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plants include the operation of two metabolic cycles (C₃ and C₄) across two photosynthetic cell types, mesophyll cells (MCs) and bundle-sheath cells (BSCs), which compartmentalize the initial carboxylation and decarboxylation reactions (Hatch, 1987; Langdale, 2011). The primary step of HCO₃⁻ fixation to phosphoenolpyruvate is catalysed by phosphoenolpyruvate carboxylase (PEP-C) to produce oxaloacetate, which is subsequently converted into C₄ acids within the MCs (Jenkins et al., 1987). These organic acids then diffuse into neighbouring BSCs where decarboxylation of C₄ acids releases CO₂. A high CO₂ concentration within the semi-gas-tight BSCs suppresses photorespiration and enhances the capacity for CO₂ fixation by Rubisco. C₄ photosynthesis has three biochemical subtypes depending on the C₄ decarboxylation enzyme: nicotinamide adenine dinucleotide phosphate malic enzyme (NADP-ME), NAD malic enzyme, and phosphoenolpyruvate carboxylase (PEP-CK) (Gutierrez et al., 1974; Kanai and Edwards, 1999). C₄ plants have been classified into one of the three subtypes based on the dominant C₄ acid decarboxylation enzyme. Specialized leaf anatomy, biochemistry, and physiology are associated with each of the C₄ subtypes (Ghannoum et al., 2005, 2011).

Nevertheless, there is evidence emerging that PEP-CK activity is more widespread among the biochemical subtypes, suggesting that a degree of flexibility within the C₄ cycle may exist depending on species or environmental conditions (Leegood and Walker, 2003; Furbank, 2011). In maize, an NADP-ME C₄ grass, 25% of the oxaloacetate produced is cycled through an alternative pathway involving the aspartate aminotransferase shuttle and the subsequent decarboxylation of oxaloacetate within the cytosol of BSCs catalysed by PEP-CK (Wingler et al., 1999; Furbank, 2011). This has been shown to exist for maize and other C₄ grasses (Gutierrez et al., 1974). Therefore, the presence of alternative decarboxylation pathways within maize provides the possibility for flexibility in the use of the decarboxylation pathways of the CCM under certain growth conditions (Leegood and Walker, 2003).

For C₄ plants, there is an additional energetic cost associated with the operation and overcycling of the CCM. Minimally, an extra two ATP molecules per CO₂ fixed are required for the regeneration of PEP from pyruvate. During C₄ photosynthesis, the C₄ cycle operates faster than the C₃ (Calvin) cycle this CO₂ is not fixed by Rubisco and ultimately leaks back into the regeneration of PEP from pyruvate. During C₄ photosynthesis, an extra two ATP molecules per CO₂ fixed are required for the operation and overcycling of the CCM. Minimally, a number of studies have examined the effects of short-term and long-term changes in environmental parameters, such as light, water stress, and salinity, yielding mixed results. A few studies have estimated leakiness from measurements of dry-matter carbon isotope, and found that leakiness was impacted by light, salinity, or water stress (Buchmann et al., 1996; Saliendra et al., 1996; Fravolini et al., 2002). When leakiness was estimated from carbon isotope discrimination measured during gas exchange, small changes in leakiness have been reported in some studies but not others in response to short- or long-term changes in the environment (Bowman et al., 1989; Kubasek et al., 2007). In particular, Bowman et al. (1989) found that leakiness changed diurnally in salt-stressed Zea mays and Andropogon glomeratus, two C₄, NADP-ME grasses, while Kubasek et al. (2007) reported that leakiness increased with low light and low temperature. Lowering light intensity during gas-exchange measurements had no effect on bundle-sheath leakiness in a number of C₄ plants (Henderson et al., 1992), and leakiness was unchanged under long-term exposure to low light (Bellasio and Griffiths, 2013a). Ubierna et al. (2013) found that the increase in leakiness commonly reported at low light (Henderson et al., 1992) was only marginally present when using the full model for carbon isotope discrimination in C₄ leaves (Farquhar and Cernusak, 2012). Leakiness depends on a number of anatomical (e.g. CO₂ diffusion path length, chloroplast position in the BSC, BSC wall conductance) and biochemical (e.g. activities of the carboxylases and decarboxylases during C₄ photosynthesis) factors (Henderson et al., 1992; von Caemmerer and Furbank, 2003). In contrast to manipulations using transgenic C₄ plants (von Caemmerer et al., 1997b; Cousins et al., 2006; Pengelly et al., 2012), few studies have investigated the effects of environmental variables on leakiness together with possible underlying biochemical mechanisms.

