuations despite the changes in carbon dioxide levels (Fig. 2). The general trend for Col-0 control samples in similar experiments is that stomatal apertures close and stomatal conductance levels decrease when exposed to elevated carbon dioxide levels while in environments with low levels of carbon dioxide, the stomatal apertures will open and accommodate an increase in stomatal conductance levels (Hsu et al., 2018). The Col-0 samples used in these experiments showed results consistent with these trends (Fig. 2).

Although the triple mutant contained the A109V point mutation in its HT1 gene which would render HT1 unable to be inhibited by MPK4/MPK12 (Y. Takahashi et al., unpublished), the *cbc1/cbc2/ht1(A109V)* triple mutant did not show a similar response to the *ht1(109V)* single mutant (Fig. 2.). Our working hypothesis was the CBC1/CBC2 kinases act downstream of HT1 and that HT1 phosphorylates CBC1/CBC2 which contributes to keeping stomatal apertures open. Seeing as the A109V point mutation in the HT1 protein inhibits MPK4/MPK12's ability to deactivate HT1 (Y. Takahashi et al., unpublished), we would expect that samples with this mutation would exhibit elevated stomatal conductance levels (Hõrak et al., 2016). The *cbc1/cbc2/ht1(A109V)* triple mutant line has a perpetually active HT1 protein and knocked out CBC1 and CBC2 genes. Whether the samples of this mutant line will exhibit stomatal response phenotypes similar to the *ht1(A109V)* samples would be dependent on whether CBC1/CBC2 is

epistatic to HT1 in the signal transduction pathway. If changes in the width of the stomatal

aperture are not dependent on phosphorylation/dephosphorylation of CBC1/CBC2 by HT1 then

we would expect that the *cbc1/cbc2/ht1(A109V)* triple mutant would have a similar open

stomatal phenotype to the *ht1(A109V)* mutant. This is because in theory, there would be no

mutated proteins downstream of the constantly active *ht1(A109V)* protein that would trigger the

closure of the stomata. However, the triple mutant samples had similar stomatal conductance

responses to changes in carbon dioxide levels when compared to the *cbc1/cbc2* mutants (Fig. 2.).

This suggests that CBC1/CBC2 are present in the carbon dioxide signal transduction pathway

and function downstream of HT1. For future experiments, I would like to take infrared thermal

images of the four genotypes used in these experiments to determine whether there is a

difference in leaf temperature due to the varying stomatal responses of each genotype. I think it

would be helpful to investigate whether the activity levels of the CBC1/CBC2 proteins

significantly change a plant's leaf temperature seeing as having more narrow stomata results in

higher leaf temperatures while more open stomata result in lower leaf temperatures (Brown and

Escombe, 1905; Huber, 1935; Raschke 1960; Jackson 1982, as cited by Jones, 2004).

It is possible that there are additional undiscovered protein(s) involved in this carbon

dioxide-induced signal transduction pathway. These potential protein(s) could be located

downstream of HT1 but upstream of CBC1/CBC2. It is also conceivable that these two proteins

do not interact directly with each other *in vivo*. Our observed results would not change if this is

the case because the phenotypes in this experiment were dependent solely on the existence of

CBC1/CBC2 in the pathway, not on if CBC1/CBC2 is interacting directly with HT1. Seeing that

recent research has shown *in vitro* protein interactions between HT1 and CBC1 there will need to

be future experiments to confirm whether HT1 and CBC1/CBC2 directly interact *in vivo* or if

there are other possible protein(s) involved (Y. Takahashi et al., unpublished). Some approaches that could be used to observe such protein interactions *in vivo* are fluorescence resonance energy transfer (FRET) microscopy or bimolecular fluorescence complementation (BiFC) as both procedures emit defined fluorescent signals when the involved proteins have close interactions.

