Carbonic Anhydrase Mutants in *Zea mays* Have Altered Stomatal Responses to Environmental Signals[1]

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Stomata regulate transpirational water loss and CO_2 uptake for photosynthesis in response to changing environmental conditions. Research investigating stomatal movement has mostly been conducted in C_3 eudicot species, which have very different CO_2 requirements for photosynthesis relative to C_4 grasses. Carbonic anhydrase (CA) catalyzes the hydration of CO_2, and its activity has been linked to stomatal aperture regulation in eudicots. The number of *Ca* genes and their evolutionary history differ between monocots and dicots, and many questions remain unanswered about potential neofunctionalization and subfunctionalization of grass *Ca* paralogs and their roles in photosynthesis and stomatal conductance. To investigate the roles of different *Ca* genes in maize (*Zea mays*), we examined stomatal responses in *ca1* and *ca2* single mutants as well as a *ca1ca2* double mutant. The *ca1* and *ca2* single mutants had 10% and 87% of the CA activity exhibited by the wild type, respectively, while *ca1ca2* had less than 5% of wild-type CA activity. The *ca* mutants had higher stomatal conductance than the wild type and slower stomatal closure in response to increases in CO_2 partial pressure. Contrary to previous reports in eudicots, *ca* mutants showed slowed stomatal closure in response to the light-dark transition and did not show differences in stomatal density compared with the wild type. These results implicate CA-mediated signaling in the control of stomatal movement but not stomatal development. Drought experiments with *ca1ca2* mutant plants suggest a role for CA in water-use efficiency and reveal that *Z. mays* is not optimized for water-use efficiency under well-watered conditions.

In C_4 photosynthesis, the enzyme carbonic anhydrase (CA) hydrates CO_2 to form bicarbonate, which is the first catalyzed step in the C_4 photosynthetic pathway (Badger, 1994). Bicarbonate and phospho*enol*pyruvate are converted into oxaloacetic acid by phospho*enol*pyruvate carboxylase (PEPC), the primary carboxylating enzyme of C_4 plants. This C_4 acid is used in the carbon-concentrating mechanism that allows C_4 species to have reduced stomatal aperture while maintaining carbon flux for photosynthesis (Sage, 2004). The reduced stomatal aperture also decreases transpirational water loss relative to rates of photosynthesis and increases water-use efficiency in C_4 relative to C_3 plants (Ehleringer and Monson, 1993). Because the carbon-concentrating mechanism of C_4 species enables a lower intercellular CO_2 concentration compared with C_3 plants with typically greater photosynthetic efficiency (von Caemmerer, 2000), it is possible that stomata of C_4 plants are not as sensitive to changes in CO_2 conditions. Thus, it is likely that stomatal CO_2-sensing and -signaling mechanisms differ between grasses and other species or between C_3 and C_4 plants.

In addition to its role in C_4 photosynthesis, CA also is involved in several cellular processes, including the generation of bicarbonate as a substrate for acetyl-CoA carboxylase during lipid metabolism, amino acid biosynthesis under low-CO_2 conditions, and stomatal regulation (Hoang and Chapman, 2002; Hu et al., 2010; DiMario et al., 2016). Therefore, a detailed characterization of each isoform of CA may provide evidence of specialization and functional redundancies. In Arabidopsis (*Arabidopsis thaliana*), specific CA isoforms have been identified as components of the CO_2 signaling pathway (Hu et al., 2010). *βca1ca4* double mutant plants have higher stomatal conductance (g_s) compared with wild-type plants (Hu et al., 2010), although neither single mutant displayed a phenotype, suggesting functional redundancies. In addition, although high CO_2 levels trigger stomatal closure in *ca1ca4* double mutant plants, the response is slower compared with the wild type. Despite these defects in the response

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to CO_2, g_s appears normal in response to abscisic acid and light treatments, suggesting that the role of CA is specific to a CO_2 signaling pathway. The CO_2 signaling pathway is particularly relevant because of the vital balance between CO_2 uptake and transpirational water loss. Furthermore, global atmospheric CO_2 increases coupled with changing climate conditions has the potential to perturb adaptive mechanisms needed for optimum growth (Ainsworth and Rogers, 2007). Although CA has been linked to CO_2 signaling in the C_3 eudicot Arabidopsis, it is currently unknown what role CA plays in stomatal regulation in C_4 monocots.

The total CA activity found in leaves varies greatly between C_4 species (Gillon and Yakir, 2000), as does the requirement for CA. In the C_4 eudicot *Flaveria bidentis*, the first-order rate constant of CA (*k*CA) is 73.96 µmol m^{-2} s^{-1} Pa^{-1}, and *ca* mutants with activities of 4.8 µmol m^{-2} s^{-1} Pa^{-1} require high CO_2 concentrations for survival (von Caemmerer et al., 2004). However, in the C_4 monocot maize (*Zea mays*), *ca* mutants have only a minor growth reduction at ambient CO_2 (Studer et al., 2014), despite the low *k*CA (1.3 µmol m^{-2} s^{-1} Pa^{-1}) of mutant plants compared with wild-type plants (*k*CA = 61 µmol m^{-2} s^{-1} Pa^{-1}). In the C_4 grass green foxtail (*Setaria viridis*), *ca* mutant plants with *k*CA = 8 µmol m^{-2} s^{-1} Pa^{-1} showed photosynthetic limitation at low CO_2, similar to what was observed in *Z. mays* (Osborn et al., 2017). Although the *ca* mutants in *Z. mays* lack a pronounced phenotype at ambient CO_2 levels, a metabolomics study in a diversity panel of *Z. mays* identified significant associations between single-nucleotide polymorphisms in genes encoding CA and metabolites related to photosynthesis (Zhang et al., 2015). Thus, while CA does not appear to be rate limiting for photosynthesis in *Z. mays* at current ambient CO_2 levels, the overabundance of CA suggests that some conditions may favor high CA levels.