Consequently, the current study was aimed at investigating the efficiency of C₄ photosynthesis in maize exposed to long-term shade and salinity, by combining measurements of leakiness with assays of the two carboxylases and decarboxylases known to operate in maize leaves. A second aim of this study was to probe the plasticity of the C₄ acid decarboxylases in response to these environmental variables. Salinity and shade were chosen because they impact on photosynthesis through contrasting effects on leaf CO₂ diffusion and fixation. Mild to moderate salinity inhibits root water uptake, thus indirectly reducing the plant water status, as detected by increased leaf water potential and reduced stomatal conductance, both of which reduce photosynthesis (Munns and Tester, 2008; Omoto et al., 2012; Shabala and Munns, 2012). Low light reduces photosynthesis mainly by reducing activity and activation of photosynthetic enzymes (Edwards et al., 1985).

**Materials and methods**

**Plant culture**

Maize seeds (Sweet Corn, Kelvedon Glory 5713) were germinated in 5 l pots (shaded plants were raised in 2 l pots) containing standard potting mix in a sunlit glasshouse during summer (December–March 2012). Nutrients were supplied through the addition of Osmocote...
and periodic watering with soluble Aquasol supplemented with magnesium sulfate. Maize plants destined for the salinity treatments were initially watered with tap water. Once seedlings were well established (2 weeks after germination), NaCl was added at increasing concentrations to the watering solution over a period of 2 weeks until the endpoint concentrations of 50 and 100 mM NaCl were reached. To minimize NaCl accumulation, pots were flushed with water once a week, and then irrigated with the desired NaCl concentration. Plants destined for shading were germinated as above in full sunlight and then placed under a shade cloth, which limited light to 20% of the ambient sunlight. At midday, the photosynthetic active radiation of full sunlight ranged between 1000 and 1800 µmol m⁻² s⁻¹ when measured at pot level during the experiment. Air temperature inside the glasshouse compartment was regulated by a temperature-control system, and day/night temperatures averaged 26/18 °C. Relative humidity was monitored and ranged between 60 and 80% during the day. There were five pots per treatments. Plants were harvested 12 weeks after germination.

Measuring leaf gas exchange

Leaf gas-exchange measurements were carried out 1–2 weeks before harvest using a portable open photosynthesis system (LI-6400XT; LI-COR, Lincoln, USA). Measurements of light-saturated photosynthetic rate ($A_{\text{max}}$) and stomatal conductance ($g_s$) were taken at 10:00 and 14:00 at ambient CO₂ (400 ppm) and under similar conditions to the spot gas exchange measurements. Photosynthetic responses to intercellular CO₂ concentration ($A/C$ curves) were measured at 10 CO₂ steps using similar conditions as described above. The $A/C$ curves were fitted using a $C_4$ photosynthesis model (von Caemmerer, 2000) to estimate maximal PEPC ($p_{\text{max}}$) and Rubisco ($V_{\text{cmax}}$) activities. The fractionation factor $\delta$ of $^{13}C$ in $\text{CO}_2$ to $\text{HCO}_3^-$ is the fraction associated with photosynthesis, respectively; $\delta_r$ ranged between 5 and 11. Leakiness $\phi$ was calculated using the model of Farquhar (1983) as modified by Pengelly et al. (2010, 2012). The formulae used are described briefly below.

$$\phi = \left(1 - t \right) - \left( a + b \right) \frac{A}{g_s C_a} - \left( b_1 - s \right) \frac{C_s}{C_a g_m}$$

where the term $t$, which represents the ternary effect, is defined as by Farquhar and Cernusak, (2012):

$$t = \frac{(1 + a')E}{2g_w}$$

where $E$ is the transpiration rate and $g_w$ the total conductance to CO₂ diffusion including boundary layer and stomatal conductance (von Caemmerer and Farquhar, 1981). The symbol $a'$ denotes the combined fractionation factor through the leaf boundary layer and through stomata:

$$a' = \frac{a (C_a - C_o) + a (C_o - C_i)}{C_a - C_i}$$

where $C_a$, $C_o$, and $C_i$ are the ambient, intercellular, and leaf surface CO₂ partial pressures, $a_i$ (2.9‰) is the fractionation occurring due to diffusion in the boundary layer, $a$ (4.4‰) is the fractionation due to diffusion in air (Evans et al., 1986), $s$ (1.8‰) is the fractionation during leakage of CO₂ out of the bundle sheath, and $a_i$ is the fractionation factor associated with the dissolution of CO₂ and diffusion through water. Here, we assume that $s = 0$.

$$b_1 = b_i - e \left( \frac{R_d}{A + R_d} - \frac{0.5R_i}{A + 0.5R_i} \right) - f \left( \frac{0.5V_c}{V_i} \right)$$

where $b_1$ is the fractionation by Rubisco (30%), $b_i$ is the combined fractionation of the conversion of CO₂ to HCO₃⁻ and PEP carboxylation (−5.74‰ at 25 °C), $f$ is the fraction associated with photosynthesis, and $V_c$ and $V_i$ are the rates of oxygenation and carboxylation, respectively. The fractionation factor $e$ associated with respiration was calculated from the difference between $\delta$ in the CO₂ cylinder (−40.5‰) used during experiments and that in the atmosphere under growth conditions (−8‰; Tazo et al., 2008). $A$ and $R_d$ denote the CO₂ assimilation rate and day respiration, respectively; $R_d$ was assumed to equal dark respiration. We assumed a mesophyll conductance ($g_m$)=1 mol m⁻² s⁻¹ bar⁻¹ for these calculations.

