RESEARCH PAPER

Effects of reduced carbonic anhydrase activity on CO$_2$ assimilation rates in *Setaria viridis*: a transgenic analysis

Hannah L. Osborn$^1$, Hugo Alonso-Cantabrana$^{1,*}$, Robert E. Sharwood$^1$, Sarah Covshoff$^2$, John R. Evans$^1$, Robert T. Furbank$^1$ and Susanne von Caemmerer$^1$

$^1$ Australian Research Council Centre of Excellence for Translational Photosynthesis, Division of Plant Sciences, Research School of Biology, The Australian National University, Acton, ACT 2601, Australia

$^2$ Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, UK

$^*$ Correspondence: Hugo.Alonso@anu.edu.au

Received 19 July 2016; Accepted 5 September 2016

Editor: Christine Raines, University of Essex

Abstract

In C$_4$ species, the major β-carbonic anhydrase (β-CA) localized in the mesophyll cytosol catalyses the hydration of CO$_2$ to HCO$_3^-$, which phosphoenolpyruvate carboxylase uses in the first step of C$_4$ photosynthesis. To address the role of CA in C$_4$ photosynthesis, we generated transgenic *Setaria viridis* depleted in β-CA. Independent lines were identified with as little as 13% of wild-type CA. No photosynthetic defect was observed in the transformed lines at ambient CO$_2$ partial pressure ($p$CO$_2$). At low $p$CO$_2$, a strong correlation between CO$_2$ assimilation rates and CA hydration rates was observed. C$^{18}$O$^{16}$O isotope discrimination was used to estimate the mesophyll conductance to CO$_2$ diffusion from the intercellular air space to the mesophyll cytosol ($g_m$) in control plants, which allowed us to calculate CA activities in the mesophyll cytosol ($C_m$). This revealed a strong relationship between the initial slope of the response of the CO$_2$ assimilation rate to cytosolic $p$CO$_2$ ($AC_m$) and cytosolic CA activity. However, the relationship between the initial slope of the response of CO$_2$ assimilation to intercellular $p$CO$_2$ ($AC_i$) and cytosolic CA activity was curvilinear. This indicated that in *S. viridis*, mesophyll conductance may be a contributing limiting factor alongside CA activity to CO$_2$ assimilation rates at low $p$CO$_2$.

Key words: Carbonic anhydrase, C$^{18}$O$^{16}$O isotope discrimination, C$_4$ photosynthesis, mesophyll conductance, *Setaria viridis*, transformation

Introduction

C$_4$ plants have evolved a CO$_2$-concentrating mechanism (CCM) that enables the elevation of CO$_2$ around the active sites of Rubisco by a combination of anatomical and biochemical specialization (Hatch, 1987). C$_4$ photosynthesis has independently evolved >60 times, providing one of the most widespread and effective solutions for remediating the catalytic inefficiency of Rubisco (Sage et al., 2012; Christin and Osborne, 2013). The key carboxylases in C$_4$ plants are localized to different cellular compartments. Phosphoenolpyruvate carboxylase (PEPC) is localized to the cytosol of mesophyll cells and Rubisco to the chloroplasts of bundle sheath cells. For the CCM to operate effectively, PEPC activity must exceed Rubisco activity to balance leakage of CO$_2$ out of the bundle sheath compartment. This maintains a high bundle sheath CO$_2$ level but prevents wasteful overcycling of the mesophyll CO$_2$ "pump" (von Caemmerer and Furbank, 2003). As PEPC utilizes HCO$_3^-$ and not CO$_2$, the first committed enzyme of the C$_4$ pathway is carbonic anhydrase (CA) which...
catalyses the reversible conversion of \( \text{CO}_2 \) and \( \text{HCO}_3^- \) in the cytosol of mesophyll cells. \( \text{C}_4 \) acids produced by PEPC then diffuse into the bundle sheath cells where they are decarboxylated, supplying \( \text{CO}_2 \) for Rubisco.

Within higher plants, there are multiple forms of the \( \alpha \)-CA, \( \beta \)-CA, and \( \gamma \)-CA families which share little sequence homology (Moroney et al. 2001). \( \beta \)-CAs are the most prevalent CA family in land plants. CA is an abundant enzyme in \( \text{C}_3 \) plants, representing up to 2% of the soluble leaf protein (Okabe et al., 1984). In \( \text{C}_4 \) plants, the role of CA is unclear (Badger and Price, 1994) as it does not appear to limit photosynthesis but does influence stomatal conductance, guard cell movement, and amino acid biosynthesis (Hu et al., 2010; DiMario et al., 2016; Engineer et al., 2016).

It has long been contended that the uncatalysed rate of \( \text{CO}_2 \) conversion to \( \text{HCO}_3^- \) is insufficient to support \( \text{C}_4 \) photosynthetic flux (Hatch and Burnett, 1990; Badger and Price, 1994). This hypothesis was supported by experiments in the \( \text{C}_4 \) dicot \( \text{Flaveria bidentis} \), where antisense plants with <10% of wild-type CA activity required high \( \text{CO}_2 \) for growth and showed reduced \( \text{CO}_2 \) assimilation rates (von Caemmerer et al., 2004; Cousins et al., 2006). However, in the \( \text{C}_4 \) monocot \( \text{Zea mays} \) mutant plants with reduced CA activity (3% of wild type), no limitation to \( \text{CO}_2 \) assimilation rates at ambient \( \text{CO}_2 \) was observed (Studer et al., 2014). CA activity has been shown to vary widely between species (Cousins et al., 2008), and it is unclear whether CA activities are limiting at high \( \text{CO}_2 \) assimilation rates, as has previously been suggested (Hatch and Burnett, 1990; Gillon and Yakir, 2000).

We examined the role of CA in the model \( \text{C}_4 \) monocot species \( \text{Setaria viridis} \) (green foxtail millet). \( \text{Setaria viridis} \) is closely related to agronomically important \( \text{C}_4 \) crops including \( \text{Z. mays} \) (maize), \( \text{Sorghum bicolor} \) (sorghum), and \( \text{Saccharum officinarum} \) (sugarcane) (Brutnell et al., 2010). It is an ideal model species due to its rapid generation time, small stature, high seed production, diploid status, and small genome that is sequenced and publicly available (Douست et al., 2009; Brutnell et al., 2010; Li and Brutnell, 2011). Here we used a stable transformation approach to examine the role of CA in \( \text{S. viridis} \) and could show that \( \text{S. viridis} \) is a useful model species that lends itself to molecular manipulation of the \( \text{C}_4 \) photosynthetic pathway. Two constructs both targeting the major leaf \( \beta \)-CA (Si003882m.g) were used to generate three independent transformed lines with reduced CA activity. A strong correlation between the \( \text{CO}_2 \) assimilation rate at low \( p\text{CO}_2 \) and CA activity was observed. Our combined measurements of mesophyll conductance and CA activity suggest that increasing mesophyll conductance may be an important way to increase the \( \text{CO}_2 \) assimilation rate at low intercellular \( p\text{CO}_2 \), as may occur under drought.

Materials and methods

Plant growth conditions

\( T_1 \) seeds were incubated in 5% liquid smoke (Wrights) for 24 h to promote germination, and germinated in garden soil mix fertilized with Osmocote (Scotts, Australia) in small containers before being transferred to individual 2 litre pots. Plants were grown in controlled environmental chambers, irradiance 500 \( \mu\text{mol} \) photons \( \text{m}^{-2} \text{s}^{-1} \), 16 h photoperiod, 28 °C day, 24 °C night, 2% \( \text{CO}_2 \). Pots were watered daily.

Construct generation

Two different constructs were used to generate three lines of reduced CA activity. First, an RNAi was targeted to the primary leaf \( \beta \)-CA Si003882m.g which generated lines 2.1 and 5.3. A region of Si003882m.g was amplified by PCR using gene-specific primers (Supplementary Table S1 at JXB online) and reverse-transcribed RNA from \( \text{S. viridis} \) leaves ligated into pENTR/D-TOPO (ThermoFisher), and verified by sequencing. The fragment was inserted via a double Gateway system LR reaction (Invitrogen) into the hairpin RNAi binary vector pSTARGATE (Greenup et al., 2010) to form a stem–loop region under the control of the ubiquitin promoter/intron (UBI) and octopine synthase (OCS) terminator to form the RNAi vector pSGiCAa.