Here, we describe the functional characterization of the most abundantly expressed CA genes in *Z. mays* leaves, *Ca1* and *Ca2*. The *Ac*/*Ds* transposable elements were used to generate *ca1* single and *ca1ca2* double mutants of these tandemly duplicated genes in *Z. mays*, and initial characterizations of the mutants were described previously (Studer et al., 2014). To fully understand the function of each gene copy, however, we generated and characterized both *ca1* and *ca2* single mutants. A screen was used to identify recombination events that generated *ca1* and *ca2* single mutant alleles, enabling a more refined genetic analysis of CA function. We utilized gas-exchange measurements of single and double *ca* mutant plants to monitor stomatal responses to changes in CO_2 and light conditions. These measurements were then compared with the morphological and biochemical attributes of the leaf. Furthermore, *Z. mays ca1ca2* double mutant plants were grown under several watering regimes to monitor water use and define a potential role for CA in drought responses. Our results show that altering CA function affects water-use efficiency in *Z. mays*.

RESULTS

Generation of *ca2* Single Mutant Plants

The *ca1* and *ca2* single mutant plants used in this study were obtained by screening a segregating population containing the *ca1ca2* alleles described previously (Studer et al., 2014). Three recombinant plants were recovered: one having a frame-shift mutation in *ca1* with a wild-type allele of *Ca2*, and the other two containing the *Ds* insertion in *ca2* without the presence of the frame-shift mutation in *Ca1*. This recombination frequency indicates that the *ca1* and *ca2* genes are located 1.6 cM apart. The newly generated *ca1* footprint mutant allele is an independent line from the two previously characterized *ca1* alleles and was caused by an imprecise *Ds* excision that left an 8-bp insertion and changed the frame of the predicted protein. The newly recovered *ca2* single mutant plants carry the original *Ds* insertions; therefore, they are stable in the absence of *Ac* transposase.

Consistent with previous reports, *ca1* mutants had 10% of the total leaf CA observed in wild-type plants, and the *ca1ca2* mutant plants only had 2% of wild-type CA levels (Fig. 1). The newly generated *ca2* single mutant plants had 87% of wild-type levels of total leaf CA. The measurements of CA levels in each single mutant and the *ca1ca2* double mutant show the additive nature of CA1 and CA2 in the leaf. This result confirms the observation that, while *Ca2* contributes 22.6% to the CA transcript pool in the leaf (Studer et al., 2014), it makes up only 13% of the total CA activity. The A/C_i measurements were consistent with *k*CA and showed that *ca1* single and *ca1ca2* double mutant plants had significant differences in the rates of photosynthesis at low CO_2, but the rates in *ca2* single mutant plants were nearly identical to those in the wild type (Fig. 1). However, only the loss of both CA1 and CA2 activities, but not CA1 or CA2 alone, resulted in a significantly lower carbon isotope signature of the leaf compared with wild-type plants (Fig. 1).

Phenotypic Characterization

The *ca* mutants were further characterized to examine the consequences of a disruption in CA activity on photosynthetic enzyme activities, biomass accumulation, and stomatal density (Table I). While there was a significant difference in CA activity between genotypes ($P < 0.0001$), no differences were observed between genotypes for total leaf PEPC and Rubisco activity ($P = 0.5232$ and 0.6397, respectively). Furthermore, while significant differences in net photosynthetic rate (A) were observed between wild-type and *ca1ca2* mutant plants, there was no significant difference in biomass between genotypes in fresh and dry weight measures ($P = 0.0674$ and 0.1449, respectively). Unlike the *βca1ca4* double mutants in Arabidopsis (Engineer et al., 2014), none of the *ca* mutants in *Z. mays* had altered stomatal density ($P = 0.4261$).

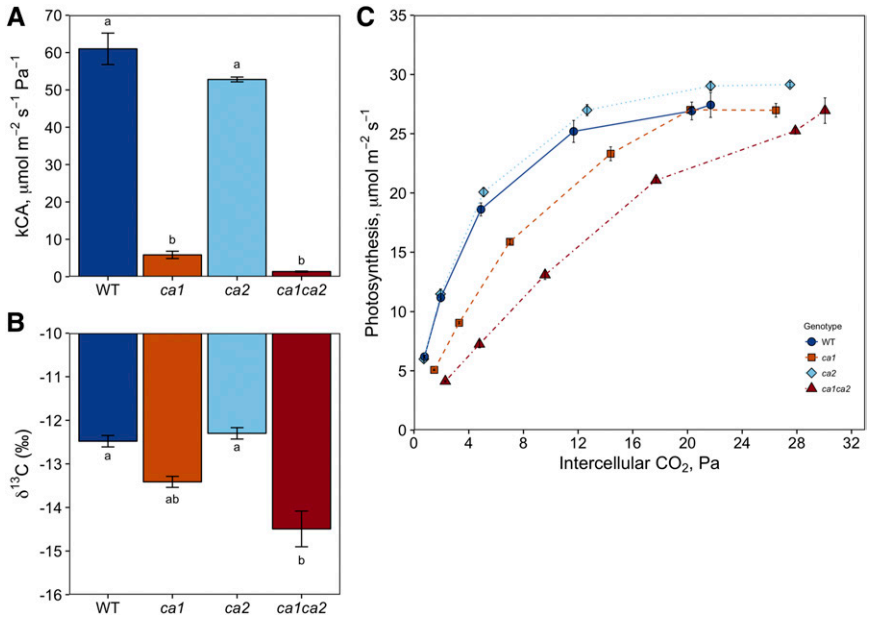

Figure 1. CA activity and stable isotope composition in *ca* mutant plants. A, CA activity expressed as *k*CA in *ca1*, *ca2*, and *ca1ca2* plants compared with wild-type (WT) plants. B, Stable isotope composition ($\delta^{13}C$) measured in wild-type and mutant leaves. Different lowercase letters denote significant differences between groups (Tukey's posthoc test, $P < 0.05$). C, Net rate of CO_2 assimilation response to intercellular CO_2 partial pressure (A/C_i curves) for each genotype measured at PPFD = 1,500 µmol m^{-2} s^{-1} and leaf temperature of 25°C. Data are presented as averages of three to four individuals ± SE.

