Carbonic anhydrase (CA) catalyzes the reversible hydration of CO$_2$ into bicarbonate (Badger and Price, 1994; Moroney et al., 2001), and multiple independently evolved families of CA are found across all kingdoms of life (Hewett-Emmett and Tashian, 1996). In land plants, the β-CAs are most abundant and have been implicated in photosynthesis (Ludwig, 2012). In photosynthetic bacteria (Fukuzawa et al., 1992; So et al., 2002; Dou et al., 2008) and green algae (Funke et al., 1997; Moroney et al., 2011), CA plays an essential role in providing CO$_2$ to the active site of Rubisco. However, the role of CA in C$_4$ plants is less clear (Badger and Price, 1994), because it does not appear to limit photosynthesis but does affect stomatal conductance and guard cell movement (Hu et al., 2010). In C$_4$ plants, CA provides HCO$_3^-$ to the initial carboxylating enzyme phosphoenolpyruvate carboxylase (PEPC), driving the production of the C$_4$ acid oxaloacetic acid. The total amount of leaf CA activity varies significantly across evolutionary lineages of C$_4$ plants (Gillon and Yakir, 2001; Cousins et al., 2008); however, there is little evidence to support a physiological role for this large natural variation in CA activity. This suggests that the importance of CA activity across C$_4$ plants may vary and may be related to the C$_4$ evolutionary lineage or the specific environment to which a particular species has adapted.

In the C$_4$ dicot *Flaveria bidentis*, which has very high leaf CA activity, the cytosolic β-CA is essential but not rate limiting for C$_4$ photosynthesis (von Caemmerer et al., 2004). In antisense *F. bidentis* with less than 20% of wild-type leaf CA activity, rates of photosynthesis were reduced, and plants with less than 10% of wild-type CA required high CO$_2$ (1–2 kPa) for growth. Alternatively, in many C$_4$ grasses, CA activity appears to almost limit the rates of photosynthesis (Hatch and Burnell, 1990; Cousins et al., 2008). For example, CA activity in maize (*Zea mays*) leaves is approximately 10% of that in wild-type *F. bidentis* (Cousins et al., 2008); however, both species have high rates of CO$_2$ assimilation typical of C$_4$ photosynthesis. While the requirement of CA for C$_4$ photosynthesis may differ between independent evolutionary C$_4$ lineages, to date, a mutant analysis has not been performed to examine the role of CA on C$_4$ photosynthesis in any monocot species.
Therefore, we used a mutational approach to test the role of CA activity in the C₄ monocot maize, which contains five annotated genes encoding β-CAs within its genome. Two homologs (Chr.2-GRMZM2G414528 and Chr.7-GRMZM2G145101) are predicted to be localized to mitochondria and are structurally conserved across grass species as single orthologs. A gene encoding β-CA on chromosome 8 (GRMZM2G094165) is a duplicated homolog unique to maize and is predicted to be chloroplast localized. Two annotated β-CAs (GRMZM2G121878 and GRMZM2G348512) are arranged as tandem duplicates on chromosome 3. The gene annotated as GRMZM2G121878 is conserved across all grass species (Ca1) and is the first gene in the tandem gene set. Although GRMZM2G348512 is annotated as a single gene in maize, RACE mapped two separate genes (sequentially Ca2 and Ca3). Thus, the tandem Ca locus is composed of three genes in maize (Ca1–Ca3) and is reported as four genes in Sorghum bicolor (Wang et al., 2009) and three in Setaria italica (Zhang et al., 2012).

The detailed genetic characterization of the Ca loci in maize allows for a directed targeting of the genes most likely involved in the first step of C₄ photosynthesis. Here, we present, to our knowledge, the first mutagenesis and physiological characterization of ca mutants in a C₄ monocot to determine whether CA is rate limiting for photosynthesis in a C₄ monocot. The low CA activity observed in maize leaves, despite the numerous genes encoding CA, provides a test for the requirement of CA for C₄ photosynthesis in a species with naturally low levels of CA. We show that Ca1 accounts for the majority of the leaf CA activity. We also show that, under low-CO₂ conditions, photosynthetic rates are compromised. In addition, photosynthetic isotope discrimination shows that the uncatalyzed hydration of CO₂ contributes significantly to bicarbonate pools in the double mutant, which has only 3% of wild-type CA activity. The data presented demonstrate that CA is not rate limiting for C₄ photosynthesis in maize under current atmospheric conditions. In addition, the interplay between stomatal and biochemical limitations for photosynthesis under different environmental conditions is discussed.