Secondly, an overexpression approach, which resulted in gene silencing, generated the third transformed line, 1.1. The coding sequence of the maize \( \beta \)-CA gene (GRMZM2G348512), \( \text{ZmCA2} \) (Studer et al., 2014), was amplified by reverse transcription–PCR (RT–PCR) from total RNA extracted from B73 maize. Total RNA was isolated using hot acid phenol and chloroform, and then treated with RNase-free DNase (Promega). The reverse transcription and PCRs were performed as per the manufacturer’s protocols with Superscript II (ThermoFisher) and Phusion High-Fidelity DNA polymerase (NEB), respectively (for primers, see Supplementary Table S1). The sequence encoding an AcV5 epitope tag (Lawrence et al., 2003) was added to the C-terminal end of \( \text{ZmCA2} \). The resulting \( \text{ZmCA2} \) ampiclon was cloned into pENTR/D-TOPO and verified by sequencing. LR Gateway cloning (ThermoFisher) was used to insert the \( \text{ZmCA2} \) coding sequence into the overexpression vector, pSC110. pSC110 was created by Gibson Assembly (Gibson et al., 2009) from two modified pmDC164 vectors (Curtis and Grossniklaus, 2003), kindly provided to us by Udo Gowik (Heinrich-Heine University, Düsseldorf, Germany). \( \text{ZmCA2} \) expression from pSC110 was driven by the B73 \( \text{ZmPEPC} \) promoter. pSC110 and pSC110/ZmCA2 were verified by sequencing.

Both constructs were transformed into \( \text{Agrobacterium tumefaciens} \) strain AGL1 for stable plant transformation.

Callus induction and plant transformation

Stable transformation of \( \text{S. viridis} \) (accession A10.1) was carried out as described by Brutnell et al. (2010). Seed coats were mechanically removed from mature \( \text{S. viridis} \) seeds to improve germination. Seeds were sterilized before plating on callus induction medium (CIM; 4.3 g l\(^{-1}\) Murashige and Skoog (MS) salts, pH 5.8, 10 ml l\(^{-1}\) 100× MS vitamins stock, 40 g l\(^{-1}\) maltose, 35 mg l\(^{-1}\) \( \text{ZnSO}_4 \), 0.6 mg l\(^{-1}\) \( \text{CuSO}_4 \), 4 g l\(^{-1}\) Gelzan, 0.5 mg l\(^{-1}\) kinetin, 2 mg l\(^{-1}\) 2,4-D). After 4 weeks in the dark at 24 °C, any seedling structures or gelatinous calli were removed and remaining calli transferred to fresh CIM. After a further 2 weeks, calli were divided and replated onto fresh CIM. One week later, transformations were performed.

AGL1 containing the construct of interest were grown in the presence of 50 \( \mu\text{g} \) l\(^{-1}\) kanamycin and 50 \( \mu\text{g} \) l\(^{-1}\) rifampicin at 28 °C to \( \text{OD}_{600} = 0.5 \) and then resuspended in CIM without Gelzan and hormones. Acetosyringone (200 mM) and spermycin [0.01% (w/v)] were added to the \( \text{Agrobacterium} \) solution before inculating the calli in the medium for 5 min at room temperature. The calli were blotted dry on sterile filter paper and incubated at 22 °C for 3 d in the dark. The calli were then transferred to selective CIM (CIM containing 40 mg l\(^{-1}\) hygromycin, 150 mg l\(^{-1}\) timentin) and incubated in the dark at 24 °C for 16 d. Calli were then transferred to selective plant regeneration medium (PRM) containing 4.3 g l\(^{-1}\) MS salts, pH 5.8, 10 ml l\(^{-1}\) 100× MS vitamins, 20 g l\(^{-1}\) sucrose, 7 g l\(^{-1}\) Phytoblend, 2 mg l\(^{-1}\) hygromycin. Calli were maintained at 24 °C under a 16 h light:8 h dark photoperiod and a light
intensity of 60 µmol photons m$^{-2}$ s$^{-1}$. Developing shoots were transferred to selective rooting medium (RM) containing 2.15 g l$^{-1}$ MS salts, pH 5.7, 10 ml l$^{-1}$ 100× MS vitamins, 30 g l$^{-1}$ sucrose, 7 g l$^{-1}$ Phytoblend, 150 mg l$^{-1}$ timentin, 20 mg l$^{-1}$ hygromycin. Shoots that survived and developed roots were genotyped using primers against the hygromycin phosphotransferase gene (Supplementary Table S1) by PCR, and positive transformants were transplanted to soil.

Selection of plants for analysis

The progeny of three independent T$_0$ transformation events were analysed for CA hydration rates (Supplementary Fig. S1). One T$_1$ plant with low CA hydration rates was selected from each transformation event (labelled 5.3, 2.1, and 1.1) and its progeny (T$_2$) used for all future analysis. Two sets of experiments were performed on the T$_2$ plants. First, gas exchange and biochemical analysis on lines 5.3, 2.1, and 1.1 (Table 1) and, secondly, gas exchange and oxygen discrimination on lines 5.3 and 1.1 (Table 2). Each T$_2$ plant was genotyped prior to experiments using primers against the hygromycin phosphotransferase gene (Supplementary Table S1). The progeny of a plant which went through the S. viridis transformation process and tested negative for the hygromycin phosphotransferase gene were used as null controls.

Insertion number estimation

DNA was isolated from a fully expanded leaf using a CTAB (cetyltrimethylammonium bromide) extraction buffer [2% CTAB (v/v), 20 mM Tris–HCl pH 8, 1.4 M NaCl, 20 mM EDTA, 1% polyvinylpyrrolidone (PVP)-40 (w/v), 0.2% (v/v) β-mercaptoethanol] followed by extraction with phenol/chloroform/isooamylalcohol (25:24:1) and ethanol clean-up. DNA quality and quantity was determined using a NanoDrop spectrophotometer (Thermo Scientific).

DNA was removed using the TURBO DNA free kit (Ambion). DNA extraction with phenol/chloroform/isoamylalcohol (25:24:1) and the IPC (the Delta Ct) was used to allocate the assayed samples into groups with the same gene copy number.

Table 1. Physiological and biochemical characteristics of CA transformants at ambient CO$_2$ conditions

|   | $A$ µmol m$^{-2}$ s$^{-1}$ | $g_s$ mol m$^{-2}$ s$^{-1}$ | $C_m$ µbar | $k_{CA}$ mol m$^{-2}$ s$^{-1}$ bar$^{-1}$ | Rubisco µmol m$^{-2}$ s$^{-1}$ | PEPC µmol m$^{-2}$ s$^{-1}$ | NADP-ME µmol m$^{-2}$ s$^{-1}$ |
|---|---|---|---|---|---|---|---|
| Null | 22.5 ± 0.6 a | 0.19 ± 0.01 a | 132.4 ± 3.3 a | 6.1 ± 0.8 a | 18.7 ± 1.5 a | 229.6 ± 19.3 a | 59.8 ± 4.3 a |
| 5.3 | 21.7 ± 2.6 a | 0.2 ± 0.02 a | 118.9 ± 13.1 a | 3.3 ± 0.2 b | 18.8 ± 1.8 a | 249.3 ± 24.6 a | 54.5 ± 5.8 a |
| 2.1 | 18.5 ± 1.9 a | 0.16 ± 0.01 a | 152.9 ± 15.2 a | 2.0 ± 0.2 b,c | 20.9 ± 2.9 a | 181.5 ± 25.4 a | 47.3 ± 2.6 a |
| 1.1 | 19.1 ± 1.2 a | 0.19 ± 0.02 a | 153.9 ± 4.4 a | 0.8 ± 0.1 c | 19.7 ± 1.8 a | 180.3 ± 18.4 a | 43.6 ± 3.9 a |

Significant differences are based on one-way ANOVA and Tukey post-hoc analysis (SPSS statistics version 22; P=0.05).