Steady-State Measurements

Transient rates of A and g_s were measured every 60 s as CO_2 partial pressure transitioned between 36.8 and 9.2 Pa (400 and 100 µL L^{-1}) for 30 min each and 60 min at 73.6 Pa (800 µL L^{-1}; Fig. 2). To minimize the confounding effects on A and g_s, the leaf temperature, concentration of water in the sample cuvette, and vapor pressure deficit (VPD) between the leaf and atmosphere were held constant (Supplemental Fig. S1). Steady-state conditions were calculated from the last 10 min under each condition.

At ambient CO_2 levels (36.8 Pa), only the *ca1ca2* double mutant showed significantly lower steady-state A compared with wild-type plants ($P = 0.019$; Fig. 2A). In addition, *ca2* mutants had significantly higher A than *ca1ca2* mutant plants ($P = 0.002$). The effect of the CA mutations became more evident at 9.2 Pa CO_2, when significant differences in A were observed for all genotypes except between wild-type and *ca2* mutant plants ($P = 0.440$). Differences in A were minimized at 73.6 Pa. The average steady-state A for *ca2* mutants was consistently higher than for wild-type plants across all CO_2 conditions; however, this trend was not statistically significant (36.8 Pa, $P = 0.344$; 9.2 Pa, $P = 0.44$; and 73.6 Pa, $P = 0.174$). Similar increases in g_s were observed in *ca1* and *ca2* single mutants at 36.8 Pa compared with wild-type plants. An additive increase in g_s was observed in *ca1ca2* double mutant plants (Fig. 2B).

CO_2 and Light Response

In addition to steady-state differences in g_s, significant differences in rates of stomatal closure also were observed between genotypes (Fig. 3; Table II). These differences were tested by calculating the time to 90% of the steady-state open or closed value. Stomatal closure was slower in *ca1ca2* double mutants compared with wild-type and *ca1* single mutant plants when the CO_2 was raised to 73.6 Pa ($P = 0.003$ and 0.0497, respectively), but it was not significantly slower than in *ca2* mutants ($P = 0.06$). Both *ca1* and *ca2* single mutants

Table I. Biochemical and physiological characteristics of CA mutants

Data are presented as averages ± SE. No significant differences were observed between genotypes.

Genotype	PEPC	Rubisco	Fresh Weight	Dry Weight	Stomatal Density
	µmol m^{-2} s^{-1}		*g*		
Wild type	277.5 ± 14.8	31.5 ± 1.4	215.6 ± 6.9	22.4 ± 1.4	49.6 ± 2.1
ca1	268.1 ± 31.4	34.4 ± 5.1	211.8 ± 14.6	21.1 ± 1.9	50.5 ± 0.5
ca2	269.9 ± 31.9	31.7 ± 1.8	209.2 ± 14.8	22.7 ± 3.2	45.8 ± 1.4
ca1ca2	307.6 ± 8.7	35.3 ± 1.6	172.9 ± 11.1	17.0 ± 1.0	48.0 ± 2.3

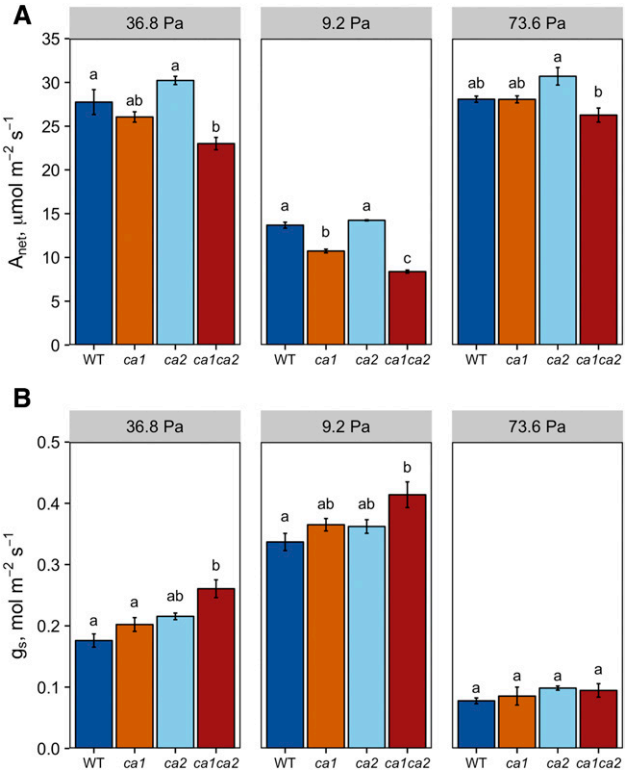

Figure 2. Steady-state photosynthesis and g_s measurements during the CO_2 response. Steady-state measurements of photosynthesis (A) and g_s (B) were calculated from the last 10 points at each CO_2 partial pressure. Bars represent averages of three to four individuals ± SE. Leaf temperature (25°C) and PPFD (1,500 µmol m^{-2} s^{-1}) were held constant in the leaf chamber. Different lowercase letters represent significant differences between groups (Tukey's posthoc test, $P < 0.05$). WT, Wild type.

showed slowed stomatal closure following a transition from low to high CO_2 (Fig. 3), but they were not significantly slower than wild-type plants. Interestingly, *ca1ca2* double mutant plants also showed slowed closure when transitioned from light to dark (Table II; Fig. 4). No difference in the rate of stomatal closing was observed in the single mutant plants compared with the wild type in response to light-dark transitions. In addition, no difference in the rate of stomatal opening was observed in response to CO_2 or light (Table II).

Effect of Reduced CA on Drought Tolerance

Because *ca1ca2* mutants showed the most striking phenotypes, with consistently lower A and higher g_s than wild-type plants, double mutant plants were grown to assess the effect of reduced CA activity on drought tolerance. Wild-type and *ca1ca2* mutant plants were grown in five different treatments: 100%, 80%, 60%, 40%, and 20% field capacity (FC), plus or minus the error associated with the plant weight over the course of the experiment. Plants were grown in pots in the greenhouse, and the water treatments were based on the FC of the soil. Whole-plant transpiration was calculated by weighing the pots daily and subtracting the average amount of water that evaporated from the empty pots from the total amount of water lost from each treatment pot. Surprisingly, no significant difference in whole-plant transpiration was observed between *ca1ca2* mutant plants and wild-type plants in any of the treatments (Table III). However, statistically significant differences were observed for plant height and dry weight biomass between the two genotypes (Table III). Furthermore, the average percentage differences between wild-type and mutant plants for plant height and dry weight were similar across all treatments (~12.3%). This resulted in a difference between wild-type and *ca1ca2* mutant plants for water-use efficiency across all treatments (Fig. 5).