RESULTS

Two Highly Expressed Ca Genes Are Correlated with C₄ Photosynthesis

The resolved Ca gene structures in maize, described above, were used with RNA-seq data from deeply sequenced maize leaf tissue to examine the expression level of each Ca gene (Fig. 1A). Unlike most RNA-seq pipelines that permit multiple mapped alignments, a stringent filter was applied (see “Materials and Methods”) so that only unique reads were aligned to a region of the 3’ untranslated region (UTR) of each Ca gene. This enables the expression profile of each Ca gene to be assayed independently, despite the high level of sequence homology within the gene family. At the tip of the developing seedling leaf (which is photosynthetically active; for a description of leaf gradient used, see Li et al., 2010), Ca1 and Ca2 encode more than 85% of the Ca transcript pool. However, Ca3 is expressed at very low levels (less than 0.5% of total reads mapped) in the leaf, indicating that it is a paralogous duplication. This result is confirmed by the available maize expression atlas data (Sekhon et al., 2011), which shows Ca3 expression mostly in non-photosynthetic tissues (Supplemental Fig. S1). Given these results, we restricted our genetic characterization to the tandemly arranged Ca1 and Ca2 genes.

Dissociation Transposition Generates Mutant Alleles in Tandemly Arranged Ca Genes

To investigate the role of CA in maize, we conducted an insertional mutagenesis to generate an allelic series of the Ca1 and Ca2 genes. Using the Activator (Ac) and Dissociation (Ds) system (Ahern et al., 2009; Vollbrecht et al., 2010), a reverse-genetic screen was performed using two donor Ds elements (B.S05.0455 and I.W06.0504) located within Ca1 in two near-isogenic W22 inbred lines. We exploited the intrinsic property of Ds to transpose locally (Alleman and Kermicle, 1993) to create tandem mutations in linked Ca genes. Ds was mobilized from Ca1, resulting in intragenic insertion alleles of Ca1 and intergenic insertion alleles in Ca2 (Fig. 1B). The donor Ds B.S05.0455 is located in the fourth intron of Ca1, but CA1 protein accumulated in homozygotes, indicating that the element is spliced efficiently from the mature mRNA (Fig. 1C). When B.S05.0455 was mobilized, an intragenic transposition event was recovered in which the Ds element was duplicated, creating an insertion into exon 12 of Ca1 while retaining a copy at the donor site. This insertion eliminates CA1 protein, creating the loss-of-function ca1 (ca1-m1::Ds) single mutant (Fig. 1C). The donor Ds I.W06.0504 is inserted into the last exon of Ca1, and plants homozygous for this Ds insertion do not accumulate CA1 protein (Fig. 1C). When I.W06.0504 was mobilized, an intragenic transposition event was recovered in which the Ds element inserted into the second exon of Ca1, leaving behind an 8-bp footprint in the last exon of Ca1. Immunoblots indicated that this allele also has no CA1 protein (Fig. 1C), resulting in a second complete loss-of-function ca1 allele (ca1-m2::Ds).

To generate calca2 double mutants, we screened for intergenic transpositions of Ds from line I.W06.0504. One insertion allele was recovered in which the donor Ds inserted into the third exon of Ca2, with a linked 8-bp excision allele in the last exon of Ca1. This 8-bp footprint causes a frame shift that adds 62 amino acids to the end of CA1 and is sufficient to eliminate CA1 protein (Fig. 1C). Therefore, from this intergenic transposition event, a double mutant was recovered (ca1-d1 ca2-m1::Ds, here-after referred to as calca2). All three insertion alleles were heritable, and plants carrying each allele were backcrossed to the reference line (B73) before characterization.
Mutant Plants Have Normal CO₂ Assimilation Rates under Ambient CO₂ Partial Pressure