MPK12 GFP Vectors

A known component in the beginning of the carbon dioxide signaling pathway are the mitogen-activated protein kinases (MAPK), in *A. thaliana* there is MPK4 and MPK12. This was suggested by a set of experiments that used mutants where a guard cell specific promotor that suppresses MPK4 was transformed into background *mpk12* plants to form a double mutant line (Tõldsepp et al., 2018). Using samples from two independent *mpk12 mpk4GC* lines this study observed normal stomatal responses when exposed to the plant stress hormone abscisic acid (ABA) but elevated stomatal conductance levels that did not fluctuate despite the atmospheric levels of carbon dioxide changing (Tõldsepp et al., 2018). A separate study also found that MPK4 and MPK12 have the ability to inhibit HT1 phosphorylation activity *in vitro* (Hõrak et al., 2016). A separate study found that an MPK12 protein with a K70R point mutation designed to remove the protein's kinase activity retained its ability to inhibit both HT1's ability to autophosphorylate and to phosphorylate other proteins, in that case, casein (Jakobson et al., 2016). Finally, recent *in vitro* phosphorylation assays with MPK12-K70R, CBC1, and HT1 showed consistent results where CBC1 phosphorylation by HT1 still occurred, but this phosphorylation is inhibited when bicarbonate is added suggesting that the presence of bicarbonate indirectly inhibits CBC1 phosphorylation (Y. Takahashi et al., unpublished). It's possible the presence of the bicarbonate affects the binding activity of MPK4/MPK12 to HT1

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and therefore prevents HT1 phosphorylation of CBC1 as *in vitro* pull-down assays showed that in the presence of bicarbonate, HT1 and MPK4 bind together more strongly (Y. Takahashi et al., unpublished). However, other experiments have shown that the protein kinase activity of MPK4 acts independently from MPK12 and was not directly affected *in vitro* by the presence of bicarbonate. This suggests that there could be an additional component upstream of the MPK proteins (Tõldsepp et al., 2018).

The goal for this set of experiments was to determine if kinase activity of MPK12 was required for MPK12 function *in vivo* by constructing genetic mutants and comparing their stomatal conductance responses. For this purpose, a vector containing the K70R point mutation was chosen when constructing the *Agrobacteria* vector that would be used in conjunction with a guard cell specific promoter (*pGC1)* and a green fluorescent protein (*GFP*) tag (Fig. 3a-b). These vectors were transformed into samples of a T-DNA *mpk12* line that has the MPK12 gene knocked out to ensure that if there were MPK12 proteins functioning in these transformed samples, they would be the result of a successful insertion by the vector (Fig. 3a-b). Samples used in stomatal conductance experiments were selected based on whether strong GFP fluorescence was visible within the guard cells of stomata indicating that the vector with the desired gene of interest, was present in the genome of that sample (Fig. 4). Samples from three independent *pGC1:MPK12(K70R)-GFP/mpk12* mutant lines and four independent *pGC1:MPK12-GFP/mpk12* were used for the stomatal conductance experiments in plants with intact leaves attached to live plants. The results were compared to responses observed in samples of Col-0 (WT) controls and the single *mpk12* T-DNA insertion mutant (Fig. 6).

The Col-0 control samples used in these experiments were consistent with general WT trends where we observed lower stomatal conductance values as the carbon dioxide

concentration increased followed by higher levels of stomatal conduction when the carbon

dioxide concentration decreased (Fig. 6) (Hsu et al., 2018). The *mpk12* samples showed an

elevated level of steady state stomatal conductance when compared to Col-0 (~0.242 mol H_2O m^{-}

^{2}s^{-1} vs ~0.135 mol H_2O m^{2}s^{-1}) and while these samples did show stomatal responses that would

increase and decrease in response to the fluctuating carbon dioxide levels, the samples

maintained an overall elevated stomatal conductance (Fig. 6). These data are consistent with

previous experiments that showed similar results in three lines that had different mutations

within the MPK12 gene that affected the MPK12 protein's ability to function (Jakobson et al.,

2016). Note that *mpk12/mpk4* double mutants are needed to more strongly disrupt the CO_2

response (Tõldsepp et al., 2018). However, this double mutant already uses the pGC1 promoter

to silence *MPK4* in guard cells (Tõldsepp et al., 2018), making this promoter less suitable for our

MPK12 complementation experiments.