Rubisco content and soluble protein determination

Following gas-exchange measurements, replicate leaf discs (0.74 cm²) were rapidly frozen in liquid nitrogen and then stored at −80 °C until analysed. Each leaf disc was extracted in 1 ml of ice-cold extraction buffer [50 mM EPPS/NaOH (pH 8.0), 5 mM dithiothreitol, 20 mM NaHCO₃, 20 mM MgCl₂, 1 mM EDTA, 4% (v/v) Protease Inhibitor Cocktail (Sigma), and 1% (w/v) polyvinyl polypyrrolidone] using a 2 ml Potter–Elvehjem glass homogenizer kept on ice. Subsamples were taken from the total extract for SDS-PAGE analysis (see below) of total leaf protein. The remaining extract was centrifuged at 16, 100g for 1 min and the supernatant used for Rubisco and soluble protein assays. Rubisco content was estimated by the irreversible binding of [¹⁴C]-carbonarabinitol bisphosphate (CABP) to the fully carbamylated enzyme (Sharwood et al., 2008). Extractable soluble proteins were measured using a Coomassie Plus kit (Pierce).

Activity of carboxylase and decarboxylase enzymes

Activity of Rubisco in maize extracts was determined by multiplying the number of Rubisco active sites determined using the [¹⁴C]-CABP binding assay by the Rubisco in vitro $k_{cat}$ (5.5 s⁻¹) determined using a CO₂ fixation assay (Sharwood et al., 2008). The activity of the PEPC
and NADP-ME enzymes were determined spectrophotometrically as described previously (Ashton et al., 1990; Pengelly et al., 2012).

The activity of PEP-CK in maize extracts was measured in the carboxylase direction using the method outlined by Walker et al. (2002). For each assay, a separate leaf disc was homogenized in extraction buffer containing 50 mM HEPES (pH 7.2), 5 mM dithiothreitol, 1% polyvinylpolypyrrolidone, 2 mM EDTA, 2 mM MnCl₂, and 0.05% Triton X-100. MgCl₂ was not added to the extraction or assay buffer to remove the possibility of interference from other enzymes. PEP-CK activity was measured in assay buffer [100 mM HEPES (pH 7.0), 4% mercaptoethanol (w/v), 100 mM KCl, 90 mM NaHCO₃, 1 mM ADP, 2 mM MnCl₂, 0.14 mM NADH, and malate dehydrogenase (6 U)] after the addition of PEP to 5 mM. The final concentration of 4 mM MnCl₂ has been shown to be sufficient for PEP-CK activity (Chen et al., 2002; Walker et al., 2002).

SDS-PAGE and immunoblot analysis of Rubisco and CCM proteins

Subsamples of total protein fractions were mixed with 0.25 vols of 4× LDS buffer (Invitrogen) containing 100 mM dithiothreitol and placed on ice until analysed within 2 h. For confirmatory visualization, protein samples were separated by SDS-PAGE in TGX Any KD (BioRad) pre-cast polyacrylamide gels buffered with 1× Tris/glycine SDS buffer (BioRad) at 200 V using a Mini-Protein apparatus at 4 °C. Proteins were visualized by staining with Bio-Safe Coomassie G-250 (BioRad) and imaged using a VersaDoc imaging system (BioRad).

For immunoblot analyses of total leaf protein, samples were separated by SDS-PAGE as outlined above and then transferred at 4 °C to nitrocellulose membranes (0.45 μm; BioRad) using a Xcell Surelock western transfer module (Invitrogen) buffered with 1× Tris/glycine SDS buffer (BioRad) at 200 V using a Mini-Protein apparatus at 4 °C. Proteins were visualized by staining with Bio-Safe Coomassie G-250 (BioRad) and imaged using a VersaDoc imaging system (BioRad).

Primary antisera raised in rabbit against tobacco Rubisco (prepared by S. M. Whitney, Australian National University, Canberra, Australia) was diluted 1:4000 in TBS before incubation at 1 h with membranes at room temperature with gentle agitation. Antisera was raised against PEP-CK was obtained from AgriSera and diluted 1:2000 with TBS. For NADP-ME and PEP-CK, synthetic peptides based on monocot amino acid sequences for each protein were synthesized with GL Biochem and antisera were raised against each peptide in rabbits. The reactive antisera were the antigen purified for use in immunoblot analysis (GL Biochem). The NADP-ME and PEP-CK antisera were diluted in TBS at 1:1000 and 1:500, respectively.