Table 2. Physiological characteristics of CA transformants at ambient CO$_2$ measured using LI-6400XT coupled to a tunable diode laser

|   | $A$ µmol m$^{-2}$ s$^{-1}$ | $g_s$ mol m$^{-2}$ s$^{-1}$ | $C_m$ µbar | $C/C_a$ µbar | $k_{CA}$ mol m$^{-2}$ s$^{-1}$ bar$^{-1}$ | $\Delta^{13}$O % | $S_m$ m$^2$ m$^{-2}$ |
|---|---|---|---|---|---|---|---|
| Null | 30.0 ± 1.4 a | 0.30 ± 0.03 a | 144.6 ± 5.9 a | 0.39 ± 0.03 a | 8.4 ± 0.7 a | 18.0 ± 1.4 a | 10.2 ± 0.4 a |
| 5.3 | 29.2 ± 0.9 a | 0.29 ± 0.02 a | 157.9 ± 10.5 a | 0.34 ± 0.01 a | 2.5 ± 0.3 b | 13.6 ± 0.7 a,b | – |
| 1.1 | 24.5 ± 1.6 a | 0.26 ± 0.03 a | 178.1 ± 13.5 a | 0.43 ± 0.02 a | 0.8 ± 0.2 b | 10.9 ± 0.6 b | 10.2 ± 0.9 a |

Significant differences are based on one-way ANOVA and Tukey post-hoc analysis (SPSS statistics version 22; P=0.05).
to the manufacturer’s instructions. Primers (Supplementary Table S1) were designed using Primer3 in Geneious R7.1.6, ensuring products spanned an intron. Primer amplification efficiencies were determined by the Ct slope method; efficiencies for all primer pairs were comparable (~95%) and no amplification was detected in the no template control. Relative fold change was calculated by the ΔΔCt method, using the average of three nulls as reference, as described by Livak and Schmittgen (2001). The geometric mean of the Ct values for three reference genes was used for normalization (Vandesompele et al., 2002). Statistics were performed with SigmaPlot (version 11.0).

**Determination of enzyme activities**

For CA activity, leaf discs (0.78 cm²) were collected from the uppermost fully expanded leaf of 5-week-old *S. viridis* plants and frozen in liquid nitrogen. Soluble protein was extracted by grinding one frozen leaf disc in ice-cold glass homogenizers (Tenbroek) in 500 μl of extraction buffer [50 mM HEPES, pH 7.8, 1% (v/v) PVP, 1mM EDTA, 10 mM dithiothreitol, 0.1% (v/v) Triton X-100, 2% (v/v) protease inhibitor cocktail (Sigma)]. Crude extracts were centrifuged at 4 °C for 1 min at 13 000 g and the supernatant collected for the soluble CA assay. Activity was measured on a membrane inlet mass spectrometer (TIMS; Finnigan MAT Delta V, Thermo Scientific, Bremen, Germany) to measure the rates of 18O exchange from labelled 1C18O2 to H218O at 25 °C (Badger and Price, 1989; von Caemmerer et al., 2004). The hydration rates were calculated as described by Jenkins et al. (1989).

For Rubisco, PEPC, and NADP-malic enzyme (ME) activities, soluble protein was extracted from fresh leaf discs collected from leaves used for gas exchange analysis. Spectrophotometric assays were then performed as described previously (Pengelly et al., 2010, 2012; Sharwood et al., 2016).

**Gas exchange measurements**

Net photosynthesis (A) was measured over a range of intercellular pCO2 (Ci) on the uppermost, fully expanded leaf of 5-week-old *S. viridis* plants using a portable gas exchange system LI-COR 6400XT (LI-COR Biosciences). Measurements were made after leaves had equilibrated at 380 μbar, flow rate 500 μmol s⁻¹, leaf temperature 25 °C, and irradiance 1500 μmol photons m⁻² s⁻¹. CO2 response curves were measured in a stepwise increase (3 min intervals) in CO2 partial pressure 380, 0, 23.75, 47.5, 71.25, 95, 142.5, 190, 285, 380, 570, 760, and 950 μbar whilst maintaining leaf temperature and irradiance conditions.

**Measurements of C18O16O discrimination (Δ18O)**

Simultaneous measurements of exchange of CO2, H2O, C18O16O2, and H16O18O were made by coupling two LI-6400XT gas exchange systems to a tunable diode laser system: TGA200A, Campbell Scientific Inc., Logan, UT, USA) to measure C18O16O and a Cavity Ring-Down Spectrometer (LI2130-1, Picarro Inc., Sunnyvale, CA, USA) to measure the oxygen isotope composition of water vapour. The system is essentially that described by Luzo et al. (2011) except that the TGA100 was replaced by TGA200A and the additional laser for water vapour measurements has been added together with a 16 port distribution manifold. To generate gas flows to the gas exchange systems, N2 and O2 were mixed by mass flow controllers (Omega Engineering Inc., Stanford, CT, USA) to generate CO2-free air with 2% O2. The humidity of incoming air was adjusted by varying the temperature of water circulating around a Nafion tube (Permupare, MH-110-12P-4) but was kept constant in this set of experiments to supply water vapour of a constant 18O composition. To supply flow to the TDL and the LI2130-1 from the sample and reference gas streams, two T junctions were inserted into the match valve tubing and in the reference line of the LI-6400XT, respectively. This allowed leaves of two plants to be measured in sequence, with each LI-6400XT sampled by the TDL at 4 min intervals for 20s at the sample and reference line. The Picarro Cavity Ring Down spectrometer sampled for 3 min, so that leaves were sampled at 6 min intervals. Supplementary Fig. 5 shows the CO2 dependence of the standard error of 18O of CO2 in the reference gas of repeated measurements on the TGA200A. The 18O isotopic composition of the CO2 calibration gas was 22.17 ± 0.04‰ for Vienna mean oceanic water (VSMOW) and was checked against standards on an Isoprime mass spectrometer. We monitored the 18O composition of water vapour of the reference air streams daily, and the values were −6.07 ± 0.08‰ and −6.34 ± 0.08‰ (VSMOW) for LI-6400XT L1 and L2 references, respectively. We attribute the small difference between the reference lines to differences in the Nafion tubing. At the end of the experiment, the calibration of the Picarro L2130-1 was confirmed by collecting water vapour samples from the gas stream of the LI-6400XT reference lines going to the Picarro as described by Cousins et al. (2006) and assaying these water samples against standards on a Picarro 1102i, which was set up to measure the 18O isotopic composition of water samples.

Gas exchange was measured on the uppermost fully expanded leaf of 5-week-old *S. viridis* plants at 25 °C, and leaves were equilibrated at ambient CO2 (380 μbar), irradiance 1500 μmol photons m⁻² s⁻¹, and 2% O2. The flow rate was 200 μmol s⁻¹. CO2 concentration was adjusted from 380 to 760, 570, 380, and 190 μbar at 1 h intervals. Immediately following gas exchange measurements, leaf discs were collected and stored at −80 °C until measurements of CA activity were made.

Calculations of C18O16O/16O (Δ18O) discrimination and mesophyll conductance (gm)

Discrimination against 18O in CO2 during photosynthesis, Δ18O, was calculated from the isotopic composition of the CO2 entering δin and exiting δout the leaf chamber and the CO2 concentration entering Ci and exiting C (all measured with the TDL) (Evans et al., 1986; Barbour et al., 2016):

$$\Delta^{18}O = \frac{\xi(\delta_{\text{out}} - \delta_{\text{in}})}{1 + \delta_{\text{out}} - \xi(\delta_{\text{out}} - \delta_{\text{in}})}$$

where $\xi = C_{\text{air}}/C_{\text{in}} - C_{\text{out}}$. Sample streams were passed through a Nafion drying tube before entering the TDL, and CO2 values presented are all at zero water vapour concentration.