   The *pGC1:MPK12-GFP/mpk12* mutant line was constructed for the purpose of acting

similar to a control as this control vector contains a wildtype MPK12 cDNA so that if

transformation was successful, the samples would show a rescue phenotype when used in the

stomatal conductance analysis experiments. These MPK12 rescue samples should show similar

stomatal responses to the Col-0 controls since the *mpk12* background plant would have received

a working MPK12 gene from the *Agrobacterium* vector (Fig. 3b). The samples from four

independent *pGC1:MPK12-GFP/mpk12* lines had similar steady state stomatal conductance

levels and responses to changes in the concentration carbon dioxide when compared to Col-0,

exhibiting a rescue phenotype (Fig. 6). The *pGC1:MPK12(K70R)-GFP/mpk12* samples whose

MPK12 proteins contained the point mutation that would remove MPK12's kinase activity had

similar steady state stomatal conductances and stomatal responses to fluctuations in carbon

dioxide to the Col-0 control samples (Fig. 6). These results suggest that the ability of MPK12 to inhibit HT1 activity was not impeded by the loss of its kinase activity.

It is possible that there is(are) some kind of additional element(s) operating upstream of the MPK4/MPK12 proteins as the protein kinase activities of MPK12 and MPK4 were not directly affected by the presence of bicarbonate *in vitro* (Tõldsepp et al., 2018). While the presence of bicarbonate does stabilize the binding between MPK4 and HT1 (Y. Takahashi et al., unpublished) it is unknown whether we would see similar results *in vivo*. Procedures like BiFC and FRET microscopy would be viable options as these procedures can be used to observe *in vivo* protein interactions. Steps to see if there are additional components operating upstream of MPK4/12 that are currently unknown would take some time but could be done by performing a forward genetic screen.

CBC1 GFP Vectors

In recent years, there has been the identification of a new component in the carbon dioxide signal transduction pathway that functions upstream of the slow anion channel-associated 1 (SLAC1) protein responsible for fluctuations in ion concentration within the guard cells of stomata by affecting the turgor pressure and overall aperture width. These two new kinase proteins were named convergence of blue light and CO_2 (CBC) 1 and 2 (Hiyama et al., 2017). Single mutant lines and a double mutant line were developed to determine how these proteins contributed to responses to changes in carbon dioxide concentrations. The *cbc1* and *cbc2* lines showed only weak stomatal opening responses as the carbon dioxide levels were lowered experimentally, but the samples were stunted and only reached ~0.6 mol H_2O $m^{-2}s^{-1}$ compared to the Col-0 wildtype control samples that reached as high as ~0.11 mol H_2O $m^{-2}s^{-1}$

(Hiyama et al., 2017). The *cbc1/cbc2* line showed no noticeable fluctuations in its stomatal

conductance levels and remained around ~0.3 mol H_2O m^{-2}s^{-1} for the entirety of the experiment

despite the carbon dioxide levels decreasing multiple times before returning to ambient levels

(Hiyama et al., 2017). These results might be explained by a model in which CBC1 and CBC2

were present in the carbon dioxide signaling pathway and this same study also found that

CBC1/CBC2 had autophosphorylation activity and that HT1 had the ability to phosphorylate

kinase-dead CBC1 and CBC2 *in vitro* but that CBC1/CBC2 could not phosphorylate HT1

(Hiyama et al., 2017). The effect and relevance of HT1-mediated phosphorylation of CBCs

remained however unknown.

The goal of the experiments was to identify potential HT1-mediated phosphorylation

sites in CBC1 that might be important in the carbon dioxide signaling pathway *in vivo* by

comparing the stomatal response in specially designed mutants to those of Col-0 and *cbc1/cbc2*.