All primary antisera were incubated with membranes at room temperature for 1 h with gentle agitation before washing three times with TBS. Secondary goat anti-rabbit antisera conjugated to horseradish peroxidase (Perkin Elmer) was diluted 1:3000 in TBS and incubated with the membranes for 1 h at room temperature followed by three washes with TBS. Immunoreactive peptides were detected using an Immun-Star WesternC kit (BioRad) and imaged using VersaDoc.

Plant biomasses, leaf water potential, and nitrogen and carbon isotope composition

Before harvest, leaf water potential (Ψₛ) was measured on a cut, matching gas-exchange leaf using a Scholander-style pressure chamber (PMS Instrument Company, Corvallis, OR, USA). At harvest, leaves were sampled and their area determined using a leaf area meter (LI-3100A; LI-COR) and roots were washed free of soil. Plant tissues were oven dried at 80 °C for 48 h, weighed, and ground to a homogenous powder in a ball mill (MM-400; Retsch).

Leaf N content was determined on the ground tissue using a CN analyser (LECO TruSpec; LECO Corp., MI, USA). For carbon isotope composition (δ¹³C), ground leaf samples were combusted in a Carlo Erba elemental analyser (Model 1108) and the CO₂ was analysed by mass spectrometry. Isotopic composition (δ) was calculated as [([Rsample – Rstandard]/Rstandard)×1000, where Rsample and Rstandard are the ¹³C/¹²C ratios of the sample and standard (Pee Dee Belemnite), respectively.

Statistical analysis

Statistical significance tests were conducted using one-way analysis of variance computed in a general linear model. Treatment means were ranked using a post-hoc Tukey test.

Results

Plant growth and leaf nitrogen

Plant leaf area was reduced by 18 and 22% for plants exposed to 50 and 100 mM NaCl, respectively, whereas plant biomass was decreased by 34 and 50% for the same treatments when compared with the control (Fig. 1A, B, Table 1). Leaf mass per area was not significantly affected by salinity (Table 1).

The impact of 80% shading (shaded plants received 20% of ambient sunlight) on the maize plants was profound. Leaf area and total plant biomass were reduced to 18 and 3% of that of the control plants, respectively, while leaf mass per area was reduced to 37% of that of the control plants (Fig. 1A, B, Table 1).

Leaf N content per unit mass decreased in the high-salt-treated plants only relative to the control. When expressed on an area basis, leaf N concentration tended to be lower in the shaded plants relative to the control (Table 1).

Leaf photosynthesis

Leaf water potential (Ψₛ) decreased in plants exposed to moderate (100 mM NaCl) but not mild (50 mM NaCl) levels of soil salinity (Table 1). Consequently, photosynthetic rates measured at ambient CO₂ (Aᵣ) decreased in maize plants exposed to the higher salinity treatment only (Fig. 1C, D, Table 1), whereas gₛ decreased in plants exposed to both salinity levels (Fig. 1D, Table 1). Plants exposed to shade underwent larger decreases in photosynthesis and gₛ. (Fig. 1C, D, Table 1). A common linear relationship related Aᵣ to gₛ (r²=0.73) in all the maize plants regardless of the treatment (Fig. 2C).

A/Cᵣ curves were fitted using the C₄ photosynthesis model (von Caemmerer, 2000) to estimate in vivo V₇₀max and V₇₀max. Both parameters decreased in the shaded plants relative to the control, while there was a small but non-significant reduction in V₇₀max in the higher salinity treatment. The ratio V₇₀max/V₇₀max (2.3–2.8) was similar for all the maize plants, regardless of the treatment (Fig. 3, Table 1).

Photosynthetic and dry-matter carbon isotope discrimination

Concurrent measurements of ¹³CO₂/¹²CO₂ discrimination and leaf gas exchange showed that photosynthetic discrimination (Δₑ) varied linearly with p/pₑ for plants in the control and
salinity treatments, yielding a common bundle-sheath $\phi$ value of 0.25 according to the carbon discrimination model for C₄ plants (Farquhar, 1983). Thus, salinity changed $p/p_s$ without affecting $\phi$. In contrast, shaded plants had lower $\Delta_p$, $p/p_s$, and $\phi$ relative to both control and salt-stressed plants (Fig. 2A).

Leaf dry-matter carbon isotope composition ($^{13}\delta$) decreased (more negative) significantly in the salt-treated plants only, while shade plants had similar leaf $^{13}\delta$ to the control plants (Table 1). Photosynthetic $\Delta_p$ and leaf dry-matter $^{13}\delta$ changed proportionately for the control and salt-treated plants.

