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 remedying 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 CO$_2$ and HCO$_3^-$ in the cytosol of mesophyll cells. C$_4$ acids produced by PEPC then diffuse into the bundle sheath cells where they are decarboxylated, supplying CO$_2$ for Rubisco.

Within higher plants, there are multiple forms of the α-CA, β-CA, and γ-CA families which share little sequence homology (Moroney et al., 2001). β-CAs are the most prevalent CA family in land plants. CA is an abundant enzyme in C$_3$ plants, representing up to 2% of the soluble leaf protein (Okabe et al., 1984). In 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 CO$_2$ conversion to HCO$_3^-$ is insufficient to support C$_4$ photosynthetic flux (Hatch and Burnell, 1990; Badger and Price, 1994). This hypothesis was supported by experiments in the C$_4$ dicot Flaveria bidentis, where antisense plants with <10% of wild-type CA activity required high CO$_2$ for growth and showed reduced CO$_2$ assimilation rates (von Caemmerer et al., 2004; Cousins et al., 2006). However, in the C$_4$ monocot Zea mays mutant plants with reduced CA activity (3% of wild type), no limitation to CO$_2$ assimilation rates at ambient 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 CO$_2$ assimilation rates, as has previously been suggested (Hatch and Burnell, 1990; Gillon and Yakir, 2000).

We examined the role of CA in the model C$_4$ monocot Zea mays (green foxtail millet). Setaria viridis is closely related to agronomically important C$_4$ crops including Z. mays (maize), Sorghum bicolor (sorghum), and 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 (Doust 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 S. viridis and could show that S. viridis is a useful model species that lends itself to molecular manipulation of the C$_4$ photosynthetic pathway. Two constructs both targeting the major leaf β-CA (Si003882mg) were used to generate three independent transformed lines with reduced CA activity.

A strong correlation between the CO$_2$ assimilation rate at low pCO$_2$ 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 CO$_2$ assimilation rate at low intercellular pCO$_2$, 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 μmol photons m$^{-2}$ s$^{-1}$, 16 h photoperiod, 28 °C day, 24 °C night, 2% 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 β-CA Si003882mg which generated lines 2.1 and 5.3. A region of Si003882mg was amplified by PCR using gene-specific primers (Supplementary Table S1 at JXB online) and reverse-transcribed RNA from 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 β-CA gene (GRMZM2G348512, 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 ZmCA2. The resulting ZmCA2 amplicon was cloned into pENTR/D-TOPO and verified by sequencing. LR Gateway cloning (ThermoFisher) was used to insert the ZmCA2 coding sequence into the overexpression vector, pSC110. pSC110 was created by Gibson Assembly (Gibson et al., 2009) from two modified pmD164 vectors (Curtis and Grossniklaus, 2003), kindly provided to us by Udo Gowik (Heinrich-Heine University, Düsseldorf, Germany). ZmCA2 expression from pSC110 was driven by the B73 ZmPEPC promoter. pSC110 and pSC110/ZmCA2 were verified by sequencing.

Both constructs were transformed into Agrobacterium tumefaciens strain AGL1 for stable plant transformation.

**Callus induction and plant transformation**

Stable transformation of S. viridis (accession A10.1) was carried out as described by Brutnell et al. (2010). Seed coats were mechanically removed from mature 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$^{-1}$ ZnSO$_4$·7H$_2$O, 0.6 mg l$^{-1}$ CuSO$_4$·5H$_2$O, 4 g l$^{-1}$ Gelzan, 0.5 mg l$^{-1}$ kanamycin, 0.5 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 μg l$^{-1}$ kanamycin and 50 μg l$^{-1}$ rifampicin at 28 °C to OD$_{500}$=0.5 and then resuspended in CIM without Gelzan and hormones. Acetosyringone (200 mM) and synperonic [0.01% (v/v)] were added to the Agrobacterium solution before inoculating 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$^{-1}$ kanamycin, 150 mg l$^{-1}$ timentin, 15 mg l$^{-1}$ hygromycin. Calli were maintained at 24 °C under a 16 h light:8 h dark photoperiod and a light
Effects of reduced carbonic anhydrase on \textit{Setaria viridis} | 301

Intensity of 60 \( \mu \)mol 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\(_2\) 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) \( \beta \)-mercaptoethanol] followed by extraction with phenol/chloroform/isoamylalcohol (25:24:1) and ethanol clean-up. DNA quality and quantity was determined using a NanoDrop spectrophotometer (Thermo Scientific).