The reduction of CA activity in \( \text{ca1} \) and \( \text{ca1ca2} \) mutants was quantified by total leaf CA activity assays (soluble plus membrane fractions) measured with a membrane inlet mass spectrometer (see “Materials and Methods”). Both \( \text{ca1} \) single mutants show similar decreases in CA activity, and although other \( \text{Ca} \) genes are transcribed, homozygous \( \text{ca1} \) mutants had approximately 10% of wild-type CA activity (Fig. 2A). Heterozygous \( \text{ca1} \) mutants had intermediate CA activity, demonstrating the additive nature of the mutation and the absence of a compensatory up-regulation of \( \text{Ca} \) genes in response to reduced total CA activity (Fig. 1C). Thus, while \( \text{Ca2} \) constitutes 22.6% of the leaf transcript pool, \( \text{Ca2} \) protein is not able to compensate for the decrease in CA activity in either \( \text{ca1} \) single mutant. These data indicate that the transcript levels of \( \text{Ca1} \) and \( \text{Ca2} \) are not directly correlated with the activity levels, suggesting a posttranscriptional regulation of CA. The \( \text{ca1ca2} \) double mutant had only 3% of wild-type CA activity (Fig. 2A).

The residual CA activity in the double mutant is likely from anaplerotic CA functioning in the mitochondria, chloroplasts, or bundle sheath cytosol and, therefore, does not significantly contribute to the carbon-concentrating mechanism of \( \text{C}_4 \) photosynthesis. The activities of PEPC and Rubisco were also assayed, but there were no differences between wild-type and mutant plants grown under 928 Pa of CO₂ (Table I). Thus, under saturating CO₂ conditions, the mutations in \( \text{Ca1} \) and \( \text{Ca2} \) do not appear to have major secondary effects on the activities of other photosynthetic enzymes or plant growth (Table I). A rate constant of leaf CA of 2.5 mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\) in maize was reported previously (Cousins et al., 2008), which corresponds to a CA activity of 88 m mol m\(^{-2}\) s\(^{-1}\) at the CO₂ partial pressure (pCO₂) used in this study. The CA activity of wild-type plants was approximately 220 to 240 m mol m\(^{-2}\) s\(^{-1}\) in the soluble fraction. Although this activity is 2.6 times higher than the value reported previously, it remains in the range of CA activity measured across \( \text{C}_4 \) lineages (Cousins et al., 2008).

To assess the physiological consequences of reduced CA activity, the rates of net CO₂ assimilation were measured in response to pCO₂ (Fig. 2B) and light intensity (Supplemental Fig. S2). Net CO₂ assimilation decreased at subambient CO₂ levels (9.3 Pa) in both \( \text{ca1} \) single mutants and \( \text{ca1ca2} \) double mutants compared with the wild type (Table I). Additionally, the in vivo maximal rate of PEPC (\( V_{\text{pmax}} \)) estimated from the initial slope of the CO₂ response curve, was significantly
lower in both \textit{ca1} and \textit{ca1ca2} mutants compared with the wild type (Fig. 2C). However, no difference in net CO$_2$ assimilation was observed between genotypes at ambient or high pCO$_2$, nor was there a difference in the quantum yield at high CO$_2$ (Table I). This suggests that under ambient and higher pCO$_2$, the amount of HCO$_3^-$ is sufficient to maintain levels of photosynthesis, but under low pCO$_2$, CA limits photosynthesis. Furthermore, CA mutants grown at elevated CO$_2$ (10,000 ppm, 928 Pa of CO$_2$) showed no difference in aboveground biomass or total leaf nitrogen compared with the wild type (Table I). However, \textit{ca1ca2} plants grown at ambient pCO$_2$ in the greenhouse were, on average, 5% (9.6 cm) shorter than wild-type plants (Fig. 2D; \( n = 3 \), \( P = 0.0193 \)). When grown at sub-ambient CO$_2$ (100 ppm, 9.3 Pa of CO$_2$), the dry weight was approximately 20% and 45% lower in single and double mutants, respectively, compared with the wild type (Fig. 2E; Supplemental Fig. S3). Therefore, under elevated pCO$_2$, there is sufficient conversion of CO$_2$ to HCO$_3^-$ to maintain rates of net CO$_2$ assimilation; however, under low CO$_2$ availability, additional CA activity is required to maintain high rates of C$_4$ photosynthesis in maize.