To determine a list of potential phosphorylation site targets for HT1 on the CBC1 protein that we

could target, data from a mass spectrometry experiment performed on *in vitro* phosphorylation

assays of wildtype and mutant inactive CBC1 protein with and without the presence of HT1 was

used as a reference (Y. Takahashi et al., unpublished). The mass spectrometry experiments

identified eight possible phosphorylation sites across the CBC1 protein and by comparing the

results to the data collected from the kinase inactive CBC1 protein experiment, the amino acid

sites of Thr-256 and Ser-280 were chosen as mutation targets as both are located near the

activation loop of CBC 1 (Y. Takahashi et al., unpublished). There were two *Agrobacterium*

vector lines constructed for this set of experiments, the first contained a wildtype copy of the

CBC1 gene while the other contained both of the previously mentioned CBC1 point mutations

(Fig. 3a). Both of these CBC1 gene constructs were placed into a vector with a guard cell

preferential *pGC1* promotor (Yang et al., 2008) to ensure expression in guard cells and a *GFP* protein at the C-terminus to ensure that there would be an observable tag that could be used for screening samples (Fig. 3a). These vectors, with their respective genes of interest, were transformed into *cbc1/cbc2* double mutant plants and samples were selected for stomatal response experiments only if the samples exhibited strong *GFP* fluorescence within the guard cells of the stomata outside of the general autofluorescence observed in the stomata of Col-0 which so as to indicate the presence of our *GFP* vector within the genome of the candidate sample (Fig. 4a-b). The presence of these vectors indicated that these plants, whose parent had no functional CBC1 gene, now contained either a wildtype or double mutant CBC1 gene that was inserted into the genome by their respective *Agrobacterium* vectors.

The two controls used in these experiments were Col-0 (WT) and *cbc1/cbc2*, the Col-0 samples had the expected responses of a decreasing stomatal conductance when carbon dioxide levels went from 400ppm to 800ppm followed by increased stomatal conductance when the carbon dioxide concentration shifted from 800ppm to 100ppm (Fig. 5). Consistent with previous research, the samples from the *cbc1/cbc2* line showed low stomatal conductance levels around 0.1 mol H_2O $m^{-2}s^{-1}$ that did not fluctuate substantially when carbon dioxide levels rose while stomatal conductance levels rose slightly when the carbon dioxide levels became low (Fig. 5) (Hiyama et al., 2017).

The *pGC1:CBC1-GFP/cbc1cbc2* lines contain a wildtype copy of the CBC1 gene and if the transformation was successful, we would expect to observe some kind of rescue phenotype that would restore the sample's ability to respond to fluctuations in carbon dioxide levels. The samples of the *pGC1:CBC1-GFP/cbc1cbc2* line that were used showed a higher steady stomatal conductance when compared to Col-0 (~0.16 mol H_2O $m^{-2}s^{-1}$ vs ~0.13 mol H_2O $m^{-2}s^{-1}$) and also

displayed more narrow stomata in high carbon dioxide and stomata opening in low carbon dioxide environment although once the conductance levels reached ~0.195 H_2O m^2s^{-1}, the conductance plateaued while the stomatal conductance of Col-0 samples continued to increase as the carbon dioxide levels remained at 100ppm (Fig. 5). The *pGC1:CBC1(T256A/S280A)-GFP/cbc1cbc2* lines contained a CBC1 gene with the two point mutations T256A and S280A that were selected based on the previously mentioned mass spectrometry data. These samples had a steady stomatal conductance comparable to the *cbc1/cbc2* mutants (~0.87 mol H_2O m^2s^{-1} vs ~0.84 mol H_2O m^2s^{-1}) and showed similar stomatal responses with the only change being a slight increase after the carbon dioxide levels decreased to 100ppm (Fig 5.) These results suggest that these two possible HT1-specfic phosphorylation sites could be crucial for CBC1 activation by HT1 seeing as the mutation of these sites resulted in a phenotype similar to plants that contained no functioning CBC1 protein.