DNA genetics (UK) performed quantitative real-time PCR (qPCR) analysis to estimate the numbers of transgene copies in the CA transformed lines following the procedure described in Bartlett \textit{et al.} (2008) with some modifications. The hygromycin phosphotransferase gene (with a FAM reporter) and the internal positive control (IPC, with a VIC reporter) were amplified together in a multiplex reaction (15 min denaturation, then 40 cycles of 15 s at 95 °C and 60 s at 60 °C) in an ABI 1900 real-time PCR machine. Fluorescence from the FAM and VIC fluorochromes was measured during each 60 °C step and the Ct values obtained. The difference between the Ct values for the hygromycin phosphotransferase gene and the IPC (the Delta Ct) was used to allocate the assayed samples into groups with the same gene copy number.

\textbf{Table 1. Physiological and biochemical characteristics of CA transformants under ambient CO\(_2\) conditions}

Net CO\(_2\) assimilation rate (\( A \)), stomatal conductance (\( g_s \)), mesophyll \( p\text{CO}_2 \) (\( C_m \)), the rate constant of CA hydration (\( k_{\text{CA}} \)), and enzyme activities were measured from the uppermost, fully expanded leaf of 5-week-old plants grown at 2% CO\(_2\). Gas exchange measurements were made at 25 °C leaf temperature, flow rate at 500 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), and irradiance of 1500 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\). Three T\(_2\) plants from three different transformation events were measured.

<table>
<thead>
<tr>
<th>( A ) ( \mu )mol m(^{-2}) s(^{-1})</th>
<th>( g_s ) ( \mu )mol m(^{-2}) s(^{-1})</th>
<th>( C_m ) ( \mu )bar</th>
<th>( k_{\text{CA}} ) mol m(^{-2}) s(^{-1}) bar(^{-1})</th>
<th>Rubisco ( \mu )mol m(^{-2}) s(^{-1})</th>
<th>PEPC ( \mu )mol m(^{-2}) s(^{-1})</th>
<th>NADP-ME ( \mu )mol m(^{-2}) s(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null</td>
<td>22.5 ± 0.6 a</td>
<td>0.19 ± 0.01 a</td>
<td>132.4 ± 3.3 a</td>
<td>6.1 ± 0.8 a</td>
<td>18.7 ± 1.5 a</td>
<td>229.6 ± 19.3 a</td>
</tr>
<tr>
<td>5.3</td>
<td>21.7 ± 2.6 a</td>
<td>0.2 ± 0.02 a</td>
<td>118.9 ± 13.1 a</td>
<td>3.3 ± 0.2 b</td>
<td>18.8 ± 1.8 a</td>
<td>249.3 ± 24.6 a</td>
</tr>
<tr>
<td>2.1</td>
<td>18.5 ± 1.9 a</td>
<td>0.16 ± 0.01 a</td>
<td>152.9 ± 15.2 a</td>
<td>2.0 ± 0.2 b</td>
<td>20.9 ± 2.9 a</td>
<td>181.5 ± 25.4 a</td>
</tr>
<tr>
<td>1.1</td>
<td>19.1 ± 1.2 a</td>
<td>0.19 ± 0.02 a</td>
<td>153.9 ± 4.4 a</td>
<td>0.8 ± 0.1 c</td>
<td>19.7 ± 1.8 a</td>
<td>180.3 ± 18.4 a</td>
</tr>
</tbody>
</table>

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

\textbf{Table 2. Physiological characteristics of CA transformants at ambient CO\(_2\) measured using LI-6400XT coupled to a tunable diode laser}