**Figure 2.** Photosynthetic measurements predict plant growth phenotypes in CA mutant plants. A, CA activity of CA single and double mutant lines grown under elevated CO$_2$ (928 Pa). Solid and hatched bars show CA activity in the soluble and membrane fractions, respectively. B, Net CO$_2$ assimilation (\( A_{\text{net}} \)) in response to changes in intercellular pCO$_2$ (P$_i$; A-P$_i$ curve) measured at an oxygen partial pressure of 18.6 kPa, leaf temperature of 25°C, and irradiance of 1,000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). C, Maximum in vivo \( V_{\text{Pmax}} \) estimated from the initial slope of the A-P$_i$ curve and plotted for each CA mutant line. D, Wild-type and homozygous CA double mutant plants grown in the greenhouse at ambient pCO$_2$. E, CA double mutant plants grown at low CO$_2$ (9.3 Pa).
Reduced CA Activity Affects Isotopic Discrimination

The low CA activity is further substantiated by large differences in leaf $^{13}$CO$_2$ ($\Delta^{13}$C) and C$^{18}$O$_2$ ($\Delta^{18}$O) isotope exchange in high pCO$_2$-grown CA mutants compared with the wild type. The $\Delta^{13}$C increased approximately 2\% and 4\% in $ca1$ and $ca1ca2$ mutants, respectively (Fig. 3A). The increase in $\Delta^{13}$C with lower CA activity is consistent with previous studies of CA mutants in $F. bidentis$ (Cousins et al., 2006a) and with models of CA activity and $\Delta^{13}$C in C$_4$ plants. Theoretically, the increase in $\Delta^{13}$C measured in the $ca1$ and $ca1ca2$ mutants could be explained by either a decrease in the efficiency of the CO$_2$ concentration mechanism (leakiness, the proportion of carbon fixed by PEPC, which subsequently leaks out of the bundle sheath cells) or an increase in the ratio of PEPC to CA activity ($V_{p}/V_{h}$; Farquhar, 1983; Cousins et al., 2006a; Ubierna et al., 2011). However, gas-exchange measurements (Fig. 2, B and C) suggest that leakiness does not increase in the CA mutants but is likely lower than in wild-type plants. For example, comparison of in vivo $V_{p,max}$ relative to the light-saturated photosynthesis indicates that, despite the delivery of 30\% and 38\% less CO$_2$ to the bundle sheath cells in $ca1$ and $ca1ca2$, respectively, net rates of CO$_2$ assimilation in mutants are comparable with those in the wild type. This suggests that leakiness in the single and double mutants is less than in the wild type and would impart a decreased $\Delta^{13}$C in the mutants.

Alternatively, higher $V_{p}/V_{h}$ values and uncatalyzed conversion of CO$_2$ to HCO$_3^-$ will increase $\Delta^{13}$C (Fig. 3B). Assuming that $V_{p}/V_{h}$ is near 0 in wild-type plants, the measured $\Delta^{13}$C is effectively modeled with a leakiness of 0.272, which is typical for many C$_4$ grasses (Henderson et al., 1992; Cousins et al., 2008). The measured $\Delta^{13}$C in the $ca1$ mutants is predicted with a $V_{p}/V_{h}$ of 1 (with some CA activity) and leakiness of 70\% of the wild type. However, for the $ca1ca2$ mutants, leakiness is estimated to be approximately 38\% of the wild type. In this case, $\Delta^{13}$C is only modeled with a $V_{p}/V_{h}$ of 1, and there is a significant nonenzymatic fractionation occurring during the hydration of CO$_2$ to HCO$_3^-$.

These data confirm that the CA in the $ca1$ single mutants is just sufficient to supply HCO$_3^-$ to PEPC. However, in the $ca1ca2$ double mutant, there is insufficient CA activity and the supply of HCO$_3^-$ is driven in part by the uncatalyzed reaction. The low CA activity in the $ca1$ and $ca1ca2$ mutants also decreased $\Delta^{18}$O by reducing the isotopic equilibrium between CO$_2$ and the isotopically enriched water within the leaf (Fig. 3, C and D), which is consistent with previous observations in $F. bidentis$ CA mutants (Cousins et al., 2006b). These large differences in isotopic discrimination without drastic effects on CO$_2$ assimilation support the hypothesis that $\Delta^{13}$C and $\Delta^{18}$O are sensitive to reduced CA activity in C$_4$ plants. Additionally, the changes in $\Delta^{13}$C and $\Delta^{18}$O further support the hypothesis that leaf-level CA activity is very low in the $ca1$ and $ca1ca2$ mutants even though the rates of net CO$_2$ assimilation are not significantly impacted at ambient pCO$_2$.