While previous experiments support the hypothesis that HT1 and CBC1 interact, it remains unconfirmed whether HT1 and CBC1 interact via direct binding interactions *in vitro* or *in vivo*. For future experiments, would be interesting to use the information gathered in these experiments to test if there are direct interactions between HT1 and CBC1. One possible approach to determine this could include pull-down assays using wildtype CBC1 and *cbc1(T356A/S280A)* to confirm first *in vitro* that there are physical interactions between these two proteins. Follow up experiments could involve using procedures such as FRET microscopy or BiFC to determine if there are direct interactions *in vivo*. Learning as much information as possible about the interactions and phosphorylation sites of proteins present in the signal transduction pathway for carbon dioxide signaling is crucial for the future of the food supply of the world. The information identified from these studies will be useful in determining potential

target genes and specific amino acid mutations that can be of use in the development of genetically modified agricultural products that will be less vulnerable to the changes in the atmospheric levels of carbon dioxide caused by climate change.

Material in this thesis has been submitted for publication of the material as it may appear in Science Advances, 2022, Bosmans, Krystal; Yohei Takahashi., Po-Kai Hsu, Karnelia Paul, Chung-Yueh Yeh, Yuh-Shuh Wang, Dmitry Yarmolinsky, Maija Sierla, Triin Vahisalu, Jaakko Kangasjarvi, Hannes Kollist, Li Zhang, Thien Trac, and Julian I. Schroeder. The thesis author was the primary investigator and author of this paper.

MATERIAL AND METHODS

1.1 Plant growth conditions.

    Arabidopsis thaliana seeds were put into a microcentrifuge tube and a solution of $800\mu L$ milli-q water, $200\mu L$ bleach, and $5\mu L$ of 20% Triton X-100 was made. The tube was shaken until bubbles were visible and left to sit for 15 minutes. The tube was put in a mini centrifuge for ~30 seconds and the supernatant was removed. 1mL of milli-q water was added to the tube to act as a wash and the contents were shaken before being centrifuged again for ~30 seconds followed by removing the supernatant. This wash step was done twice. In a hood under sterile conditions, ~$100\mu L$ of 0.05% agar was added to the tube to suspend the seeds. Individual seeds were then placed on a ½ Murashige and Skooge (Sigma) plate that had a pH of 5.7/5.8. The plate was then placed in a $4^{o}C$ environment for 3 days before being placed into a growth room with 16 hours of day and 8 hours of night conditions, if the samples were grown for use in gas exchange experiments then the plates were placed into a plant growth chamber (AR-41L2; Percival Scientific, Perry, Iowa, USA). The settings on the growth chamber were 12 hours of light and 12 hours of dark with ~111 μmol m^{-2} s^{-1} light, CO_2 levels ~600 p.p.m., 55-65% relative humidity, and a temperature of 21°C. After 7-10 days, germinated seedlings were transferred to individual small pots with sterilized soil for continued growth.

1.2 Hygromycin Selection Assays.

Approximately 80mg of seeds (1,000-1,500 seeds) were put into a microcentrifuge tube along with 1mL of 70% ethanol and 5μL of 10% SDS. The tube was shaken to allow for mixing and the contents were left to incubate for 20 minutes. The contents were spun down for ~30 seconds using a minicentrifuge and the supernatant was removed. The contents were washed using 1mL of 100% ethanol, spun down, and the supernatant removed. This ethanol washing step was done twice. The tube was then placed in a sterilized environment inside a hood for 60-90 minutes to allow the seeds to dry out. The seeds were then spread onto selection plates, the plates used were ½ Murashige and Skooge (Sigma) with 100 μg/mL hygromycin. The plates were wrapped in aluminum foil and left in a 4ºC environment for 3 days before being moved into the growth room with growing conditions of 16 hours of day and 8 hours of night. After 7-10 days seedlings that had germinated were transferred into individual small pots filled with sterilized soil to allow for further growth.