Following the derivation by Barbour et al. (2016) and Farquhar and Cernusak (2012) photosynthetic Δ18O discrimination was used to calculate pCO2 in the mesophyll cytosol, Cm, with the assumption that $C_m$ is equal to the $pCO_2$ at the site of $CO_2$–H2O exchange and assuming that cytosolic CO2 is in full isotopic equilibrium with local cytosolic water. This allowed g_m to be calculated from

$$g_m = A / (C_i - C_m)$$

$$C_m = C \left( \frac{\delta_i - a_{\delta_i} - \delta_\Lambda (1 + a_{\delta_i})}{\delta_i - a_{\delta_i} - \delta_\Lambda (1 + a_{\delta_i})} \right)$$

Equation 3 is the same as equation 21 of Barbour et al. (2016), and is a rearrangement of equation 18 of Farquhar and Cernusak (2012) using their notation. The oxygen isotope ratios are expressed relative to the standard, (VSMOW) ($\delta_i = \left( \frac{^{18}O/^{16}O}{^{18}O/^{16}O}_{\text{VSMOW}} \right) - 1$).

Intercellular $pCO_2$ is denoted by $C_i$, and $a_\delta$ is the discrimination against $C^{18}O$ during liquid phase diffusion and dissolution (0.8‰).

The isotopic composition of CO2 being assimilated, $\delta_\Lambda$, is given by

$$\delta_\Lambda = \delta_{\text{out}} - \Delta^{18}O / 1 + \Delta^{18}O.$$
where $\delta_a$ is the isotopic composition of ambient air (in our case $\delta_a=\delta_{out}$).

The oxygen isotope composition of CO$_2$ in the intercellular airspaces, $\delta_c$, including ternary corrections proposed by Farquhar and Cernusak (2012), is given by

$$\delta_c = \delta_i + \left[ \frac{\delta_A C + 1}{C_i} - \delta_{c} C_C \right] \frac{1 + t}{1 + t}$$  \hspace{1cm} (5)

where $C_a$ is the $p$CO$_2$ in the ambient air. The ternary correction factor, $t$, is given by

$$t = \frac{1 + \alpha_{lbs}}{1000} E$$

where $g_{lbs}$ is the total conductance to CO$_2$, $E$ the transpiration rate, and $\alpha_{lbs}$ is the weighted discrimination of C$^{18}$O$^3$HO diffusion across the boundary layer and stomata in series given by:

$$\alpha_{lbs} = \frac{(C_a - C_i)\alpha_{lbs} - (C_a - C_i)\alpha_{lbs}}{(C_a - C_i)}$$  \hspace{1cm} (7)

where $C_a$ is the $p$CO$_2$ at the leaf surface and $\alpha_{lbs}$ and $\alpha_{lbs}$ are the discriminations against C$^{18}$O$^3$HO through stomata and the boundary layer (8‰ and 5.8‰, respectively).

The isotopic composition of intercellular CO$_2$ ignoring ternary corrections is given by

$$\delta_t = \delta_A C + 1 - \delta_{c} C_C \frac{\delta_i - \alpha_{lbs}}{1}$$  \hspace{1cm} (8)

To calculate $C_m$, we assume that the isotopic composition of CO$_2$ in the cytosol, $\delta_c$, is the isotopic composition of CO$_2$ equilibrated with cytosolic water, $\delta_w$, and

$$\delta_c = \delta_w + \epsilon_w$$  \hspace{1cm} (9)

where $\delta_w$ is the stable oxygen isotope composition of water in the cytosol at the site of evaporation and $\epsilon_w$ is the isotopic equilibrium between CO$_2$ and water (dependent on temperature $T_K$ in K (Barbour et al., 2016, and references therein)).

$$\epsilon_w (‰) = \frac{17604}{T_K} - 17.93$$  \hspace{1cm} (10)

Calculation of the isotopic composition of water at the site of evaporation from the isotopic composition of transpired water

The isotopic composition of water at the site of evaporation, $\delta_w$, can be estimated from the Craig and Gordon model of evaporative enrichment (Craig and Gordon, 1965; Farquhar and Lloyd, 1993)

$$\delta_w = \delta_i + \epsilon^* + \epsilon_k + \frac{\epsilon_i}{\epsilon_k} (\delta_{ns} - \delta_k)$$  \hspace{1cm} (11)

where $\epsilon^*$ is the equilibrium fractionation during evaporation, $\epsilon_k$ is the kinetic fractionation during vapour diffusion in air, $\delta_i$ is the oxygen isotopic composition of transpired water, $\epsilon_i/\epsilon_k$ is the ratio of ambient to intercellular vapour pressure, and $\delta_{ns}$ is the isotopic composition of ambient air. $\epsilon^*$ is dependent on stomatal and boundary layer conductances and associated fractionation factors (Barbour et al., 2016, and references therein):

$$\epsilon_k = \frac{28g_{lbs}^{-1} + 19g_{lbs}^{-1}}{g_{lbs}^{-1}}$$  \hspace{1cm} (13)

The isotopic composition of transpired water $\delta_i$ can be calculated from mass balance knowing the isotopic composition of the water entering $\delta_{in}$ and exiting $\delta_{out}$ the leaf chamber (measured with the Picarro) and the water vapour concentration entering $w_{in}$ and exiting $w_{out}$ (measured with the LI-6400XT):

$$\delta_i = \left( \frac{\delta_{w}w_{in} - \delta_{w}w_{out}}{w_{out} - w_{in}} \right)$$

Calculation of the proportion of mesophyll cytosolic CO$_2$ in equilibration with leaf water, $\theta$

If $C_m$ is known, it is possible to calculate the isotopic composition of cytosolic CO$_2$ from measurements of $\Delta^{18}$O using equation 18 from Farquhar and Cernusak (2012):

$$\delta_c = \delta_A C + 1 + a_w + a_{lbs} \frac{C_a - C_i}{C_m}$$  \hspace{1cm} (15)

This can then be compared with $\delta_c$ (Equation 9), the isotopic composition of CO$_2$ in equilibrium with water at the site of evaporation. We calculated mesophyll conductance, $g_m$, in the S. viridis null plants assuming that $\delta_m=\delta_{ns}$ and then used this $g_m$ to estimate $C_m$ in the S. viridis transgenics to calculate the proportion of cytosolic CO$_2$ in equilibrium with leaf water, $\theta$ using equations developed by Cernusak et al. (2004)

$$\theta = \frac{\delta_c - \delta_w + a_w \frac{C_a - C_i}{C_m}}{\delta_w - \delta_{ns} + a_{lbs} \frac{C_a - C_i}{C_m}}$$  \hspace{1cm} (16)

where $a_{lbs}$ is the weighted discrimination of C$^{18}$O$^3$HO diffusion across the boundary layer, stomata, and the liquid phase in series given by:

$$a_{lbs} = a_w \frac{(C_a - C_i) + a_i (C_a - C_i) + a_{lbs} (C_a - C_m)}{(C_a - C_m)}$$  \hspace{1cm} (17)

Leaf anatomical measurements and estimation of $g_m$ from anatomical measurements

Fully expanded leaves from 5-week-old T$_2$ plants, null and line 1.1, were collected and cut into ~0.5×2 mm pieces. Leaf slices were fixed in 2.5% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde, 0.1 M phosphate buffer, and 0.01% (v/v) Tween-20 under vacuum for 20 min, then replaced with buffer containing no Tween-20 and fixed overnight at 4 °C. Leaf pieces were washed in phosphate buffer and post-fixed in 1% (w/v) osmium tetroxide for 2 h. Fixed leaf pieces were then dehydrated in an ethanol series (10, 30, 50, 70, 80, 95, 100%) followed by infiltration with LR white. Leaf sections were finally placed in moulds filled with resin and baked at 60 °C for 24 h. Sections of 0.5 µm thickness were cut using glass knives on a Reichert ultramicrotome, stained with toluidine blue, and heat fixed to glass slides. Slides were viewed using a Zeiss Axioskop light microscope at ×400 magnification. Three images were taken from each slide for analysis, each containing a leaf cross-section in the same orientation and showing at least two vascular bundles. Fiji quantification
software (Schindelin et al., 2012) was used to select regions of interest. Mesophyll surface area exposed to intercellular airspace to leaf area ratio \( (S_m) \) was calculated using Equation 18 where CCF is the curvature correction factor of 1.43 (Evans et al., 1994).

\[
S_m = \frac{\text{Length of mesophyll cells exposed to intercellular airspace}}{\text{Intervinal distance}} \times \text{CCF} \quad (18)
\]

The values of \( S_m \) together with measurements of cell wall thickness and cytosol thickness were used to derive an estimate of \( g_m \) from anatomical parameters. The cell wall thickness (0.113 ± 0.005 μm) was kindly estimated from transmission electron micrographs of \( S. \ viridis \) grown under similar conditions by Florence Danila (Danila et al., 2016). Calculations followed equations 1–5 of von Caemmerer and Evans (2015) using the membrane permeability of Gutknecht for a lipid bilayer of \( 3.5 \times 10^{-10} \text{ m s}^{-1} \) since only the plasma membrane needs to be transversed for diffusion of \( CO_2 \) from the intercellular airspace to mesophyll cytosol (Gutknecht et al., 1977) and a cytosol thickness of 0.3 μm (von Caemmerer and Evans, 2015). These calculations give a \( g_m \) of 0.68 mol m⁻² s⁻¹ bar⁻¹.