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**Table 1. Summary of plant growth, leaf chemistry, leaf gas exchange and photosynthetic enzyme activity determined for maize plants grown in full sunlight and irrigated with normal water (control), 50 mM NaCl (Salt-50) or 100 mM NaCl (Salt-100)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Shade</th>
<th>Salt-50</th>
<th>Salt-100</th>
<th>Model $P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plant and leaf traits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total leaf area (cm² plant⁻¹)</td>
<td>512 ± 61b</td>
<td>94 ± 23a</td>
<td>418 ± 47b</td>
<td>397 ± 4b</td>
<td>0.0012</td>
</tr>
<tr>
<td>Plant dry mass (g plant⁻¹)</td>
<td>28.2 ± 4.6c</td>
<td>0.75 ± 0.5a</td>
<td>18.5 ± 4.3bc</td>
<td>14.0 ± 2.7b</td>
<td>0.0031</td>
</tr>
<tr>
<td>Leaf mass per area (g m⁻²)</td>
<td>71 ± 5b</td>
<td>26 ± 2a</td>
<td>69 ± 6b</td>
<td>59 ± 15b</td>
<td>0.0343</td>
</tr>
<tr>
<td>Leaf water potential, $\Psi_L$ (MPa)</td>
<td>0.32 ± 0.07a</td>
<td>ND</td>
<td>0.53 ± 0.10a</td>
<td>1.08 ± 0.09b</td>
<td>0.0000</td>
</tr>
<tr>
<td>Leaf N content (mg g⁻¹)</td>
<td>36 ± 2b</td>
<td>38 ± 0b</td>
<td>33 ± 2ab</td>
<td>29 ± 1a</td>
<td>0.0054</td>
</tr>
<tr>
<td>Leaf N content (mmol m⁻²)</td>
<td>184 ± 10a</td>
<td>95 ± 25a</td>
<td>132 ± 24a</td>
<td>124 ± 28a</td>
<td>0.1027</td>
</tr>
<tr>
<td>Leaf C isotope composition, $^{13}\delta$ (%)</td>
<td>−14.68 ± 0.24a</td>
<td>−14.38 ± 0.19a</td>
<td>−15.43 ± 0.05b</td>
<td>−15.85 ± 0.07b</td>
<td>0.0006</td>
</tr>
<tr>
<td><strong>Leaf gas exchange</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photosynthesis, $A_{\text{sat}}$ (µmol m⁻² s⁻¹)</td>
<td>33.7 ± 1.0c</td>
<td>13.0 ± 0.7a</td>
<td>32.6 ± 1.1c</td>
<td>28.3 ± 1.6b</td>
<td>0.0000</td>
</tr>
<tr>
<td>Stomatal conductance, $g_s$ (mol m⁻² s⁻¹)</td>
<td>0.285 ± 0.017c</td>
<td>0.088 ± 0.007a</td>
<td>0.191 ± 0.012b</td>
<td>0.172 ± 0.020b</td>
<td>0.0000</td>
</tr>
<tr>
<td>in vivo $V_{\text{crus}}$ (µmol m⁻² s⁻¹)</td>
<td>40 ± 10b</td>
<td>19 ± 6a</td>
<td>40 ± 1b</td>
<td>33 ± 1b</td>
<td>0.0125</td>
</tr>
<tr>
<td>in vivo $V_{\text{crus}}$ (µmol m⁻² s⁻¹)</td>
<td>104 ± 6b</td>
<td>45 ± 5a</td>
<td>94 ± 1b</td>
<td>94 ± 1b</td>
<td>0.0012</td>
</tr>
<tr>
<td>$V_{\text{crus}}/V_{\text{cmax}}$</td>
<td>2.6 ± 0.2a</td>
<td>2.5 ± 0.4a</td>
<td>2.3 ± 0.1a</td>
<td>2.8 ± 0.1a</td>
<td>0.4882</td>
</tr>
<tr>
<td>Photosynthetic $\Delta_p$ (%)</td>
<td>3.66 ± 0.13b</td>
<td>2.46 ± 0.36a</td>
<td>3.90 ± 0.04b</td>
<td>4.23 ± 0.11b</td>
<td>0.0040</td>
</tr>
<tr>
<td>Leaikiness, $\phi$</td>
<td>0.26 ± 0.02ab</td>
<td>0.13 ± 0.04a</td>
<td>0.24 ± 0.02ab</td>
<td>0.31 ± 0.01b</td>
<td>0.0125</td>
</tr>
<tr>
<td><strong>Photosynthetic enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rubisco content (g m⁻³)</td>
<td>0.31 ± 0.04b</td>
<td>0.14 ± 0.03a</td>
<td>0.23 ± 0.01ab</td>
<td>0.24 ± 0.03ab</td>
<td>0.0141</td>
</tr>
<tr>
<td>Soluble proteins (g m⁻³)</td>
<td>4.2 ± 0.2b</td>
<td>2.5 ± 0.2a</td>
<td>3.2 ± 0.2ab</td>
<td>3.9 ± 0.4b</td>
<td>0.0057</td>
</tr>
<tr>
<td>Rubisco activity (µmol m⁻² s⁻¹)</td>
<td>27.5 ± 1.4c</td>
<td>11.5 ± 2.0a</td>
<td>18.1 ± 0.8b</td>
<td>19.5 ± 2.1b</td>
<td>0.0034</td>
</tr>
<tr>
<td>PEPC activity (µmol m⁻² s⁻¹)</td>
<td>107 ± 7d</td>
<td>21 ± 4a</td>
<td>52 ± 4b</td>
<td>72 ± 4c</td>
<td>0.0000</td>
</tr>
<tr>
<td>PEPC/Rubisco</td>
<td>3.9 ± 0.10c</td>
<td>1.8 ± 0.08a</td>
<td>2.9 ± 0.13b</td>
<td>3.3 ± 0.15b</td>
<td>0.0000</td>
</tr>
<tr>
<td>NADP-ME activity (µmol m⁻² s⁻¹)</td>
<td>53 ± 8b</td>
<td>32 ± 5a</td>
<td>18 ± 0.1a</td>
<td>19 ± 3a</td>
<td>0.0073</td>
</tr>
<tr>
<td>PEP-CK activity (µmol m⁻² s⁻¹)</td>
<td>12.4 ± 1.6c</td>
<td>3.0 ± 0.4a</td>
<td>7.6 ± 0.7b</td>
<td>8.2 ± 1.2b</td>
<td>0.0021</td>
</tr>
</tbody>
</table>
plants (Fig. 2B). In contrast, the shade plants fell outside the common relationship because their photosynthetic $\Delta p$ decreased but not their leaf $\delta_{13}$ relative to the control plants (Fig. 2B).