**DISCUSSION**

The genetic and physiological analysis of CA mutants in maize presented here provides, to our knowledge, the first genetic analysis of the catalytic
Figure 3. Decreased CA activity impacts leaf discrimination against $^{13}\text{CO}_2$ and $^{18}\text{O}_2$ in high-p$\text{CO}_2$-grown plants. A and B, Leaf discrimination against $^{13}\text{CO}_2$ ($\Delta^{13}\text{CO}_2$) in relation to CA activity (A) and the ratio of intercellular to ambient p$\text{CO}_2$ (P/$P_a$; B). The solid and dashed lines represent the theoretical relationship between $\Delta^{13}\text{CO}_2$ and P/$P_a$ (see Eq. 3) assuming CA-catalyzed hydration or uncatalyzed hydration, respectively, with $V_r/V_i = 0$ (see Eq. 4). The gray areas delineate theoretical $\Delta^{13}\text{CO}_2$ assuming $V_r/V_i = 1$ and a leakiness ($\phi$) of 0.272 (calculated from wild-type [WT] plants; top limit) or 0.161 (expected in mutants due to lower $V_{p_{\max}}$; bottom limit). Solid and hatched gray areas delineate theoretical $\Delta^{13}\text{CO}_2$ assuming CA-catalyzed hydration or uncatalyzed hydration, respectively (see Eq. 4). C and D, Photosynthetic discrimination against C$^{18}$O$_2$ ($\Delta^{18}\text{O}_2$) in relation to CA activity (C) and the ratio of intercellular to ambient p$\text{CO}_2$ (D).

The result is surprising given that maize has one of the lowest CA activity levels measured (Gillon and Yakir, 2001; Cousins et al., 2008). Thus, by perturbing CA activity in maize, we provide evidence for a limited catalytic requirement of CA in this C$_4$ monocot. This result contrasts with observations in a dicot C$_4$ species with high CA activity, and it remains to be seen how the findings from maize can be extended to other C$_4$ grasses.

Previous studies in maize have used inhibitors to assess the impact of CA on rates of photosynthesis (Badger and Pfanz, 1995; Salama et al., 2006). By limiting zinc availability, a CA limitation can be imposed, as zinc is required for CA catalysis (Salama et al., 2006). However, zinc deficiency also results in a general reduction in photosynthesis and total protein in chickpea (Cicer arietinum; a C$_3$ plant), suggesting that the pleiotropic effects of zinc deficiency, not specifically CA limitation, are responsible for reduced rates of CO$_2$ fixation. Ethoxyzolamide inhibition of CA activity has also been attempted in leaf peels of maize, resulting in reduced photosynthetic oxygen evolution (Badger and Pfanz, 1995). Interestingly, the authors noted that “the reduction in photosynthesis is less than would be expected from the theoretical requirement for CA” (Badger and Pfanz, 1995), suggesting that the ethoxyzolamide inhibition of CA was inefficient. However, despite the nature of the assay, there are several possible explanations for this result, including a limited requirement for CA to provide substrate to PEPC for photosynthesis. These earlier studies highlight the need for genetic mutants that provide a specific reduction in CA activity, in an otherwise wild-type background, without indirectly inhibiting other aspects of photosynthesis.
assimilation when CO₂ delivery into the leaf is reduced. For example, at current pCO₂, CA activity in C₄ monocots may be limiting when the diffusion of CO₂ into the leaf is restricted under environmental stresses such as high temperature and drought. Rates of C₄ photosynthesis in the grasses are especially sensitive to stomatal conductance in these species (von Caemmerer, 2000; von Caemmerer and Furbank, 2003; von Caemmerer et al., 2008). This likely explains why the net CO₂ assimilation in response to changes in intercellular pCO₂ curves of mutant plants grown under elevated CO₂ had normal rates of CO₂ assimilation at ambient CO₂ levels but ca1ca2 mutants had a mild growth phenotype when grown in pots at ambient pCO₂ in the greenhouse, where stomatal limitation is more likely. Because the requirement for CA may depend on stomatal responses, increased levels of CA activity may have a large effect on photosynthetic water use efficiency.

Indeed, a critical role for CA in maintaining photosynthesis under drought may continue to drive selection for CA activity in C₄ grasses. Natural variation in CA activity is potentially beneficial for plant breeders who are tasked with improving yield under stress conditions, such as the record drought seen throughout the midwestern United States in 2012. An analysis of CA variation in maize, especially in drought-tolerant varieties, would reveal if selection has already inadvertently been applied for high CA activity in current breeding programs. Regardless, the examination of the role of CA under water stress will be the focus of future experimentation.