With these data, we were also able to investigate the water requirement for normal plant growth in this experiment. There was no difference in plant growth between wild-type plants given 100% and 80% FC (Fig. 6). Thus, 80% of the pot's water-holding capacity is sufficient for normal growth. However, plants under 100% FC conditions transpired significantly more water

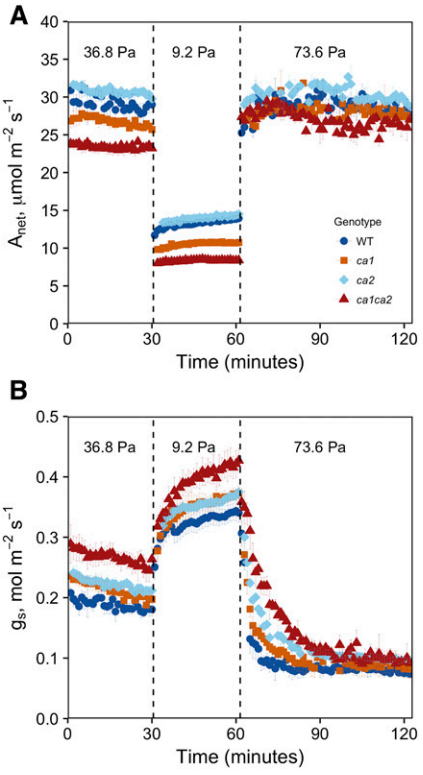

Figure 3. Gas-exchange measurements during the CO_2 response. Photosynthesis (A) and g_s (B) were measured in response to CO_2 in wild-type (WT) and mutant plants. Points represent averages of three to four individuals ± SE. Leaf temperature (25°C) and PPFD (1,500 µmol m^{-2} s^{-1}) were held constant in the leaf chamber. Leaf-to-air VPD was maintained between 1.3 and 1.5 kPa across all conditions by manually controlling water concentration in the leaf chamber (~20 mmol mol^{-1}; Supplemental Fig. S1).

Table II. Rates of stomatal response

Data are presented as averages ± SE. Values represent time (min) to 90% open or closed response.

Genotype	CO_2 Open[a]	CO_2 Close[b]	PAR Open[a]	PAR Close[b]
Wild type	13 ± 2	6 ± 2 a	18 ± 1	7 ± 1 a
ca1	13 ± 2	14 ± 4 a	17 ± 1	7 ± <1 a
ca2	10 ± 2	15 ± 5 a,b	16 ± 1	6 ± <1 a
ca1ca2	14 ± 2	27 ± 2 b	15 ± 2	16 ± 2 b

[a]No significant differences were observed between genotypes.

[b]Lower case letters show significant differences between genotypes.

(~10%; $P = 0.016$) over the course of the experiment than plants grown at 80% FC to achieve similar growth (Fig. 6). An identical trend was seen for *ca1ca2* mutant plants, suggesting that *Z. mays* plants do not operate at their maximum water-use efficiency under FC conditions. This is also supported by the observed trend across treatments for the amount of water needed to produce 1 g of dry weight biomass. Under 100% FC conditions, 213 mL of water was required, but under 20% FC conditions, only 137 mL of water was used (Fig. 5).

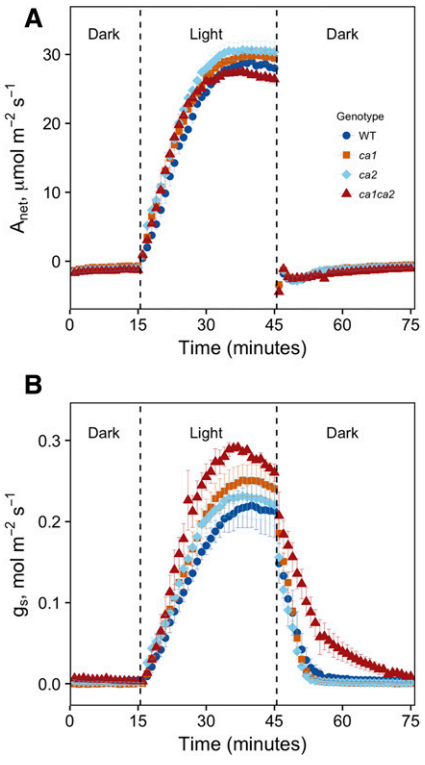

Figure 4. Gas-exchange measurements during the light response. Photosynthesis (A) and g_s (B) were measured in response to dark-light and light-dark transitions. Points represent averages of three to four individuals ± SE. Leaves were clamped in an unilluminated LICOR chamber and allowed to acclimate. Lights were turned on (PPFD = 1,500 µmol m^{-2} s^{-1}) for 30 min, then turned off to measure stomatal closure. Measurements were taken at constant CO_2 in the leaf chamber (36.8 Pa) and leaf temperature (25°C). Leaf-to-air VPD was maintained around ~1.5 kPa across all conditions by manually controlling water concentration in the sample (~18–19 mmol mol^{-1}). WT, Wild type.

DISCUSSION

CA1 and CA2 account for more than 95% of the total CA activity in *Z. mays* leaves (Studer et al., 2014). Although stomatal movement differences were observed in *ca* single mutants (Fig. 3), a double *ca* mutant was necessary to observe significant gas-exchange differences compared with wild-type *Z. mays* under standard growth conditions. This result is likely due to the presence of multiple CA isoforms with redundant function in the same cell type. Based on these results, we hypothesize that the tandemly duplicated *Ca* genes are at least partially subfunctionalized with respect to their roles in stomatal movement, photosynthetic rates, and carbon isotope composition.