1.3 Genome extraction from plants.

When samples were 2-3 weeks old, one or two small leaves were cut from the parent plant and placed into a labeled microfuge tube. $50\mu L$ of an autoclaved DNA extraction buffer made of 100mL of 1M Tris-HCl (pH 7.5), 25mL of 5M NaCl, 25mL of 0.5M EDTA Disodium (pH 8.0), 25mL of 10% SDS, and 325mL of double distilled water (ddH_2O) was added to the microfuge tube and a disposable pestle was used to lightly grind the leaves. An additional $150\mu L$ of the DNA extraction buffer was added to the tube and the leaves were grinded even further using the pestle until there were no visible pieces left. The pestle was sterilized with 100% ethanol before being used to grind each sample. The tube was centrifuged at 14,500 rotations per minute (RPM) for five minutes, during this time $150\mu L$ of 100% 2-propanol was added to a new microcentrifuge tube. When the sample was ready, $150\mu L$ of the grinded sample was added to the tube with the 2-propanol and homogenized. Samples were placed in a -20 ^{o}C freezer for ~15 minutes then centrifuged at 4°C with a speed of 12,000 RPM for 15 minutes. Upon removal from the centrifuge, all of the supernatant was removed and $50\mu L$ of milli-q water was added to the tube with the genomic DNA and placed on a Vortex Genie 2 (Fisher) for a couple of seconds to ensure that the water reached all areas where genomic DNA material could be concentrated. Samples were then stored in a -20 ^{o}C freezer until they were needed.

1.4 Crossing Arabidopsis thaliana samples.

Plants were placed in medium sized pots with four seedlings in each one and grown in an open growth room that was kept at 70 °F with a light cycle of 16 hours of day and 8 hours of night until samples were flowering. Samples used for crossing included *cbc1/cbc2* and *ht1(A109V)* and all parent plants were confirmed via PCR to be the correct genotype before being used in crossing. To start, a flower bud on one parent plant that had not bloomed yet would be taken apart using tweezers until only the pistil remained. Then, on a parent plant of the other genotype, a blooming flower would have all parts removed until the stamen with the pollen could be removed. Several stamens would then be tapped onto the pistil from the first parent plant so that the pollen could enter the pistil and fertilize the plant. After each fertilization event was done, a small colored thread was tied around the branch so that artificially fertilized pistils could be easily located later. This process would be repeated until every parent plant had 5-7 fertilization sites and were then returned to the growth room. After ~2 weeks of continued growth, successful fertilization attempts will have produced seeds that were harvested and used a starting point for the isolation of higher order mutants in the next generation.

1.5 E. coli transformation.

To begin, competent *E. coli* cells were removed from a -80°C freezer and put on ice for two minutes. After these two minutes the reaction product containing the genomic product intended to be inserted into the *E. coli* was added to the cells and continued to be incubated on ice for an additional 30 minutes. During these 30 minutes, 500μL of lysogeny broth (LB) medium was placed in a microfuge tube and incubated at 37°C. After the 30 minutes of incubation, the microfuge tube with the cells and product were incubated at 42°C for 50 seconds before the 500μL of 37°C LB medium was added. This mixture was then placed on a shaker inside a room heated to 37°C for one hour. The cells were then taken to a hood so that they could be spread over an LB plate with 100μg/mL of whatever antibiotic the plasmid had resistance for in a sterile environment. Once the LB medium containing the *E. coli* cells had dried on the plate, it was taken back to the 37°C room overnight before the plate was checked for any colony growth the following morning.