Net CO\(_2\) assimilation rate (\( A \)), stomatal conductance (\( g_s \)), mesophyll \( p\text{CO}_2 \) (\( C_m \)), the ratio of intercellular to ambient \( p\text{CO}_2 \) (\( C/C_m \)), the rate constant of CA hydration (\( k_{\text{CA}} \)), online \( \Delta^{13}\text{CO}_2 \) discrimination, and the length of mesophyll cells exposed to intercellular airspace (\( S_m \)) were measured from the uppermost, fully expanded leaf of 5-week-old plants grown at 2% CO\(_2\). Gas exchange measurements were made at 2% \( O_2 \), 25 °C leaf temperature, flow rate at 500 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), and irradiance of 1500 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\). Three T\(_2\) plants from two different transformation events were measured.

<table>
<thead>
<tr>
<th>( A ) ( \mu )mol m(^{-2}) s(^{-1})</th>
<th>( g_s ) ( \mu )mol m(^{-2}) s(^{-1})</th>
<th>( C_m ) ( \mu )bar</th>
<th>( C/C_m )</th>
<th>( k_{\text{CA}} ) mol m(^{-2}) s(^{-1}) bar(^{-1})</th>
<th>( \Delta^{13}\text{CO}_2 )</th>
<th>( S_m ) m(^{2}) m(^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null</td>
<td>30.0 ± 1.4 a</td>
<td>0.30 ± 0.03 a</td>
<td>144.6 ± 5.9 a</td>
<td>0.39 ± 0.03 a</td>
<td>8.4 ± 0.7 a</td>
<td>18.0 ± 1.4 a</td>
</tr>
<tr>
<td>5.3</td>
<td>29.2 ± 0.9 a</td>
<td>0.29 ± 0.02 a</td>
<td>157.9 ± 10.5 a</td>
<td>0.34 ± 0.01 a</td>
<td>2.5 ± 0.3 b</td>
<td>13.6 ± 0.7 a, b</td>
</tr>
<tr>
<td>1.1</td>
<td>24.5 ± 1.6 a</td>
<td>0.26 ± 0.03 a</td>
<td>178.1 ± 13.5 a</td>
<td>0.43 ± 0.02 a</td>
<td>0.8 ± 0.2 b</td>
<td>10.9 ± 0.6 b</td>
</tr>
</tbody>
</table>

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 mRNAs 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, 1 mM 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 (Laudon and Barbour, 2016) to measure the rates of 18O exchange from labelled 18C18O2 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 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, C18O16O, and H18O were made by coupling two LI-6400XT gas exchange systems to a tunable diode laser (TDL: TGA200A, Campbell Scientific Inc., Logan, UT, USA) to measure C18O16O and a Cavity Ring-Down Spectrometer (L2130-i, Picarro Inc., Sunnyvale, CA, USA) to measure the oxygen isotope composition of water vapour. The system is essentially that described by Tazoe et al. (2011) except that the TGA100 was replaced by a 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., Stamford, 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 (Permapure, 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 L2130-i 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 20 s 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 δ18O 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-i 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 (Δ18O) discrimination and mesophyll conductance (gma)

Discrimination against 16O in CO2 during photosynthesis, Δ18O, was calculated from the isotopic composition of the CO2 entering δi and exiting δo the leaf chamber and the CO2 concentration entering Cm and exiting C out (all measured with the TDL) (Evans et al., 1986; Barbour et al., 2016):

\[
\Delta^{18}O = \frac{\delta_o - \delta_i}{1 + \frac{\delta_o - \delta_i}{\delta_m - \delta_i}}
\]

where \(\delta = C_{m,i} - C_{o,i}\). 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 Cm is equal to the pCO2 at the site of CO2–H2O exchange and assuming that cytosolic CO2 is in full isotopic equilibrium with local cytosolic water. This allowed gma to be calculated from

\[
gma = A/(C_i - C_m)
\]

\[
C_m = C_i \left( \frac{\delta_i - a_{w} - \delta_A (1 + a_{w})}{\delta_i - a_{w} - \delta_A (1 + a_{w})} \right)
\]

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) (δi = [(18O/16O)i - 1] × 1000). Intercellular pCO2 is denoted by Ci, and a is the discrimination against C18O16O during liquid phase diffusion and dissolution (0.8‰).