In Arabidopsis and rice (*Oryza sativa*) *ca* mutants, stomatal closure defects are only observed in response to elevated CO_2, suggesting that the role of CA is specific to the CO_2 signaling pathway (Hu et al., 2010; Chen et al., 2017). However, *Z. mays ca* mutant plants exhibit slower stomatal closure in response to both high CO_2 and light-dark transitions. This result indicates that, unlike in Arabidopsis and rice, CA may function in stomatal closure in response to light or in stomatal movement downstream of the convergence point between the CO_2 and light signaling pathways. It is interesting that stomata of the *ca1ca2* double mutant opened rapidly in response to light, and then conductance decreased after reaching a maximum (Fig. 4). We hypothesize that rapid stomatal opening after exposure to light cannot be modulated properly in the *ca1ca2* mutant plants because of slowed stomatal closure. Thus, the plants initially increase g_s too much and need time to reach the appropriate steady-state g_s.

The role of C_i as a signal in stomatal responses to light has been debated in the literature: low C_i has been suggested to increase g_s, and high C_i would reduce g_s (Lawson et al., 2014). Furthermore, some have speculated that light-to-dark stomatal responses may be mediated by an increase in C_i as photosynthesis decreases and respiration increases (Roelfsema et al., 2002; Hashimoto et al., 2006). However, the data presented here are not consistent with C_i as a signal for stomatal closure. For example, the *ca* mutants tended to have higher C_i than the wild type due to decreased photosynthetic rate and increased g_s (Supplemental Fig. S2). Therefore, if stomatal closure in the dark is driven by elevated C_i, then the *ca1ca2* mutants would be expected to have a more rapid stomatal closure.

Table III. Drought stress phenotypes

Data are presented as averages ± SE. Days, Number of days under treatment conditions; N, replicates; E, transpiration. Asterisks indicate significance when comparing the wild type with the mutant using pairwise Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Genotype	Treatment	Days	N	E	Plant Height	Dry Weight
				L	*cm*	*g*
Wild type	100%	19	7	10.9 ± 0.4	120 ± 2.2	51.4 ± 1.6
ca1ca2	100%	19	7	11.4 ± 0.2	109 ± 2.6**	45.6 ± 1.0*
Wild type	80%	19	8	9.6 ± 0.3	118 ± 1.6	51.2 ± 1.1
ca1ca2	80%	19	8	9.7 ± 0.2	105 ± 2.4***	44.6 ± 0.9***
Wild type	60%	18	8	7.8 ± 0.2	103 ± 1.4	46.9 ± 1.0
ca1ca2	60%	18	7	7.8 ± 0.1	86 ± 2.3***	40.0 ± 0.7***
Wild type	40%	16	8	4.6 ± 0.1	76 ± 2.6	34.0 ± 0.9
ca1ca2	40%	16	7	4.8 ± 0.4	66 ± 3.1*	29.7 ± 1.4*
Wild type	20%	11	7	0.9 ± 0.2	61 ± 1.8	24.0 ± 0.8
ca1ca2	20%	11	7	1.1 ± 0.1	54 ± 1.0**	21.2 ± 0.4*

While Arabidopsis *ca* mutants have alterations in both stomatal density and aperture, there is no difference in stomatal density in *Z. mays ca* mutants. The lack of stomatal density differences also was observed recently in *ca* mutants in rice (Chen et al., 2017). This discrepancy may be indicative of different developmental trajectories in monocots and dicots: the patterning of stomatal density in eudicots occurs later in leaf development and is more subject to environmental perturbation, whereas patterning of stomatal density occurs earlier in leaf development in monocots and is less sensitive to environmental cues. Indeed, many of the known stomatal patterning genes are expressed in a narrow developmental window at the base of a developing leaf blade in both *Z. mays* and rice (Li et al., 2010; Wang et al., 2014). This apparent insensitivity to short-term environmental variability suggests that grasses are more dependent on the regulation of stomatal aperture to control g_s.

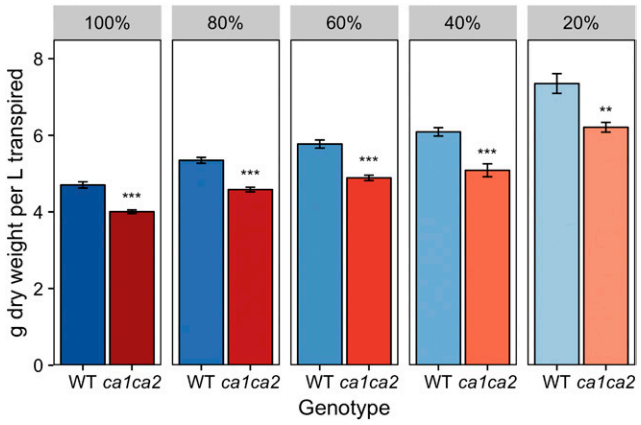

Figure 5. Water-use efficiency of wild-type (WT) and *ca1ca2* plants grown under different drought conditions. Dry weight biomass was measured at the end of the experiment. Water treatments were determined based on the water-holding capacity of the soil. Transpiration was calculated over the course of the treatments by weighing each pot daily and controlling for evaporation using empty control pots. Data are presented as averages of seven to eight individuals ± SE. **, $P < 0.01$ and ***, $P < 0.001$.

The *ca2* single mutant detailed here provides some insights into the role of CA2 in *Z. mays*. The discrepancy between the transcript levels and the enzyme activity of the CA isoforms suggests posttranscriptional differences between *Ca1* and *Ca2* transcripts or differences in the kinetic properties of the enzyme isoforms. Furthermore, the increase in g_s observed in *ca2* mutant plants was not proportional to the CA2 contribution to the total CA activity, suggesting that it may be concentrated in a specific cellular or subcellular location that maximizes its role in the CO_2 response. Although we consistently observed higher A and g_s in *ca2* mutants under all CO_2 conditions, differences between *ca2* mutant and wild-type plants were not statistically significant with respect to A, g_s, and A/g_s even under low-CO_2 conditions. These results suggest that the 13% reduction in total leaf CA observed in the *ca2* mutant is not sufficient to limit A, even under low CO_2.