Statistical analysis
One-way ANOVAs with post-hoc Tukey test analyses were performed for all measurements of gas exchange and enzyme activities with \( P=0.05 \) using the IBM SPSS Statistics 22 package.

Results

Generation of transgenic \( S. \ viridis \) with reduced β-CA

In \( S. \ viridis \) we identified four β-CA genes: Si002140m.g (with one other isoform Si002148m), Si002669m.g, Si030616m.g (with two other isoforms Si030928m and Si030803m), and Si003882m.g. There is very low sequence identity between these β-CA genes, ~37% (Supplementary Fig. S2). Si003882m.g has been shown to be the major leaf β-CA (Christin et al., 2013; John et al., 2014).

Three independent transformation events resistant to hygromycin and with reduced CA activity were generated using two different approaches. First, one line (1.1) was generated through gene suppression upon transformation with the overexpression construct pSC110/ZmCA2. The coding sequences of \( ZmCA2 \) and Si003882m.g show 87% identity (Supplementary Fig. S3). Most probably, expression of \( ZmCA2 \) therefore caused suppression of the primary \( S. \ viridis \) β-CA gene, resulting in reduced CA activity in line 1.1. The second approach was to target Si003882m.g using the RNAi construct pSG/CAa which generated stably transformed lines from two different events (2.1 and 5.3). Plants were grown at high \( pCO_2 \) for all experiments.

To determine the specificity of the RNAi construct and check which β-CA was suppressed in line 1.1, RT–qPCR was performed against the β-CA genes in \( S. \ viridis \). Expression of the primary leaf β-CA Si003882m.g was significantly down-regulated, between 83% and 96%, in lines from all three transformation events (Fig. 1A). Transcript levels of Si030616m.g and Si002140m.g were unchanged relative to expression in the null plants (Fig. 1B, C) while Si002669m.g transcript was undetectable in all samples (data not shown). Therefore, expression of only the target β-CA gene was affected in the three transformed lines.

qPCR was used to estimate the number of insertions in the transgenic plants, based on the number of copies of the hygromycin phosphotransferase gene. Three \( T_2 \) plants of the
three lines were analysed and there were two, four, and more than four transgene insertions detected for plants of line 5.3, 2.1, and 1.1, respectively. The high copy number in the over-expressing line of 1.1 is the likely cause of the suppression of transcript accumulation.

CA and photosynthetic enzyme activity and leaf anatomy

T1 progeny of the three independent transformation events showed a range of CA hydration rates as measured on the soluble leaf fraction on a membrane inlet mass spectrometer. Compared with the null control, lines 1.1, 2.1, and 5.3 had on average (n=7 T2 plants) an 87, 70, and 50% reduction of CA activity, respectively (Fig. 2). The CA hydration rate in the null plants was 934±92 μmol m⁻² s⁻¹ as calculated at a mesophyll pCO₂ (Cᵅ) of 140 μbar (Equation 2).

The activities of the photosynthetic enzymes Rubisco, PEPC, and NADP-ME were unchanged in lines 5.3, 2.1, and 1.1 compared with the nulls (Table 1) and showed no correlation with CA hydration rates (one-way ANOVA and Tukey post-hoc analysis; SPSS statistics version 22; P=0.05).

No significant differences were observed for the surface area of mesophyll cells exposed to intercellular airspace per unit leaf area (Sₑ) in embedded leaf sections of nulls (10.22±0.35 m² m⁻²) and plants from line 1.1 (10.18±0.95 m² m⁻²). These anatomical measurements were used to estimate an anatomical gₑ of 0.68 mol m⁻² s⁻¹ bar⁻¹ (see the Materials and methods).

CA activity and CO₂ assimilation rates

The response of CO₂ assimilation rate (A) to increasing intercellular pCO₂ (Cᵅ) was investigated to examine the effect of reduced CA activity on CO₂ assimilation rates (Fig. 3). There were no statistical differences in the maximum rate of CO₂ assimilation under ambient or high CO₂ conditions between null control and progeny of transformant lines. At low pCO₂, CO₂ assimilation rates were reduced to varying degrees in the progeny of the transformed lines compared with the null control. Individuals of line 1.1 with the lowest CA hydration rate had the lowest initial slopes of the ACᵅ curves. The initial slopes of the ACᵅ and ACᵅ curve were plotted against the CA hydration rate constant (kᵦ; Fig. 4). Mesophyll cytosolic pCO₂, Cᵅ, was calculated from Equation 2, using the average null gₑ (0.9 mol m⁻² s⁻¹ bar⁻¹) since there was no difference in Sₑ. A strong correlation between the initial slope from the ACᵅ curve and kᵦ was observed, with the initial slope increasing as CA hydration rates increase (R²=0.845; Fig. 4). There was a curvilinear response between the initial slope of the ACᵅ curves indicating other limitations. No difference in stomatal conductance (gₛ) was observed across a range of intercellular pCO₂ between null controls and any of the transformed lines during the rapid measurements of CO₂ responses (Fig. 5).

![Image](https://academic.oup.com/jxb/article-abstract/68/2/299/2628091 by guest on 16 March 2020)
Oxygen isotope discrimination measurements

Oxygen (Δ^{18}O) isotope discrimination and CO₂ assimilation rates were measured in response to changes in pCO₂ using a LI-6400XT coupled to a tunable diode laser to measure C^{18}O^{16}O and a Cavity Ring-Down Spectrometer to measure the oxygen isotope composition of water vapour. Transformed plants with reduced CA hydration rates had lower Δ^{18}O compared with the nulls, but only line 1.1 was significantly lower (Table 2).

In the null controls, measurements of Δ^{18}O were used to estimate conductance of CO₂ from the intercellular airspace to the sites of CO₂ and H₂O exchange in the cytosol (gₘ) with the assumption that CO₂ was in full isotopic equilibrium with leaf water in the cytosol (Equation 2; Fig. 6). Although gₘ appeared to increase with decreasing Cᵢ, there were no significant differences between gₘ estimated at the different Cᵢ, and the average value was 0.94 ± 0.06 mol m⁻² s⁻¹ bar⁻¹ (Fig. 6B). Cᵢ–Cₘ indicates the drawdown of CO₂ from the intercellular airspace to the site of fixation, and for the null controls there is an increasing gradient of pCO₂ as Cᵢ increases (Fig. 6C).

Δ^{18}O at ambient pCO₂ showed statistically significant differences between line 1.1 (with the lowest CA activity) and null plants (Table 2). When plotted against Cₘ/Cᵢ, Δ^{18}O measurements closely correspond to theoretical curves representing θ (Equation 16) under different scenarios either where cytosolic CO₂ is at full isotopic equilibrium with the cytosolic water (null lines) or where there is only partial equilibrium (such as line 1.1; Fig. 7). Calculated values for line 5.3 which showed a 50% reduction in CA activity relative to the null controls fell in between these two theoretical lines. This is illustrated again with theta (θ) of lines 1.1 and 5.3 over a range of Cₘ (Fig. 8). When CO₂ is at full isotopic equilibrium with the cytosolic water, θ would be 1, whereas in lines 1.1 and 5.3 (with reduced CA hydration rates relative to the null control) θ is <1. There was no CO₂ dependence of θ over the range of pCO₂ measured.