**Activity of photosynthetic enzymes**

Leaf Rubisco content and Rubisco activity calculated from $k_{cat}$ and the irreversible binding of the transition state analogue $[14C]CABP$ decreased by 25 and 50% in the salt-treated and shaded plants, respectively. As expected for C_4 leaves, Rubisco activity was equivalent to $A_{sat}$ for the control and shade leaves; this was not the case for the salt-treated leaves (Fig. 4A, Table 1). Leaf soluble proteins changed together with Rubisco such that Rubisco constituted a constant fraction of soluble proteins under all treatments (Table 1). PEPC activity measured in leaf extracts was reduced by 80% in the shaded plants and by 30–50% in the salt-treated plants relative to the control treatment (Fig. 4B, Table 1). Generally, changes in Rubisco and PEPC activities were reflected by the immunoblots probed with antibodies raised against each of the carboxylase enzymes (Fig. 5). Shading reduced PEPC activity to a greater extent than Rubisco activity, and consequently halved the PEPC/Rubisco activity ratio relative to the control treatment. The PEPC/Rubisco ratio was not significantly affected by salinity in the maize plants (Table 1). It is worth noting that *in vivo* and *in vitro* estimates of Rubisco ($V_{cmax}$) and PEPC ($V_{pmax}$) activities did not closely correlate in this study. Reconciling these parameters requires more detailed parameterization of C_4 photosynthesis model (von Caemmerer, 2000).

The activity of the primary decarboxylase NADP-ME and its relative content determined by immunoblot analysis showed 35% reductions across both salinity treatments. NADP-ME activity declined by 60% in the shaded relative to the control plants (Figs 4C and 5, Table 1). Activity and protein expression of the secondary decarboxylase, PEP-CK, was detected in the leaf extracts of all maize plants (Figs 4D

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**Fig. 2.** Leaf gas exchange and carbon isotope discrimination in maize plants exposed to salinity and shade. Photosynthetic carbon isotope discrimination, $\Delta$, measured during the gas exchange of maize leaves as a function of intercellular to ambient CO$_2$ ratio (A) or leaf dry matter carbon isotope composition, $^{13}\delta$ (B). Photosynthetic rates as a function of stomatal conductance are also shown (C). In (A), the solid line is the solution for the C$_4$ discrimination model (Farquhar, 1983) using a leakiness ($\Phi$) value of 0.25. Leaf gas exchange was measured at high light (1800 µmol m$^{-2}$ s$^{-1}$), ambient CO$_2$ (400 µl l$^{-1}$) and 26 °C. In (B), the solid line is the linear regression of all data points excluding the shade treatment. In (C), the solid line is the linear regression of all data points. Maize plants were grown in full sunlight and irrigated with water (control, filled circle), 50 mM NaCl (Salt-50, half-filled triangle), or 100 mM NaCl (Salt-100, filled triangle), or grown in 20% sunlight (shade, open circle).