Based on our results, we suggest a model for the subfunctionalization of the duplicated *Ca2* gene copy. We speculate that the ancestral gene that encodes CA1 has both stomatal signaling and photosynthetic functions, whereas the newly duplicated CA2 copy may have a specialized function in guard cells. From our data, we cannot conclusively determine the extent to which CA2 functions in photosynthesis. It is clear that, when CA1 is mutated, CA2 has the ability to provide bicarbonate for photosynthesis and, thus, is not present exclusively in guard cells. However, because there is no indication of a decrease in A in *ca2* single mutants, this could suggest that the difference between *ca1* and *ca1ca2* mutant plants is the extent to which CA2 is redundant. From this work, we are unable to determine the cellular or subcellular localization of these CA isoforms. Because both CA1 and CA2 can contribute bicarbonate for photosynthesis at some level, this would suggest that, at least in mesophyll cells, these proteins are located in the cytosol. However, it remains unknown if either isoform is localized to the plasma membrane or in chloroplasts, as seen in Arabidopsis (Fabre et al., 2007; Hu et al., 2015).

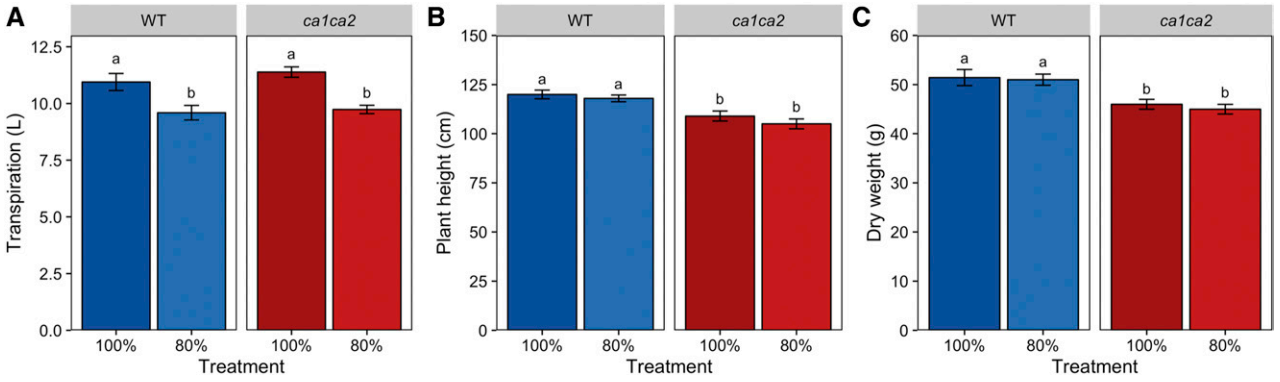

Figure 6. Transpirational water loss compared with plant growth of wild-type (WT) and *ca1ca2* plants under well-watered conditions. Transpiration (A), plant height (B), and dry weight (C) are shown for plants grown under 100% and 80% FC. Water treatments were determined based on the water-holding capacity of the soil. Data are presented as averages of seven to eight individuals ± SE. Different lowercase letters represent significant differences between groups (pairwise Student's *t* test, $P < 0.05$).

Although a significant difference in g_s was observed in *ca1ca2* double mutants in *Z. mays*, no differences in g_s were observed in *S. viridis ca* mutants (Osborn et al., 2017). One possible explanation for this result may be that *Z. mays* and *S. viridis*, which have independent origins of C_4 photosynthesis, have different requirements for CA activity in the leaf even though they have similar leaf CA rate constants (*S. viridis k*CA = 61–84 µmol m^{-2} s^{-1} Pa^{-1}; Osborn et al., 2017). Alternatively, the impact on g_s may relate to the severity of the *ca* knockdowns. The most dramatic knockdowns in *S. viridis* have *k*CA = 8 µmol m^{-2} s^{-1} Pa^{-1}, which is approximately 6 times more activity than what was observed in the *Z. mays ca1ca2* double mutants (*k*CA = 1.3 µmol m^{-2} s^{-1} Pa^{-1}). Another possibility is that there is a CA isoform involved in stomatal signaling that was not affected by the knockdown construct used in *S. viridis*. This is quite possible given that there are three tandem *Ca* genes in *S. viridis* and no clear ortholog to *Ca2* (Studer et al., 2016).

Higher g_s in *ca* mutant plants would seem to indicate a higher whole-plant transpirational loss, which would be detrimental under water-limiting conditions. However, the difference in g_s between mutant and wild-type plants did not translate into a significant difference in water losses due to whole-plant transpiration. It is possible that the reduction in biomass, and therefore the reduced surface area for transpiration, balances the difference in g_s. It is also possible that the high-temperature and low-humidity conditions under which the drought experiment was conducted increased VPD to the point that the signal to close stomata through the abscisic acid pathway overrode the signal to open stomata in response to low C_i. This would indicate that *Z. mays* is more sensitive to high VPD stress than to intercellular CO_2 limitations, which is consistent with correlations between climate change and *Z. mays* yield losses in the Midwest (Lobell et al., 2014). Regardless, the difference in water-use efficiency (Fig. 5) between wild-type and mutant plants indicates a limitation for photosynthesis that resulted in a reduction in biomass.

An important observation in the drought experiment was a significant difference in transpiration rates of wild-type plants under 100% and 80% FC conditions. It was not surprising that plants grown at 100% and 80% FC would be morphologically similar and produce the same amount of biomass. This observation indicates that water is not limiting for growth at 80% FC. However, it is intriguing that plants grown at 100% FC used 10% more water to achieve the same growth as plants grown at 80% FC. This result indicates that, under these growth conditions, *Z. mays* is not water-use efficient when well watered. This provides a potential target for crop improvement and merits testing under field conditions. Conserving water under well-watered conditions would build up water reserves in the soil that could be used later in the season during dry periods. While most studies look at water-use efficiency under drought conditions, further investigation into the water-use efficiency under well-watered conditions could provide insight into other ways of optimizing water use in *Z. mays*.

In the experiments presented here, *ca1ca2* mutant plants tended to produce less biomass than wild-type plants, consistent with previous measurements (Studer et al., 2014). However, this difference is only significant for plants grown for the drought experiment, which were 6.5 weeks old, compared with 4-week-old plants grown for the stomatal response experiments. Very low CA activity likely has a small but cumulative effect on photosynthesis and, thus, would be more evident toward the end of the plant's life cycle. While these results highlight the relatively subtle effects of CA on plant performance, CA function may be important for achieving optimum efficiency over an entire growing season. This function may help explain the large amount of variation in CA activity observed between species (Gillon and Yakir, 2000). If levels of CA

correlated with the sensitivity of stomata, variable conditions, such as those experienced in a field environment, may require CA to fine-tune stomatal responses in a dynamically changing environment.