Discussion

Setaria viridis as a model species to study photosynthetic physiology in a C₄ monocot

Flaveria bidentis, a readily transformable model C₄ dicot, has been successfully used to study the regulation of C₄ photosynthesis using antisense and RNAi technology (Furbank et al., 1997; Matsuoka et al., 2001; von Caemmerer et al., 2004; Pengelly et al., 2012). This work has been crucial in quantifying the rate-limiting steps in the C₄ pathway by ‘titrating’ out levels of target enzymes by gene suppression and observing
the effects on physiological characteristics of the resultant transgenics (Furbank et al., 1997). There are, however, important differences between C₄ dicots and the C₃ monocots which make up the majority of agriculturally important C₄ species. Setaria viridis has emerged as a new model grass to

study C₄ photosynthesis in crops and related bioenergy species. Setaria viridis is an appropriate biochemical model species for Z. mays and S. bicolor as all three use NADP-ME as the primary decarboxylation enzyme. We generated transgenic S. viridis plants with reduced CA activity to compare the effect with previous results obtained with F. bidentis and Z. mays (von Caemmerer et al., 2004; Studer et al., 2014) and to explore the effect that a reduction in CA activity has on the initial slope of the ACᵢ and ACᵢᵢ curves. In these lines, only the major leaf isoform of β-CA was reduced (Fig. 1). The transgenic plants had a range of different CA activities (Fig. 2), but showed no changes in PEPC and Rubisco activity (Table 1) or anatomical parameters (Table 2), making these plants ideal for exploring the role of CA activity in S. viridis.

Models of C₄ photosynthesis suggest that the initial slope of the ACᵢ curve is determined by three possible limitations: (i) the mesophyll conductance to CO₂ diffusion from the intercellular airspace to the mesophyll cytosol (gᵢ); (ii) the rate of CO₂ hydration by CA; and (iii) the rate of PEP carboxylation (von Caemmerer, 2000). However, it is not readily known which is the major limitation in C₄ species. Studies with PEPC mutants from the C₃ dicot Amaranthus edulis indicate that PEPC activity may not be the major limitation as a 60% reduction in PEPC leads to only a 20% reduction in the CO₂ assimilation rate at ambient pCO₂ accompanied by a small reduction in initial slope for the ACᵢ curves (Dever et al., 1992; Dever, 1997; Cousins et al., 2007). This study with S. viridis confirms that substantial reductions in CA activity are possible before a reduction in steady-state CO₂ assimilation rate and initial slope of the ACᵢ curve are observed. This is in accordance with previous observations in F. bidentis and Z. mays (von Caemmerer et al., 2004; Studer et al., 2014).

The Michaelis–Menten constant for CO₂ for CA is >2 mM (~5% CO₂) which makes it appropriate to quantify CA activity by its first-order rate constant (Jenkins et al., 1989; Hatch and Burnell, 1990) and simplifies species comparisons. In S. viridis, the lowest rate constant recorded was 0.8 mol m⁻² s⁻¹ bar⁻¹ compared with values of 0.1 for the calca2 double mutant in Z. mays and 0.47 for transgenic F. bidentis (von Caemmerer et al., 2004; Studer et al., 2014). With this low rate constant, F. bidentis had very low CO₂ assimilation rates and the CO₂ response curves did not saturate at high CO₂. In contrast, for both S. viridis transgenics and Z. mays mutants, CO₂ assimilation rates were only slightly less than in the controls, suggesting that S. viridis is more similar to Z. mays in its CA requirements. This suggests that these two monocot species can make better use of leaf CA activity or that in vivo CA activity is greater than that estimated in vitro.

**Mesophyll conductance and the initial slope of ACᵢᵢ curves**

Next, we used recently established techniques that utilize 18O discrimination measurements to quantify gᵢ in our null controls (Fig. 6B; Barbour et al., 2016). This estimates the

![Fig. 7. Oxygen isotope discrimination (Δ₁⁸O) as a function of the ratio of mesophyll pCO₂ to ambient pCO₂ (Cᵢ/Cᵢᵢ) in null and lines 5.3 and 1.1. Each point represents a measurement made on an individual leaf of a T₂ plant. Triangular symbols represent measurements made at low pCO₂. Theoretical curves represent the scenario where cytosolic CO₂ is at full isotopic equilibrium with cytosolic water (θ=1, black) or under partial equilibrium (θ=0.5, grey) of ¹⁸O in the leaf. The equations for the curves are given by Δ₁⁸O = Δ₀ + Cᵢ/Cᵢᵢ(δᵢ − δᵢᵢ) and Δ₀ = 5.85‰ and δᵢ − δᵢᵢ = 33‰ at full equilibration or Δ₀ = 5.1‰ and δᵢ − δᵢᵢ = 15‰ (Farquhar and Lloyd, 1993).](https://academic.oup.com/jxb/article-abstract/68/2/299/2628091)

![Fig. 8. Average isotopic equilibrium (theta, θ) over a range of mesophyll pCO₂ in two reduced CA lines 5.3 (grey) and 1.1 (white). Measured values of θ were determined from Δ₁⁸O using Equation 16. Each point represents the average measurement of three T₂ plants.](https://academic.oup.com/jxb/article-abstract/68/2/299/2628091)
diffusion of CO₂ from the intercellular airspace through the cell wall, plasma membrane, and cytosol to the sites of CA activity. At ambient pCO₂, the gₘ observed for the null plants were similar to those reported by Barbour et al. (2016). A key assumption for the calculation of gₘ is that CA activity is not limiting and that CO₂ is in isotopic equilibrium with HCO₃⁻; consequently gₘ was not measured in the transgenic lines with reduced CA activity. In C₃ species, gₘ (in this instance from the intercellular airspace to the chloroplast stroma) has been shown to be proportional to the chloroplast surface area of Sₘ. A relationship was observed with comparative measurements of gₘ (Evans et al., 1994). Evans and von Caemmerer (1996) hypothesized that in C₄ species gₘ may correlate with the mesophyll surface area exposed to intercellular airspace per unit leaf area (Sₘ). Since Sₘ was similar between the nulls and line 1.1 plants, we assumed that gₘ may also be similar between the plants. In C₃ species, gₘ has been shown to, in some instances, increase with decreasing pCO₂ (Flexas et al., 2007; Tazoe et al., 2011; Alonso-Cantabrana and von Caemmerer, 2016). These changes to gₘ which may be important in regulating and maintaining photosynthesis were also observed here in the S. viridis null plants, with gₘ increasing slightly at low pCO₂. However, because the differences in gₘ at different pCO₂ were not significant, we used the average gₘ estimated for the null plants to calculate mesophyll cytosolic pCO₂ (Cₘ) in the transgenics.

As shown in Fig. 4, a strong almost linear relationship was found between ACₘ and kₐ, whereas a saturating relationship was observed with ACₜ. This indicates that the CO₂ assimilation rate is limited by cytosolic CA activity, with the relationship becoming clearer after accounting for gₘ. It is tempting to speculate that the differences between the two monocot species and F. bidentis relate to differences in limitations imposed by gₘ which affects cytosolic pCO₂ and hence in vivo CA activity, but this is not borne out by comparative measurements of gₘ made by Barbour et al. (2016). CA activity increases with increasing pH, so variation in cytosolic pH can also contribute to variations in in vivo CA activity; however, these effects are not large (Jenkins et al., 1989). The interaction of β-CA and a CO₂-permeable aquaporin in Arabidopsis thaliana has indicated that CA can be localized near the plasma membrane rather than dispersed throughout the mesophyll cytosol (Wang et al., 2016). This may also impact on CA activity and result in another difference between the C₄ species. Other possibilities pertain to differences in anatomical characteristics of leaves. Both CA and PEPC are cytosolic enzymes, and differences in Sₘ may affect the efficiency with which CA is used. Our results suggest that increasing gₘ may be an important way to increase the CO₂ assimilation rate at low intercellular pCO₂, a scenario that may, for example, occur under drought.