**Fig. 3.** A/C$_i$ response curves for leaves of maize exposed to salinity and shade. Responses of assimilation rates to intercellular CO$_2$ (A/C$_i$) curves measured at a light intensity of 1800 µmol m$^{-2}$ s$^{-1}$ and a leaf temperature of 26 °C. Data points are the average of two replicates. Lines are the mathematical fits using the C$_4$ photosynthesis model (von Caemmerer, 2000). Maize plants were grown in full sunlight and irrigated with water (control, filled circle), 50 mM NaCl (Salt-50, half-filled triangle), or 100 mM NaCl (Salt-100, filled triangle), or grown in 20% sunlight (shade, open circle).
capacity, salinity reduced stomatal conductance with small effects on photosynthetic rates of the maize plants. Salinity inhibited plant growth to a lesser extent than shade (Table 1).

The evolution of a CCM in higher plants represents a key step to improving photosynthesis under environmental conditions favouring photorespiration by circumventing the inefficiency of Rubisco. The efficient operation of C₄ photosynthesis requires close coordination between the C₄ and C₃ cycles, which is achieved through the distinct cellular compartmentalization of the initial and final carboxylases PEPC (in MCs) and Rubisco (in BSCs), respectively, as well as the localization of the decarboxylases (NADP-ME and PEP-CK for maize) within the BSC. In addition, the maintenance of a high PEPC/Rubisco activity ratio is critical for the build-up of CO₂ within the BSCs. Importantly, regulating the balance between Rubisco, PEPC, NADP-ME, and other enzymes of the C₃ and C₄ cycles allows the dynamic regulation of C₄ efficiency that other features such as BSC wall conductance or CO₂ diffusion path length cannot offer in the short to medium term (Hatch, 1987; von Caemmerer and Furbank, 2003). Perturbation of the PEPC/Rubisco ratio by genetically suppressing PEPC results in C₃ plants unable to grow effectively in air (Dever et al., 1997; Cousins et al., 2007). Leakiness of CO₂ from the BSCs as determined from measurements of ¹³C/¹²C carbon isotope discrimination represents a key surrogate indicator of the coordination between the C₃ and C₄ cycles (Farquhar, 1983). Combining measurements of leakiness with activities of the key enzymes in the C₃ and C₄ cycles can elucidate the regulation and efficiency of C₄ photosynthesis under different environments (Evans et al., 1986; Henderson et al., 1992). Below, we demonstrate that shade, but not salinity, can perturb CCM efficiency as evidenced by changed leakiness.

Mild to moderate salinity impacts on carbon isotope discrimination through stomatal conductance without affecting leakiness

In maize, mild salinity (50 mM NaCl) reduced leaf gₛ but not Aₛ, while moderate salinity (100 mM NaCl) reduced both gₛ and Aₛ. Hence, reduced photosynthetic rates were largely caused by increased resistance to CO₂ diffusion under both salinity treatments, and this was born out in the lower p∕pₛ ratio and more negative dry-matter ¹³δ observed in the leaves of salt-treated maize plants (Fig. 2). Reduced stomatal conductance and leaf ¹³δ in response to salinity is commonly reported in C₃ (Seemann and Critchley, 1985; Brugnoli and Lauteri, 1991) and C₄ (Bowman et al., 1989; Meinzer et al., 1994; Meinzer and Zhu, 1999) plants. In maize, reduced photosynthetic rates, especially at the highest salinity treatment was also caused by the lower Rubisco and PEPC activities. This reduction was observed in the spectrophotometric assays and the immunodetection of the expressed proteins. Reduced expression of Rubisco under salinity was part of a general reduction in soluble proteins and leaf N. Leaf N is known to decline under salinity due to Cl⁻ interference with nitrate uptake by roots (Munns and Termaat, 1986).