The isotopic composition of CO2 being assimilated, δi, is given by

\[
\delta_A = \frac{\delta_i - \Delta^{18}O}{1 + \Delta^{18}O}
\]

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

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

$$\delta = \delta_a + t \left( \delta_{\text{aq}} \left( \frac{C_s - C_i}{C_i} \right) - \delta_{\text{aq}} \frac{C_s - C_i}{C_i} \right)$$

where $C_s$ is the pCO$_2$ in the ambient air. The ternary correction factor, $t$, is given by

$$t = \frac{1 + a_{\text{ths}}}{1000} E$$

where $g_{ac}$ is the total conductance to CO$_2$, $E$ the transpiration rate, and $a_{\text{ths}}$ is the weighted discrimination of C$^{18}$O/H$_2$O diffusion across the boundary layer and stomata in series given by:

$$a_{\text{ths}} = \frac{(C_s - C_a) a_{\text{sth}} - (C_s - C_i) a_{\text{th}}}{(C_s - C_i)}$$

where $C_a$ is the pCO$_2$ at the leaf surface and $a_{\text{sth}}$ and $a_{\text{th}}$ are the discriminations against C$^{18}$O/H$_2$O through stomata and the boundary layer (8% and 5.8‰, respectively).

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

$$\delta_{\text{in}} = \delta_a \left( 1 - \frac{C_i}{C_a} \right) \left( 1 + a_{\text{ths}} \right) - \delta_a a_{\text{ths}}$$

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, $\delta_{\text{cyt}}$.

$$\delta_{\text{cyt}} = \delta_a + \epsilon_w$$

where $\delta_a$ is the stable oxygen isotopic 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).

$$\epsilon_w (\%) = \frac{17604}{T_k} - 17.93$$

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, $\delta_{\text{w}}$, can be estimated from the Craig and Gordon model of evaporative enrichment (Craig and Gordon, 1965; Farquhar and Lloyd, 1993)

$$\delta_{\text{w}} = \delta_0 + e^* + e_k + \frac{\epsilon_w}{e_i} (\delta_{\text{cyt}} - \delta_{\text{w}})$$

where $e^*$ is the equilibrium fractionation during evaporation, $e_k$ is the kinetic fractionation during vapour diffusion in air, $\delta_0$ is the oxygen isotopic composition of transpired water, $e_i/e_s$ is the ratio of ambient to intercellular vapour pressure, and $\delta_{\text{cyt}}$ is the isotopic composition of ambient air. $e^*$ is dependent on temperature:

$$e^* = 2.644 - 3.206 \left( \frac{10^3}{T_k} \right) + 1.534 \left( \frac{10^6}{T_k^2} \right)$$
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).

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

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 ($0.113 \pm 0.005 \mu m$) 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 $3.5 \times 10^{-20} m^{-3} s^{-1}$ 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 $0.3 \mu m$ (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 $\beta$-CA

In S. viridis we identified four $\beta$-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 $\beta$-CA genes, $\sim37\%$ (Supplementary Fig. S2). Si003882m.g has been shown to be the major leaf $\beta$-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 $\beta$-CA gene, resulting in reduced CA activity in line 1.1. The second approach was to target Si003882m.g using the RNAi construct pSG1/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 $\beta$-CA was suppressed in line 1.1, RT–qPCR was performed against the $\beta$-CAs in S. viridis. Expression of the primary leaf $\beta$-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 $\beta$-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 \(934\pm92\) \(\mu\)mol \(\text{m}^{-2}\) \(\text{s}^{-1}\) as calculated at a mesophyll \(p\text{CO}_2\) \((C_m)\) of 140 \(\mu\)bar (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 \((S_m)\) in embedded leaf sections of nulls \((10.22\pm0.35\) \(\text{m}^2\) \(\text{m}^{-2}\)) and plants from line 1.1 \((10.18\pm0.95\) \(\text{m}^2\) \(\text{m}^{-2}\)). These anatomical measurements were used to estimate an anatomical \(g_m\) of 0.68 \(\mu\)mol \(\text{m}^{-2}\) \(\text{s}^{-1}\) \(\text{bar}^{-1}\) (see the Materials and methods).