Oxygen isotope discrimination and the CO₂ dependence of isotopic equilibrium

As had previously been observed, Δ¹⁸O decreased with reductions in CA activity as CA facilitates the exchange of O₂ between cytosolic water and CO₂ (Fig. 7; Williams et al., 1996; Cousins et al., 2006). Previous reports, which have estimated the proportion of cytosolic CO₂ in equilibrium with leaf water (θ) in C₄ species, have generally assumed a relatively large gₘ value and this then led to lower estimates of θ (Cousins et al., 2006, 2008). Here we assumed that in the S. viridis null plants there is sufficient CA for isotopic equilibrium to be reached, as discussed by Barbour et al. (2016). For comparison, we also estimated gₘ from anatomical estimates of Sₘ, and cell wall and cytosolic thickness following calculations outlined by von Caemmerer and Evans (2015). This gives a gₘ value of 0.68 mol m⁻² s⁻¹ bar⁻¹ which is less than the value of 0.9 mol m⁻² s⁻¹ bar⁻¹ calculated from Δ¹⁸O measurements and highlights the anatomical constraints for CO₂ diffusion dictated by the photosynthetic pathway in leaves of C₄ plants (von Caemmerer et al., 2007).

Reduction in CA activity led to significant reductions in θ but it is interesting to note that θ did not vary significantly with pCO₂. This is explained by the fact that CA activity increases linearly with pCO₂ so that although there is more CO₂ that needs to equilibrate with leaf water, there is also proportionally more CA activity. The fact that neither transgenic line showed a CO₂ dependence suggests that the decrease in the ratio of CA activations to PEP carboxylations is not affecting the isotopic equilibration of CO₂ with leaf water. These results have important implications for the interpretation of the δ¹⁸O signature of atmospheric CO₂ (Yakir and Sternberg, 2000; Gillon and Yakir, 2001; Wingate et al., 2009).

Reduction of CA in S. viridis does not alter the stomatal response to CO₂

The CO₂ regulation of stomatal conductance remains an open question (Engineer et al., 2016). It has been previously shown that in the cal1ca4 double mutant of A. thaliana, the degree of stomatal closure in response to increasing pCO₂ was reduced (Hu et al., 2010; Wang et al., 2016). It is clear that CA is part of a complex signal transduction network. However, nothing is currently known about the role of CA in stomatal CO₂ responses in C₄ species. In our study, where only one β-CA isoform was reduced, we found no change in the response of stomatal conductance to CO₂. The S. viridis β-CA reduced here (Si003882m.g) has low sequence identity (<50%) to all of the Arabidopsis β-CAs, but we would predict that multiple reductions in β-CA isoforms would be required to observe a similar stomatal phenotype in S. viridis.

Conclusion

Under current atmospheric conditions, CA activity was not rate limiting for C₄ photosynthesis in S. viridis. At lower C₄, which may, for example, occur under conditions of drought, our results suggest that gₘ may pose a greater limitation than CA activity. However, it is important to investigate the role of CA on C₄ photosynthesis under a range of environmental conditions such as high temperatures which have recently been suggested to deactivate CA activity in S. viridis (Boyd et al., 2015). Here we have shown that S. viridis is a useful
model monocot C₄ species that lends itself to molecular manipulation of the C₄ photosynthetic pathway.

**Supplementary Data**

Supplementary data are available at JXB online.

Table S1. Primers used in this study

Figure S1. CA hydration rates at mesophyll pCO₂ in the T₁ plants.

Figure S2. Very low sequence identity (~37%) between the four main S. viridis β-CAs.

Figure S3. High sequence identity (87%) of Si003882m.g to the ZmCA2 (GRMZM2G348512).

Figure S4. CO₂ assimilation rate of the TDL experiment.

Figure S5. Standard error of δ¹⁸O in the reference gas of repeated measurements with the TGA200A.

**Acknowledgements**

We thank Jasper Pengelly for assisting with construct generation, Xueqin Wang for assisting with S. viridis transformations, Soumi Bala for help with biochemical assays, gas exchange, and TDL measurements, and Murray Badger for making the MIMS available for measurements of CA activity. We thank Hilary Stuart-Williams for calibrating standard gases and water samples, and Joyce van Eck and Tom Brutnell for helpful discussions regarding S. viridis transformations. We thank Joanne Lee and the Centre for Advanced Microscopy at ANU for technical assistance with microscopy. This research was supported by the Bill and Melinda Gates Foundation’s funding for the C₄ Rice consortium and by the Australian Research Council Centre of Excellence for Translational Photosynthesis (CE140100015). RES is funded by ARC DECRA (DE130101760).

**References**

Alonso-Cantabrana H, von Caemmerer S. 2016. Carbon isotope discrimination as a diagnostic tool for C₄ photosynthesis in C₄–C₃ intermediate species. Journal of Experimental Botany 67, 3109–3121.

Badger MR, Price GD. 1989. Carbonic anhydrase activity associated with the cyanobacterium Synechococcus PCC7942. Plant Physiology 89, 51–60.

Badger MR, Price GD. 1994. The role of carbonic anhydrase in photosynthesis. Annual Review of Plant Biology 45, 369–392.

Barbour MM, Evans JR, Simonin KA, von Caemmerer S. 2016. Online CO₂ and H₂O oxygen isotope fractionation allows estimation of mesophyll conductance in C₄ plants, and reveals that mesophyll conductance decreases as leaves age in both C₄ and C₃ plants. New Phytologist 210, 875–889.

Bartlett JG, Alves SC, Smedley M, Snape JW, Harwood WA. 2008. High-throughput Agrobacterium-mediated barley transformation. Plant Methods 4, 1–12.

Boyd RA, Gandin A, Cousins AB. 2015. Temperature response of C₄ photosynthesis: biochemical analysis of Rubisco, phosphoenolpyruvate carboxylase and carbonic anhydrase in Setaria viridis. Plant Physiology 160, 1850–1861.

Brutnell TP, Wang L, Swartwood K, Goldschmidt A, Jackson D, Zhu X-G, Kellogg E, Van Eck J. 2010. Setaria viridis: a model for C₄ photosynthesis. The Plant Cell 22, 2537–2544.

Cernusak LA, Farquhar GD, Wong SC, Stuart-Williams H. 2004. Measurement and interpretation of the oxygen isotope composition of carbon dioxide respired by leaves in the dark. Plant Physiology 136, 3390–3393.

Christin PA, Boxall SF, Gregory R, Edwards EJ, Hartwell J, Osborne CP. 2013. Parallel recruitment of multiple genes into C₄ photosynthesis. Genome Biology and Evolution 5, 2174–2187.

Christin PA, Osborne CP. 2013. The recurrent assembly of C₄ photosynthesis, an evolutionary tale. Photosynthesis Research 117, 163–175.

Cousins AB, Badger MR, von Caemmerer S. 2006. A transgenic approach to understanding the influence of carbonic anhydrase on C¹³O₂ discrimination during C₄ photosynthesis. Plant Physiology 142, 662–672.

Cousins AB, Badger MR, von Caemmerer S. 2008. C₄ photosynthetic isotope exchange in NAD-ME- and NADP-ME-type grasses. Journal of Experimental Botany 59, 1695–1703.

Cousins AB, Baroli I, Badger MR, Ivakov A, Lea P, Leegood RC, von Caemmerer S. 2007. The role of phosphoenolpyruvate carboxylase during C₄ photosynthetic isotope exchange and stomatal conductance. Plant Physiology 145, 1–12.

Craig H, Gordon LL. 1965. Deuterium and oxygen 18 variations in the ocean and the marine atmosphere. In: Tongioj E, ed. Proceedings of a Conference on stable isotopes in oceanographic studies and paleotemperatures. Pisa, Italy: Consiglio Nazionale delle Ricerche, Laboratorio di Geologia Nucleare, 9–130.

Curtis MD, Grossniklaus U. 2003. A Gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiology 133, 462–469.

Danila F, Quick WP, White RG, Furbank RT, von Caemmerer S. 2016. The metabolic pathway between bundle sheath and mesophyll: quantification of plasmodesmata in leaves of C₄ and C₃ monocots. The Plant Cell 28, 1461–1471.

Dever LV. 1997. Control of photosynthesis in Amaranthus edulis mutants with reduced amounts of PEP carboxylase. Australian Journal of Plant Physiology 24, 469–476.

Dever LV, Lea PJ, Blackwell RD, Leegood RC. 1992. The isolation of mutants of C₄ photosynthesis. In: Murata N, ed. Research in Photosynthesis. Vol. 111. Kluwer Academic Publishers, 891–894.