1.6 Cloning of MPK12 and CBC vectors.

To mutate an MPK12 gene to contain the *K70R* point mutation, mutagenesis was performed. A reaction of 6.6μL of milli-q water, 1μL of 10x pfu Turbo buffer (Agilent), 1μL of 2.5mM dNTPs, 0.5μL of an *mpk12* plasmid, 0.3μL of MPK12_K70R_Fw primer, 0.3μL of MPK12_K70R_Rv primer, and 0.3μL of PfuTurbo DNA Polymerase (Agilent) was made (Table 3). After the solution was made, 0.5μL of the restriction enzyme DpnI (New England BioLabs) was added to the reaction and incubated at 37$^\mathbf{O}$C for 90 minutes. After incubation, the reaction product was used in the transformation of the JM109 *E. coli* strain and plated on a lysogeny broth (LB) plate that contained 100μg/mL of Kanamycin. The plate was left overnight at 37$^\mathbf{O}$C and colonies were purified using a GeneJET Plasmid Miniprep Kit (Thermo Scientific). Plasmids that contained the WT and mutated genes of interest (GOI) were amplified using forward and reverse primers specific to the genes of interest and their intended use in USER cloning (Table 3). Each reaction contained 28μL of milli-q water, 10μL of 5x Phusion HF Buffer (Thermo Scientific), 5μL of 2.5mM dNTPs, 3μL of 10μM forward primer, 3μL of 10μM reverse primer, 0.5μL of Phusion DNA polymerase (Thermo Scientific) and 0.5μL of the plasmid acting as the genetic material source. 10μL of 6x loading dye (2μL of loading dye for every 10μL of reaction) was added to the reaction after the PCR program was complete and 10μL of the dyed reaction was placed in a 1% Agarose A (Bio Pioneer) gel and run at ~90mV for 10-15 minutes for gel electrophoresis. If the length of the band looked appropriate, the rest of the reaction was purified using a NucleoSpin Gel and PCR Clean-Up Kit (Macherey-Nagel) and DNA concentrations and quality measured with a Nandodrop 1000 Spectrophotometer (Thermo Scientific) before use in USER cloning (Table 4). The USER cloning reactions contained 7.5μL of 200131 35S:USER-

GFP PaCI, 0.5μL of the purified fragment of the GOI, 1μL of 10x CutSmart Buffer (New

England BioLabs), and 1μL of USER Enzyme (New England BioLabs). Reactions were

incubated at 37°C for 25 minutes then 25°C for 25 minutes then transformed into competent

cells from the *E. coli* strain JM109 and plated onto a lysogeny broth (LB) plate with 100μg/mL

of Carbenicillin. After being left in 37°C overnight, colonies were purified using the GeneJET

Plasmid Miniprep Kit (Thermo Scientific) and submitted to Retrogen for sequencing to ensure

the GOIs had the correct sequences before being amplified (Table 3). The same 50μL

amplification reaction mixture was used but with a forward primer specific to the GOI and

pGC1, a reverse primer specific to Nos, and newly purified plasmids as the source of genetic

material. Gel electrophoresis, DNA purification, and nanodrop analysis were all performed using

the same steps previously mentioned for amplification before the purified GOIs were used in

SLiCE cloning. For SLiCE cloning, the reactions contained 4μL of 170523 pGC1:Nos PC1300

Bam, 4μL of the purified GOI-GFP fragment, 1μL of 10x SLiCE buffer prepared as previously

described (Motohashi, 2015), and 1μL of SLiCE enzyme prepared as described previously

(Motohashi, 2015). These reactions were incubated for 30 minutes at 37°C before being

transformed into the competent *E. coli* cell strain JM109 and plated onto an LB plate with

100μg/mL of Kanamycin. Colonies that formed after the plate was incubated at 37°C overnight

were purified using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) and sequenced by

Retrogen. Once the genetic sequencing was confirmed to be correct these plasmids were used for

the transformation of *Agrobacterium tumefaciens* competent cells.

1.7 Agrobacterium tumefaciens transformation.

The vectors transformed into *A. tumefaciens* cells contained a guard cell specific promoter (*pGC1*) on the 5' end with a GFP tag placed at the 3' end. To start, competent cells were removed from a -80°C freezer and left in room temperature for 3 minutes to thaw. 5μl of the *E. coli* plasmid with the GOI was added to the competent cells before the microfuge tube containing the sample was thrown into liquid nitrogen to flash freeze it. The sample was removed and incubated at 37°C for ten minutes then 400μl of lysogeny broth (LB) medium was added before placing the tube on a shaker inside a 28°C room for two hours. After two hours the sample was plated onto an LB plate with 50μg/mL Gentamicin and 100μg/mL of Kanamycin and placed back into the 28°C room for two days before being checked for any colony growth. Colonies were selected for use in transforming *A. thaliana* background plants via floral dipping.