CA activity and \(\text{CO}_2\) assimilation rates

The response of \(\text{CO}_2\) assimilation rate \((A)\) to increasing intercellular \(p\text{CO}_2\) \((C_i)\) was investigated to examine the effect of reduced CA activity on \(\text{CO}_2\) assimilation rates (Fig. 3). There were no statistical differences in the maximum rate of \(\text{CO}_2\) assimilation under ambient or high \(\text{CO}_2\) conditions between null control and progeny of transformant lines. At low \(p\text{CO}_2\), \(\text{CO}_2\) 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 \(A_C\) curves.

The initial slopes of the \(A_C\) and \(A_{C_m}\) curve were plotted against the CA hydration rate constant \((k_{CA};\) Fig. 4). Mesophyll cytosolic \(p\text{CO}_2\), \(C_m\), was calculated from Equation 2, using the average null \(g_m\) \((0.9\) \(\mu\)mol \(\text{m}^{-2}\) \(\text{s}^{-1}\) \(\text{bar}^{-1}\)) since there was no difference in \(S_m\). A strong correlation between the initial slope from the \(A_{C_m}\) curve and \(k_{CA}\) was observed, with the initial slope increasing as CA hydration rates increase \((R^2=0.845;\) Fig. 4). There was a curvilinear response between the initial slope of the \(A_C\) curves indicating other limitations.

No difference in stomatal conductance \((g_s)\) was observed across a range of intercellular \(p\text{CO}_2\) between null controls and any of the transformed lines during the rapid measurements of \(\text{CO}_2\) responses (Fig. 5).
Oxygen isotope discrimination measurements

Oxygen (Δ¹⁸O) isotope discrimination and CO₂ assimilation rates were measured in response to changes in pCO₂ using a LI-6400XT coupled to a TDL trace gas analyser to measure C¹⁸O₁⁹O and a Cavity Ring-Down Spectrometer to measure the oxygen isotope composition of water vapour. Transformed plants with reduced CA hydration rates had lower Δ¹⁸O compared with the nulls, but only line 1.1 was significantly lower (Table 2).

In the null controls, measurements of Δ¹⁸O 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).

Δ¹⁸O 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, Δ¹⁸O 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
Effects of reduced carbonic anhydrase on Setaria viridis

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 ACᵢ 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.

### Initial slope of ACᵢ curves in C₄ plants

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 >2mM (~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 ACᵢ curves

Next, we used recently established techniques that utilize \(^{18}\text{O}\) discrimination measurements to quantify \(gᵢ\) in our null controls (Fig. 6B; Barbour et al., 2016). This estimates the
diffusion of $\text{CO}_2$ from the intercellular airspace through the cell wall, plasma membrane, and cytosol to the sites of CA activity. At ambient $p\text{CO}_2$, the $g_m$ observed for the null plants were similar to those reported by Barbour et al. (2016). A key assumption for the calculation of $g_m$ is that CA activity is not limiting and that $\text{CO}_2$ is in isotopic equilibrium with HCO$_3^-$. Consequently, $g_m$ was not measured in the transgenic lines with reduced CA activity. In $C_3$ species, $g_m$ (in this instance from the intercellular airspace to the chloroplast stroma) has been shown to be proportional to the chloroplast surface area pressing the intercellular airspace per unit leaf area ($S_m$). Since $S_m$ was similar between the nulls and line 1.1 plants, we assumed that $g_m$ may also be similar between the plants. In $C_3$ species, $g_m$ has been shown to, in some instances, increase with decreasing $p\text{CO}_2$ (Flexas et al., 2007; Tazoe et al., 2011; Alonso-Cantabrana and von Caemmerer, 2016). These changes to $g_m$ which may be important in regulating and maintaining photosynthesis were also observed here in the $S.\ viridis$ null plants, with $g_m$ increasing slightly at low $p\text{CO}_2$. However, because the differences in $g_m$ at different $p\text{CO}_2$ were not significant, we used the average $g_m$ estimated for the null plants to calculate mesophyll cytosolic $p\text{CO}_2$ ($C_m$) in the transgensics.