DiMario RJ, Quebedeaux JC, Longstreth D, Dassanayake M, Hartman MM, Moroney JV. 2016. The cytoplasmic carbonic anhydrases iCA2 and iCA4 are required for optimal plant growth at low CO₂. Plant Physiology 171, 280–293.

Doust AN, Kellogg EA, Devos KM, Bennetzen JL. 2009. Foxtail millet: a sequence-driven grass model system. Plant Physiology 149, 137–141.

Engineer CB, Hashimoto-Sugimoto M, Negi J, Israelsson-Nordstrom M, Azoulay-Shemer T, Rappel WJ, Iba K, Schroeder JL. 2016. CO₂ sensing and CO₂ regulation of stomatal conductance: advances and open questions. Trends in Plant Science 21, 16–30.

Evans J, Sharkey T, Berry J, Farquhar G. 1986. Carbon isotope discrimination measured concurrently with gas exchange to investigate CO₂ diffusion in leaves of higher plants. Functional Plant Biology 13, 281–292.

Evans JR, Caemmerer S, Setchell BA, Hudson GS. 1994. The relationship between CO₂ transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. Functional Plant Biology 21, 475–485.

Evans JR, von Caemmerer S. 1996. Carbon dioxide diffusion inside leaves. Plant Physiology 110, 339–346.

Farquhar GD, Cernusak LA. 2012. Ternary effects on the gas exchange of isoploilogues of carbon dioxide. Plant, Cell and Environment 35, 1221–1231.

Farquhar G, Lloyd J. 1993. Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In: Etheringer JR, Hall AE, Farquhar G, eds. Stable isotopes and plant carbon–water relations. New York: Academic Press, 47–70.

Flexas J, Diaz-Espejo A, GalmES J, Kaldenhoff R, Medrano H, Ribas-Carbo M. 2007. Rapid variations of mesophyll conductance in response to changes in CO2 concentration around leaves. Plant, Cell and Environment 30, 1284–1298.

Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanian SJ, von Caemmerer S, Ashton AR. 1997. Genetic manipulation of key photosynthetic enzymes in the C₄ plant Flaveria bidentis. Australian Journal of Plant Physiology 24, 477–485.

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA 3rd, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods 6, 343–345.
Gillon JS, Yakir D. 2001. Influence of carbonic anhydrase activity in terrestrial vegetation on the \(^{18}\)O content of atmospheric CO\(_2\). Science 291, 2584–2587.

Gillon JS, Yakir D. 2000. Naturally low carbonic anhydrase activity in C\(_4\) and C\(_3\) plants limits discrimination against \(^{18}\)O\(_2\) during photosynthesis. Plant, Cell and Environment 23, 903–915.

Greenup AG, Sasani S, Oliver SN, Talbot MJ, Dennis ES, Hemming MN, Trevaskis B. 2010. ODDSOC2 is a MADS box floral repressor that is down-regulated by vernalization in temperate cereals. Plant Physiology 153, 1062–1073.

Gutknecht J, Bisson MA, Tosteson FC. 2014. Evolutionary convergence of cell-specific gene expression in independent lineages of C\(_4\) grasses. Plant Physiology 165, 62–75.

Hatch MD. 1987. C\(_4\) photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochimica et Biophysica Acta 895, 51–106.

Hatch MD, Burnell JN. 1989. Mechanism of C\(_4\) photosynthesis: a model describing the inorganic carbon pool in bundle sheath cells. Plant Physiology 93, 825–828.

Hu H, Boisson-Dernier A, Israelsson-Nordstrom M, Bohmer M, Xue S, Ries A, Godoski J, Kuhn JM, Schroeder JI. 2010. Epitope tagging: a monoclonal antibody specific for recombinant fusion proteins in plants. Plant, Cell and Environment 33, 145–153.

Lawrence SD, Novak NG, Slack JL. 2003. Epitope tagging: a monoclonal antibody specific for recombinant fusion proteins in plants. Biotechniques 35, 488–492.

Li P, Brutnell TP. 2011. Setaria viridis and Setaria italica, model genetic systems for the Panicoid grasses. Journal of Experimental Botany 62, 3031–3037.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)-Delta Delta C(T) Method. Methods 25, 402–408.

Matsuoka M, Furbank RT, Fukayama H, Miyao M. 2001. Molecular engineering of C\(_4\) photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology 52, 297–314.

Moroney JV, Bartlett SG, Samuelsson G. 2001. Carbonic anhydrases in plants and algae. Plant, Cell and Environment 24, 141–153.

Okabe K, Yang S-Y, Tsuzuki M, Miyachi S. 1984. Carbonic anhydrase: its content in spinach leaves and its taxonomic diversity studied with anti-spinach leaf carbonic anhydrase antibody. Plant Science Letters 33, 145–163.

Pengelly JJL, Sirault XRR, Tazoe Y, Evans JR, Furbank RT, von Caemmerer S. 2010. Growth of the C\(_4\) dicot Flaveria bidentis: photosynthetic acclimation to low light through shifts in leaf anatomy and biochemistry. Journal of Experimental Botany 61, 4109–4122.

Pengelly JJL, Tan J, Furbank RT, von Caemmerer S. 2012. Antisense reduction of NADP-malic enzyme in Flaveria bidentis reduces flow of CO\(_2\) through the C\(_4\) cycle. Plant Physiology 160, 1070–1080.

Sage RF, Sage TL, Kocicnar F. 2012. Photorespiration and the evolution of C\(_4\) photosynthesis. Annual Review of Plant Biology 63, 19–47.

Schindelin J, Arganda-Carreras I, Frise E, et al. 2012. Fiji: an open-source platform for biological-image analysis. Nature Methods 9, 67–68.

Sharwood RE, Sonawane BV, Ghannoum O, Whitney SM. 2016. Improved analysis of C\(_4\) and C\(_3\) photosynthesis via refined in vitro assays of their carbon fixation biochemistry. Journal of Experimental Botany 67, 3137–3148.

Studer AJ, Gandin A, Kolbe AR, Wang L, Cousins AB, Brutnell TP. 2014. A limited role for carbonic anhydrase in C\(_4\) photosynthesis as revealed by a ca1ca2 double mutant in maize. Plant Physiology 165, 608–617.

Tazoe Y, von Caemmerer S, Estavillo GM, Evans JR. 2011. Using tunable diode laser spectroscopy to measure carbon isotope discrimination and mesophyll conductance to CO\(_2\). Photosynthesis dynamically at different CO\(_2\) concentrations. Plant, Cell and Environment 34, 580–591.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT–PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, Research0034.

von Caemmerer S. 2000. Biochemical models of leaf photosynthesis. Collingwood, Australia: CSIRO Publishing.

von Caemmerer S, Evans JR. 2015. Temperature responses of mesophyll conductance differ greatly between species. Plant, Cell and Environment 38, 629–637.

von Caemmerer S, Evans JR, Cousins AB, Badger MR, Furbank RT. 2007. C\(_4\) photosynthesis and CO\(_2\) diffusion. In: Sheehy JE, Mitchell PL, Hardy B, eds. Charting new pathways to C\(_4\) photosynthesis: a transgenic analysis. Plant, Cell and Environment 27, 697–703.

Wang C, Hu H, Qin X, Zeise B, Xu D, Rappel W-J, Boron WF, Schroeder JI. 2016. Reconstitution of CO\(_2\) regulation of SLAC1 anion channel and function of CO\(_2\)-permeable PIP2;1 aquaporin as CARBONIC ANHYDRASE4 interactor. The Plant Cell 28, 568–582.

Williams TG, Flanagan LB, Coleman JR. 1996. Photosynthetic gas exchange and discrimination against \(^{13}\)CO\(_2\) and \(^{18}O\) in tobacco plants modified by an antisense construct to have low chloroplastic carbonic anhydrase. Plant Physiology 112, 319–326.

Wingate L, Ogee J, Cuntz M, et al. 2009. The impact of soil microorganisms on the global budget of delta \(^{18}\)O in atmospheric CO\(_2\). Proceedings of the National Academy of Sciences, USA 106, 22411–22415.

Yakir D, Sternberg LDL. 2000. The use of stable isotopes to study ecosystem gas exchange. Oecologia 123, 297–311.