In contrast with modern CA requirements, it is likely that high CA activity in the leaf was essential during the Oligocene epoch, when many C₁ plants likely arose because of low atmospheric pCO₂ (Sage and Coleman, 2001; Edwards et al., 2010; Sage et al., 2012). This is highlighted by the reduction in growth when CA mutants were grown under low pCO₂, (Fig. 2E; Supplemental Fig. S3). Unlike a controlled growth chamber experiment, a continuing rise in global atmospheric pCO₂ will likely be accompanied by climatic changes (drought and temperature) with considerable regional variability (Intergovernmental Panel on Climate Change, 2013), making it difficult to speculate on the requirement for CA in the future. It has been reported that maize is unlikely to benefit from rising pCO₂, except under drought stress (Leakey et al., 2006). This again highlights the importance of investigating the role of CA under temperature and drought stress conditions, which may be a part of future climatic shifts.

The results presented here also impact efforts aimed at engineering C₄ traits into C₃ monocots, which include the introduction of a cytosolic CA into rice (Oryza sativa; Hibberd and Covshoff, 2010; Sage et al., 2012). Not only does this mutant analysis suggest that an increase in CA may not be achieved through transcriptional overexpression due to posttranscriptional regulation, because Ca2 transcript levels do not correlate with CA2 protein accumulation, but it also raises questions about the optimal level of CA. Our results suggest that a low level of CA could be maintained under current ambient pCO₂, but it would be beneficial to have an inducible increase in CA under low-CO₂ conditions. This would allow the plant to conserve resources under moderate growth conditions and respond to stomatal closure with high levels of CA.

MATERIALS AND METHODS

Ds Reverse Genetic Screen

Populations for identifying Ds insertions into Ca genes were generated by test crossing female maize (Zea mays) plants (W22 inbred) lacking Ac with males carrying the Ds donor and Ac-in (Conrad and Brutnell, 2005). A total of 540 purple and spotted test-cross kernels were planted in 10 flats (9 columns by 6 rows; Hummert 11-0770) filled with MetroMix 360 (Hummert 10-0356-1) and Turface MVP (Hummert 10-2400) mixed in a 3:1 ratio by volume. Flats were grown under standard greenhouse conditions at the Boyce Thompson Institute in Ithaca, NY. After approximately 10 d, a single one-inch-thick slice of tissue was harvested from each plant. The tissue from 18 individual plants was combined in a single tube, resulting in a total of 30 pools. DNA was then extracted from these pools using the cetyltrimethyl-ammonium bromide protocol for DNA isolation (available upon request). A two-primer strategy was used to identify new Ds insertions by PCR, where a single target gene primer was paired with a Ds-specific primer. Phire Taq (Thermo Scientific F-122L) was used for amplification following the recommended standard reaction and cycling conditions. PCR products were resolved using standard agarose gel electrophoresis. Pools containing putative insertions were deconvoluted to a single plant using the Phire Plant Direct PCR Kit (Thermo Scientific F-130) following the manufacturer's instructions. Tissue was collected from a leaf other than the one used for the first collection. Finally, the exact location of the new insertion was determined by Sanger sequencing of the amplified insertion product using the Ds end primer.

Mapping Unique Reads to the 3' UTR of Maize Ca Genes

RNA-seq reads from maize leaf tip tissues were obtained as described previously (Wang et al., 2011). Nearly 290 million reads from leaf tips were used to align 125 bp of all 3' UTR sequences of the six maize Ca genes. Alignments were performed using the Burrows-Wheeler Aligner algorithm with default settings (Li and Durbin, 2009). The alignment results were then processed using a custom Perl script to identify unique mapped reads (available upon request). Final counts were normalized by the total number of all reads mapped before filtering.