CONCLUSION

Characterization of the newly generated *ca* mutants has enabled us to investigate the contribution of specific *CA* genes to photosynthesis and stomatal movement. Our data show that, in *Z. mays*, CA plays a role in stomatal movement in response to CO_2 and light; however, it does not affect stomatal patterning. Differences observed between the *ca1* and *ca2* single mutants lead us to speculate that CA1 could be the main isoform providing bicarbonate for C_4 photosynthesis, while CA2 may be more specialized and required for normal guard cell movement. Drought experiments revealed that *ca1ca2* double mutants did not transpire more than the wild type but had reduced biomass, presumably due to a limitation of photosynthesis. Collectively, these results indicate that there is a benefit to high levels of CA, which enables plants to maintain high rates of photosynthesis under CO_2-limiting conditions and to fine-tune stomatal movements to changing environmental conditions.

MATERIALS AND METHODS

Screen for *ca2* Single Mutant Alleles

Maize (*Zea mays*) plants heterozygous for the *cis ca1ca2* mutations (Studer et al., 2014) were self-pollinated and produced progeny that segregated 1:2:1 for the mutated *ca* locus. From this ear, 192 kernels were planted, and DNA was extracted from each seedling using a CTAB DNA extraction protocol. An approximately 2.5-cm^2 piece of leaf tissue was harvested from the youngest leaf of 8- to 11-d-old *Z. mays* seedlings. Tissue samples were placed in 96 plastic racks (Fisher Scientific, catalog no. 50-823-921) of 1.4-mL tubes (Fisher Scientific, catalog no. 50-823-825) containing two one-eighth-inch Grade 1000 Type 430 Stainless Steel Balls (Abbott Ball) on ice. Tubes were capped with eight-well strip caps (VWR, catalog no. 82006-694). After collection, liquid nitrogen was used to freeze the plates containing the tissue. The tissue was homogenized using a paint shaker (Harbil, model no. 5G-HD) set to shake for 2 min. The plates were then removed and again placed in liquid nitrogen for 1 min. This was repeated until the tissue was fully homogenized (two to four rounds). One hundred milliliters of CTAB buffer was prepared by mixing 50 mL of water, 10 mL of 1 M Tris-HCl (pH 8), 2 g of CTAB, 0.2 g of sodium bicarbonate, 4 mL of 0.5 M EDTA (pH 8), and 28 mL of 5 M sodium chloride. Water was then added to the solution up to 99 mL, and 1 mL of β-mercaptoethanol was added immediately before use. The strip caps were removed with forceps, and 400 µL of CTAB buffer was added to each sample. The caps were replaced, and the plates were shaken by hand until the tissue was suspended in the CTAB buffer. The plates were spun in a centrifuge (Eppendorf, model no. 5410R) with a swinging-bucket rotor at 2,000 rpm for 2 min. The samples were then incubated for 30 min in a water bath at 65°C.

After incubation, the plates were removed and allowed to cool at room temperature for 10 min. The caps were removed and discarded. Then, 400 µL of chloroform:isoamyl alcohol (24:1) was added to each sample and mixed by pipetting up and down using wide-bore tips. A mat (Fisher Scientific, catalog no. 50-823-876) was used to cover the tubes, and the plates were centrifuged at 4,000 rpm for 15 min at room temperature. A total of 100 µL of the aqueous layer was removed from each sample and placed on a new plate (VWR, catalog no. 89049-178) containing 100 µL of ice-cold 100% isopropanol. The samples were mixed by pipetting up and down. The plate was then covered with a plastic seal (VWR, catalog no. 89091-702) and placed at 4°C for 1 h. After DNA precipitation, the plate was centrifuged at 4,000 rpm for 15 min at room temperature. The isopropanol was removed by carefully inverting the plate, and the DNA pellet was washed with 500 µL of ice-cold 70% (v/v) ethanol. The plate was again covered with a plastic seal and centrifuged at 4,000 rpm for 15 min at room temperature. The ethanol was removed by inverting the plate, and the DNA pellet was dried at 30°C using an Eppendorf Vacufuge for 10 min. The DNA pellet was resuspended in 100 µL of water and placed at 4°C overnight. DNA concentrations were determined using a Nanodrop 2000C spectrophotometer and then diluted for downstream applications. Individuals were tested for the presence of the *Ds* element in *ca2* using standard PCR (genic-genic primers AJS007 [5′-GGCTGCTTATTGTCCCATGT-3′] and AJS097 [5′-CTGGAGTCTGGATATACCATTCC-3′]; genic-*Ds* primers AJS097 [5′-CTGGAGTCTGGATATACCATTCC-3′] and AJSR05 [5′-CGTCCCGCAAGTTAAATATGA-3′]) and visualized using agarose gel electrophoresis. The presence of the 8-bp *Ds* footprint in *ca1* was detected based on the difference in melting temperatures (wild type = 80.7°C–81.7°C; mutant = 83.2°C–84.3°C) of the PCR product amplified by two genic primers (AJS405 [5′-AATGGGACCCTCAAGCTGAT-3′] and AJS406 [5′-TGTGAGGAACTCTCCTGAGACA-3′]) that span the 8-bp insertion. Fragment amplification and melt curves were produced by using LightCycler 480 SYBR Green I Master Mix and a Roche LightCycler 480 instrument following the manufacturer's recommendations. Analysis of the melt curve was performed using the Tm calling option of the Roche software and manual checking.

Plant Growth Conditions

Seeds of wild-type, *ca1*, *ca2*, and *ca1ca2* plants were planted in 2-gallon pots filled with LC-1 Sunshine mix (Sun Gro Horticulture) and grown in a greenhouse at Washington State University with 14-h days at 27°C and 10-h nights at 20°C, with supplemental lighting to provide a fluence of at least 600 W m^{-2}. All plants were fertilized weekly (Peters 20-20-20), treated with a slow-release fertilizer (21-5-5), and supplemented with Spring 330 iron chelate (BASF).