As shown in Fig. 4, a strong almost linear relationship was found between $AC_m$ and $k_{CA}$, whereas a saturating relationship was observed with $AC_i$. This indicates that the $\text{CO}_2$ assimilation rate is limited by cytosolic CA activity, with the relationship becoming clearer after accounting for $g_m$. It is tempting to speculate that the differences between the two monocot species and $F.\ bidentis$ relate to differences in limitations imposed by $g_m$ which affects cytosolic $p\text{CO}_2$ and hence in vivo CA activity, but this is not borne out by comparative measurements of $g_m$ 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 $\beta$-CA and a $\text{CO}_2$-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 $C_4$ species. Other possibilities pertain to differences in anatomical characteristics of leaves. Both CA and PEPC are cytosolic enzymes, and differences in $S_m$ may affect the efficiency with which CA is used. Our results suggest that increasing $g_m$ may be an important way to increase the $\text{CO}_2$ assimilation rate at low intercellular $p\text{CO}_2$, a scenario that may, for example, occur under drought.

### Oxygen isotope discrimination and the $\text{CO}_2$ dependence of isotopic equilibrium

As had previously been observed, $\Delta^{18}\text{O}$ decreased with reductions in CA activity as CA facilitates the exchange of $\text{O}_2$ between cytosolic water and $\text{CO}_2$ (Fig. 7; Williams et al., 1996; Cousins et al., 2006). Previous reports, which have estimated the proportion of cytosolic $\text{CO}_2$ in equilibrium with leaf water ($\theta$) in $C_4$ species, have generally assumed a relatively large $g_m$ value and this then led to lower estimates of $\theta$ (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 $g_m$ from anatomical estimates of $S_m$, and cell wall and cytosolic thickness following calculations outlined by von Caemmerer and Evans (2015). This gives a $g_m$ 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 $\Delta^{18}\text{O}$ measurements and highlights the anatomical constraints for $\text{CO}_2$ diffusion dictated by the photosynthetic pathway in leaves of $C_4$ plants (von Caemmerer et al., 2007).

Reduction in CA activity led to significant reductions in $\theta$ but it is interesting to note that $\theta$ did not vary significantly with $p\text{CO}_2$. This is explained by the fact that CA activity increases linearly with $p\text{CO}_2$ so that although there is more $\text{CO}_2$ that needs to equilibrate with leaf water, there is also proportionally more CA activity. The fact that neither transgenic line showed a $\text{CO}_2$ dependence suggests that the decrease in the ratio of CA hydrations to PEP carboxylations is not affecting the isotopic equilibration of $\text{CO}_2$ with leaf water. These results have important implications for the interpretation of the $^{18}\text{O}$ signature of atmospheric $\text{CO}_2$ (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 $\text{CO}_2$

The $\text{CO}_2$ regulation of stomatal conductance remains an open question (Engineer et al., 2016). It has been previously shown that in the $ca1/ca4$ double mutant of $A.\ thaliana$, the degree of stomatal closure in response to increasing $p\text{CO}_2$ 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 $\text{CO}_2$ responses in $C_4$ species. In our study, where only one $\beta$-CA isoform was reduced, we found no change in the response of stomatal conductance to $\text{CO}_2$. The $S.\ viridis$ $\beta$-CA reduced here (Si003882m.g) has low sequence identity (≤50%) to all of the Arabidopsis $\beta$-CAs, but we would predict that multiple reductions in $\beta$-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 $C_4$ photosynthesis in $S.\ viridis$. At lower $C_a$ which may, for example, occur under conditions of drought, our results suggest that $g_m$ may pose a greater limitation than CA activity. However, it is important to investigate the role of CA on $C_4$ 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 Advanced Microscopy at ANU for technical assistance with microscopy.

References