CA Immunoblot Analysis

Maize seedlings were grown in a BDW-40 chamber (Conviron) with a 12-h day/night period. The chamber was set for 31°C/22°C day/night temperatures with a light intensity of 550 μmol m⁻² s⁻¹ and relative humidity of 50%. The plants were watered as needed and grown at ambient pCO₂. Two-week-old leaf blade tissue was flash frozen in liquid nitrogen and ground in a Fluid Management Harbil 5G-HD paint shaker. Protein extracts were prepared in buffer containing 50 mM HEPES-KOH (pH 7.5), 5 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride. Extracts were centrifuged at 17,000 g for 15 min, and the resulting protein concentration was measured using the Qubit 2.0 Fluorometer (Life Technologies). Samples were normalized using SDS sample buffer containing 5% (v/v) β-mercaptoethanol, and 1 μg of total protein of each sample was loaded onto NuPAGE Novex Bis-Tris Gels (Life Technologies). Proteins were transferred to nitrocellulose membranes at 200 V for 1 h and challenged overnight with a primary polyclonal antibody (from rabbit) made against rice (Oryza sativa) CA at a dilution of 1:10,000 (generously provided by Jim Burnell). The secondary anti-rabbit IgG horseradish peroxidase-linked whole antibody (from donkey; GE Healthcare) was used at a dilution of 1:50,000. Amersham ECL Prime Western Blotting Detection Reagents (GE Life Sciences) were used, and labeled proteins were visualized with the Bio-Rad Gel Doc XR+ System.
Maize Carbonic Anhydrase Mutants

Photosynthetic isotope discrimination against $^{13}$CO$_2$ was modeled according to the C$_4$ isotope model (Farquhar, 1983), including the ternary effect (Farquhar and Cerанс, 2012):

$$\Delta^{13}C_{\text{iso}} = \frac{1}{1 - (1 - \frac{P_e - P_a}{P_e - P_b})} \left( \frac{P_e - P_a}{P_e - P_b} \right) \left( c_a + \phi \right) \frac{P_l - P_a}{P_a}$$

$$+ \frac{1}{1 - (\frac{P_e - P_a}{P_e - P_b})} \left( \frac{P_e - P_a}{P_e - P_b} \right) \left( c_a + \phi \right) \frac{P_l - P_a}{P_a}$$

where $P_e$, $P_a$, $P_b$, and $P_l$ are the pCO$_2$ in the atmosphere, intercellular space, bundle sheath cells, and mesophyll cells, respectively, and $c_a$ (1.1‰), $a_o$ (2.9‰), $a_i$ (4.4‰), $a_l$ (0.7‰), and $s$ (1.8%) are the fractionations associated with CO$_2$ diffusion through the dissolution, leaf boundary layer, diffusion in air, aqueous diffusion, and leakage from the bundle sheath cells, respectively. Rubisco fractionation ($b_i$) is calculated as $b_i = \frac{(P_l - P_e) + (P_e - P_f)}{V_o}$ with $P_l$ (30‰), $e$ (9%), and $f$ (11.6‰) as the fractionation associated with Rubisco, respiration, and photorespiration, respectively, and $R_a$, $V_o$, and $V_e$ as the rates of respiration, Rubisco oxygenation, and carboxylation, respectively (von Caemmerer, 2000). The fractionation of PEP, respiration, and the isotopic equilibrium during the dissolution of CO$_2$ and the conversion to HCO$_3^-$ ($b_i$) is calculated (Farquhar, 1983; Cousins et al., 2006a) as:

$$b_i = (b_e + c_o + c_l) \left( \frac{V_o - V_e}{V_o} \right) \left( 1 - \frac{V_e}{V_o} \right)$$

where $b_e$ (2.2‰) is the fractionation by PEPC, O’Leary, 1981. $c_o$ (1.1‰) is the fractionation as CO$_2$ dissolves (O’Leary, 1984), and $c_l$ (1.9‰) is the equilibrium fractionation factor of the CA-catalyzed hydration/dehydration reactions of CO$_2$ and HCO$_3^-$ (Mook et al., 1974). Alternatively, during the hydration/dehydration reactions, the uncatalyzed equilibrium fractionation factor $c_l$ is $-7.8‰$ (Marler and O’Leary, 1984). The fractionation when CO$_2$ and HCO$_3^-$ are not at equilibrium is dependent on the rate of CA-mediated CO$_2$ hydration ($V_o$), the rate of PEPC ($V_p$), $c_l$, and the catalyzed fractionation during CO$_2$ hydration ($h$). The catalyzed hydration reaction has a fractionation factor of 1.1‰ (calculated by summing the catalyzed CO$_2$ and HCO$_3^-$ equilibrium fractionation factor $-9.0‰$ and the catalyzed dehydration fractionation factor 10.1‰; Mook et al., 1974; Paneth and O’Leary, 1965), whereas the uncatalyzed reaction has a 6.9‰ fractionation factor (Marler and O’Leary, 1984). The fractionation attributed to mitochondrial respiration is $r$ at a rate of mesophyll CO$_2$ release of $R_m$.