Gas Exchange

Photosynthesis and g_s were measured on the uppermost, fully expanded leaf of 4-week-old plants (leaf 5 or 6) using a LICOR-6400XT. Measurements were performed by controlling leaf temperature at 25°C and manually adjusting water concentrations to maintain a leaf-to-air VPD between 1 and 1.5 under all conditions. Each plant was acclimated to the measurement conditions for 15 to 30 min. Four plants were measured for the wild type and *ca1ca2*, and three plants were measured for *ca1* and *ca2* single mutants.

The stomatal response to CO_2 was assessed by maintaining constant PPFD (1,500 µmol photons m^{-2} s^{-1}) and controlling the CO_2 concentration in the sample cuvette. Each plant was initially measured for 30 min at 36.8 Pa CO_2. After 30 min, the CO_2 partial pressure was decreased to 9.2 Pa, and measurements were taken every 1 min for 30 min. Finally, the CO_2 concentration was increased to 73.6 Pa, and data were collected for 60 min. At the completion of these measurements, a short CO_2 response curve was taken by controlling the CO_2 partial pressure entering the cuvette at 73.6, 55.2, 36.8, 18.4, 9.2, and 4.6 Pa.

The stomatal response to light was assessed by maintaining a constant 36.8 Pa CO_2 in the sample cuvette. Plants were acclimated in a dark cuvette. Once stomata had closed, measurements were taken every 1 min for 15 min. Then, PPFD was set to 1,500 µmol photons m^{-2} s^{-1}, and data were logged every 1 min for 30 min. After 30 min, the light source was turned off and measurements were taken for 30 min to monitor the stomatal closure response.

Statistically significant differences in rates of stomatal opening and closing were assessed by calculating the time required for each plant to reach 90% of the open or closed response value for each condition. This method of calculating differences in response has been used to assess changes in g_s in response to sunflecks (Leakey et al., 2005). The last conductance value measured prior to changing conditions was used as the starting value for each condition, and the steady-state open/closed value was determined by averaging the final 10 points in each condition. The point at which each plant crossed its threshold of 90% open/closed response was applied to a one-way ANOVA, and subsequent pairwise comparisons were performed using Tukey's posthoc test.

Stomatal Density

Leaf material for measuring stomatal density was taken from the same portion of the leaf measured for gas exchange. Samples were immediately frozen in liquid nitrogen and stored at −80°C. Frozen leaf discs were used to measure

stomatal density. A Nanofocus µsurf explorer optical topometer with a 20× lens and a 0.6 numerical aperture was used to capture 0.8-mm × 0.8-mm images of both the abaxial and adaxial leaf surfaces. Two images were taken per side per disc. Two to three leaf discs were measured per plant replicate, and three to four replicates were used for each genotype. Thus, stomatal density for a given genotype was calculated from a minimum of 24 images.

Carbon Isotope Composition

Samples for carbon isotope composition were dried at 60°C. Approximately 2 to 2.5 mg of dried leaf material was placed in tin capsules and combusted in a hydrogen/carbon/nitrogen elemental analyzer (ECS 4010; Costech Analytical) to determine stable carbon isotope ratio. Aboveground biomass was harvested and weighed to determine fresh weight, then dried for several weeks at 60°C to determine dry weight.

Enzyme Assays

Leaf samples for enzyme activity assays were collected as described above for stomatal density. Enzyme assays were performed at 25°C and pH 8 as described previously (Studer et al., 2016).

Drought Experiment

Two hundred *Z. mays* kernels segregating 1:2:1 (wild type:heterozygous:-homozygous *ca1ca2*) from a single ear were planted in deep-well plug trays in MetroMix 360 with Turface (3:1 ratio). Seedlings were germinated in the greenhouse with 14-h days at 28°C and 10-h nights at 22°C with 50% relative humidity. Natural light was supplemented with a mixture of high-pressure sodium and metal halide fixtures. DNA for genotyping was extracted using a CTAB method on a 2-cm^2 piece of tissue from the first leaf of 10-d-old seedlings. Wild-type and homozygous *ca1ca2* mutant plants were transplanted after 2 weeks into 2.5-gallon black plastic pots. Before transplanting, pots were filled with Fafard 51 Custom Mix and dried at 60°C for 1 week. The pots were then removed from the dryer and immediately normalized to 2 kg. This was done so that a uniform amount of soil was added to each pot. Pots were then soaked in deionized water until saturated to determine the FC. Plants were transplanted and watered to saturation. A granulated starter fertilizer, consisting of one teaspoon of granulated fertilizer (600 mL [one part] Osmocote [15-9-12], 600 mL [one part] Tomato Maker [4-2-6 ca mg], 50 mL [one-twelfth part] Sprint, and one tablespoon of ferrous sulfate) was added as a top dressing to each pot after transplanting. Pots containing seedlings were then moved to a greenhouse with 14-h days at 32°C and 10-h nights at 22°C with 30% relative humidity for the duration of the experiment. Natural light was supplemented with metal halide fixtures at an intensity of 590 µmol m^{-2} (light measurement taken at the pot level). For the first 3 weeks of the experiment, all pots were well watered with tempered water supplemented with 200 µL L^{-1} 15:5:15 (N:P:K) calmag fertilizer. Eight to 10 replicates were grown per genotype in each treatment. Five representative pots were weighed at saturation to determine the well-watered (FC) weight as 4.5 kg or gravimetric water content of 1.25 g water g^{-1} soil. Three weeks after planting, each pot was watered to FC; then, the pots were allowed to dry down to begin the drought treatment. Pots were weighed daily, and water was added on an individual pot basis. Daily transpiration was measured as the difference in pot weight between measurements, taking into account soil evaporation (differences in pot weight without a plant).

Accession Numbers

Sequence data referred to in this article can be found in the Gramene or Phytozome databases under accession numbers GRMZM2G121878 (*Ca1*) and GRMZM2G348512 (*Ca2*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Water concentration and leaf-to-air VPD during stomatal response measurements.

Supplemental Figure S2. Steady-state gas-exchange measurements during the CO_2 response.

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