1.8 Arabidopsis thaliana transformation with Agrobacterium vectors via floral dipping.

The first step to this three-day process was to make a starting culture made of 3mL of autoclaved lysogeny broth (LB) medium with 3μl of 50μg/mL Gentamicin and 3μl of 100μg/mL Kanamycin within a 10 mL glass test tube in a sterile environment. A colony was taken from a plate and place inside the test tube, a cap was added, and the test tube was placed on a shaker within a room heated to 28°C overnight. The next day, this culture was upscaled. A 500mL glass container is filled with 200mL of LB medium was autoclaved and once it cooled, 200μl of 50μg/mL Gentamicin and 200μl of 100μg/mL Kanamycin was added so anything that was not the bacteria with the resistance genes could not grow. From the previous day's culture, 1mL was added into the 200mL of LB medium and the container was placed back on the shaker within the 28°C room overnight. On the third day, the 200mL solution was centrifuged at 4000 rotations per minute (RPM) for 10 minutes at room temperature. The supernatant was discarded and 200 mL of an autoclaved solution of ½ Murashige and Skooge (Sigma) with 5% sucrose was combined with the centrifuged cells and mixed until the mixture was homogenized. 40μl of Bioworld SILWET L-77 (Fisher Scientific) was added to the solution then poured into a container. Before the dipping began, all plants (~4 weeks old) had all fertilized flowers/seed containers removed with scissors so only unopened buds and unpollinated flowers remained. These samples were then dipped into the solution for 50 seconds, removed for 30 seconds, and dipped again for 50 seconds before being placed on their sides inside a try. Once all the samples had been dipped and placed in the tray, the tray is wrapped in aluminum foil and placed in the growth room to incubate for two days. Once the incubation period was over, the foil was removed, samples placed right side up, and water was added to the tray so regular growth conditions were continued until the plants were old enough to have their seeds harvested for selection assays.

1.9 Confocal microscopy imaging.

When GFP vector-expressing candidate plant samples were 2-3 weeks old, a small flat piece of a leaf was removed with scissors. This slice was then placed upside down onto a droplet of 30% glycerol on 25 x 75 x 1mm Premium Clipped Corner Frosted Microscope Slides (Fisher Scientific) and covered with a 12-545-102 Microscope Cover Glass (Fisherbrand). This sample was examined at x100 using a spinning-disk confocal-microscope that was set up as previously described (Paredez et al., 2006) using both brightfield and a GFP filter in conjunction with a Model HB-10103AF mercury lamp (Nikon) to see if there was any strong fluorescence within the guard cells independent from general autofluorescence levels observed in stomata. Images were taken with the Version 7.7.7.0 MetaMorph program with exposure set on 4 second intervals and image processing to isolate the green color was done with ImageJ version 1.53f51.

1.10 Li-COR gas exchange experiments.

Plants grown specifically for use in stomatal conductance experiments were grown in individual pots with sterilized potting soil and grown in a plant growth chamber (AR-41L2; Percival Scientific, Perry, Iowa, USA). The growth chamber settings were set to 12 hours of light and 12 hours of dark with ~111 μmol m^{-2} s^{-1} light, CO_2 levels ~600 p.p.m., 55-65% relative humidity, and a temperature of 21°C. Analyses were done using a portable gas-exchange system (LI-6400 or LI-6400XT; LI-COR, Lincoln, Nebraska, USA) each with their own LED light source that was set to 150 μmol m^{-2} sec^{-1}. The relative humidity for all experiments was monitored so that the value remained between 60-70%, the air flow was set to 400 μmol sec^{-1}, and the leaf temperature within the chamber was set to 21°C. Before each stomatal response experiment began, each leaf sample was given time to acclimate to the environment within the chamber where CO_2 levels were set to ~400 p.p.m. until the stomatal conductance was stable, this usually took about one hour. Then, after the program began, the sample's stomatal conductance was recorded every 30 seconds until the program was complete. Both programs used began with carbon dioxide levels of 400 p.p.m. to simulate the approximate current ambient levels of the atmosphere, followed by 800 p.p.m. for high carbon dioxide levels, and concluded with 150 p.p.m. for low carbon dioxide levels.

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