Rubisco and PEPC Activity Assays

Leaf discs were extracted similarly to the CA assays described above. However, Rubisco activity was spectrophotometrically measured according to Waller et al. (2013) in 100 µL 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid, pH 8.0, 20 mM MgCl$_2$, 1 mM EDTA, 1 mM ATP, 5 mM creatine phosphate, 20 mM NaHCO$_3$, 0.5 mM ribulose 5-phosphate, 0.2 mM NADH, 12.5 units mL$^{-1}$ creatine phosphate kinase, 250 units mL$^{-1}$ CA, 22.5 units mL$^{-1}$ phosphoglycerokinase, 20 units mL$^{-1}$ glyceraldehyde-3-phosphodehydrogenase, 56 units mL$^{-1}$ triose isomerase, and 20 units mL$^{-1}$ glycerol-3-phosphodehydrogenase. PEPC activity was assayed in 100 µL 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid, pH 8.0, 20 mM MgCl$_2$, 1 mM EDTA, 5 mM NaHCO$_3$, 0.2 mM NADH, 5 mM Glc-6-P, 12 units mL$^{-1}$, 1-malate dehydrogenase, and 4 µM phosphoenolpyruvate. NADH consumption was monitored at 340 nm.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression profile of the tandemly duplicated CA genes in Z. mays.

Supplemental Figure S2. Net rates of CO$_2$ assimilation in response to changes in PAR.

Supplemental Figure S3. Dry weight biomass of mutant plants grown at low CO$_2$.

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An error in the activity assay calculation resulted in the values of all samples in this article being underestimated by 5. As a result, the statement in the second paragraph on p. 610 should read: “The CA activity of wild-type plants was approximately 1,100 to 1,200 μmol m⁻² s⁻¹ in the soluble fraction.” The revised Figures 2A and 3, A and C, included on the following pages, have corrected axes.
Figure 2. Photosynthetic measurements predict plant growth phenotypes in CA mutant plants. A, CA activity of CA single and double mutant lines grown under elevated CO$_2$ (928 Pa). Solid and hatched bars show CA activity in the soluble and membrane fractions, respectively. B, Net CO$_2$ assimilation ($A_{\text{net}}$) in response to changes in intercellular pCO$_2$ ($P_i$; $A$-$P_i$ curve) measured at an oxygen partial pressure of 18.6 kPa, leaf temperature of 25°C, and irradiance of 1,000 $\mu$mol m$^{-2}$ s$^{-1}$. C, Maximum in vivo $V_{\text{pmax}}$ estimated from the initial slope of the $A$-$P_i$ curve and plotted for each CA mutant line. D, Wild-type and homozygous CA double mutant plants grown in the greenhouse at ambient pCO$_2$. E, CA double mutant plants grown at low CO$_2$ (9.3 Pa).
Figure 3. Decreased CA activity impacts leaf discrimination against $^{13}$CO$_2$ and C$^{18}$O$_2$ in high-pCO$_2$-grown plants. A and B. Leaf discrimination against $^{13}$CO$_2$ ($\Delta^{13}$CO$_2$) in relation to CA activity (A) and the ratio of intercellular to ambient pCO$_2$ ($P_i/P_a$; B). The solid and dashed lines represent the theoretical relationship between $\Delta^{13}$CO$_2$ and $P_i/P_a$ (see Eq. 3) assuming CA-catalyzed hydration or uncatalyzed hydration, respectively, with $V_p/V_h = 0$ (see Eq. 4). The gray areas delineate theoretical $\Delta^{13}$CO$_2$ assuming $V_p/V_h = 1$ and a leakiness ($\phi$) of 0.272 (calculated from wild-type [WT] plants; top limit) or 0.161 (expected in mutants due to lower $V_p$; bottom limit). Solid and hatched gray areas delineate theoretical $\Delta^{13}$CO$_2$ assuming CA-catalyzed hydration or uncatalyzed hydration, respectively (see Eq. 4). C and D. Photosynthetic discrimination against C$^{18}$O$_2$ ($\Delta C^{18}$O$_2$) in relation to CA activity (C) and the ratio of intercellular to ambient pCO$_2$ (D).