Discussion

Contrasting impacts of salinity and shade on maize

The main objective of this study was to investigate the regulation of C₄ photosynthesis subjected to environmental manipulations that are known to have contrasting impacts on the processes of CO₂ assimilation and diffusion. One of the main acclimation responses to shade is the downregulation of leaf photosynthetic capacity (Boardman, 1977). In contrast, mild to moderate salinity will primarily reduce stomatal conductance by negatively impacting on soil, and hence on leaf water potential. Compared with water stress, salinity has the added advantage of providing a steady stress while avoiding the complications associated with controlling soil water supply (Neumann et al., 1988; Chaves et al., 2009). To this end, both treatments used in this study achieved their goals. While shade markedly reduced plant growth and photosynthetic
The activity of both carboxylases declined to the same extent under salinity conditions such that the PEPC/Rubisco ratio was indistinguishable from that of the control leaves. This may explain why leakiness was unaffected by salinity in maize leaves despite the changes in photosynthetic $\Delta_p$ and leaf $^{13}\delta_p$, which were caused by reduced $p/A_p$ (Fig. 2). In line with these results, when the C4 shrub *Atriplex lentiformis* was exposed for 4 weeks to salinity levels equivalent to those used in the current study, photosynthesis and stomatal conductance decreased, while the PEPC/Rubisco ratio remained unchanged until the salinity increased above 120 mM. The same study also reported that leakiness, estimated from leaf $^{13}\delta_i/a$ and derived from leaf $^{13}\delta$, was not significantly affected (Fig. 2). Similarly to *Atriplex*, salinity reduced photosynthesis and increased $p/A_p$ and $\phi$ values in sugarcane genotypes. Changes in $\phi$ derived from leaf $^{13}\delta$ were also related to the PEPC/ Rubisco ratio in sugarcane (Meinzer et al., 1994).

The discrepancy between the studies using *Atriplex* and sugarcane with the current study using maize may be related to a number of factors, the main one being the salinity level and the basis for leakiness calculation. Meinzer and Zhu (1999) found that mild salinity mainly affected $g_a$ and had little impacts on $\phi$ (a similar scenario to the current maize study), and that $\phi$ and the PEPC/Rubisco ratio were affected at high salinity, indicating profound damage of the photosynthetic apparatus by the accumulating salt, unlike the treatments used in the current maize study. In addition, the difference between leaf $^{13}\delta$ and photosynthetic $\Delta_p$ have not been reconciled yet for C4 plants. Post-photosynthetic fractionation of $^{13}$C/$^{12}$C may be important in C4 leaves, thus representing a source of uncertainty in leakiness calculations based on leaf $^{13}\delta$ (Henderson et al., 1992).

In maize, both salinity treatments reduced the activity of the primary (NADP-ME) and secondary (PEP-CK) carboxylases. These observations, together with reduced PEPC activity, suggest that the CCM was down regulated in response to salinity. Results obtained with enzyme activity and immunoblot analysis indicated that the decarboxylases were inhibited more than the carboxylases under salinity. Evidence from transgenic Flaveria plants with reduced amounts of NADP-ME have indicated that this decarboxylase is in excess, as photosynthesis was not impacted until activity was reduced to less than 40% of that of wild type (Pengelly et al., 2012). In summary, salinity treatments reduced photosynthesis primarily by reducing $g_a$ and secondarily by reducing Rubisco and PEPC activities. The balance between the C3 and C4 cycles was unaffected, as indicated by a similar leakiness between the salt-treated and control maize plants.

**Shade profoundly reduces photosynthetic capacity and leakiness, thus perturbing the coordination between the C3 and C4 cycles**

The shade treatment used in this study had profound impacts on the growth and photosynthesis of the maize plants (Table 1). In particular, shade reduced the photosynthetic capacity measured in terms of *in vivo* $V_{cmax}$ and $V_{pmax}$ estimated from the $A/C_i$ curves and in terms of enzyme activity of the carboxylases and decarboxylases. In contrast to salinity, shade had two significantly distinct effects on leaf photosynthesis. Firstly, decreased photosynthetic capacity was mediated by a general downregulation of the activity and protein expression of all measured photosynthetic enzymes. Secondly, the PEPC/Rubisco ratio, photosynthetic $\Delta_p$, and its derived leakiness decreased relative to those of the control plants, while leaf $^{13}\delta$ was not significantly affected (Fig. 2).

The responses of C4 photosynthesis to low light vary depending on whether the condition is transient or a short-term acclimation. Under low light ($<200 \mu$mol quanta m$^{-2}$ s$^{-1}$), $\phi$ may increase, possibly as a result of decreased Rubisco activation or increased Rubisco oxygenation due to the low BSC CO$_2$ concentration. These factors decrease CO$_2$ fixation by Rubisco more than by PEPC, thus maintaining a higher supply of CO$_2$ to the BSCs than Rubisco can fix (Henderson et al., 1992; Kromdijk et al., 2008, 2010; Tazoe et al., 2008). However, $\phi$ in maize leaves was unaffected under conditions

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**Fig. 5.** Immunoblots of carboxylases and decarboxylases in maize plants exposed to salinity and shade. Immunoblots of total leaf proteins probed with antisera raised against the four photosynthetic enzymes Rubisco large subunit, PEPC, NADP-ME, and PEP-CK, as described in Materials and methods. The analysis was undertaken separately for the salinity-treated (A) and shade-treated (B) maize plants. Changes in immunoblot densitometry were calculated relative to the control treatment (C).
of short-term acclimation to low light (Bellasio and Griffiths, 2013a, b).

In contrast to these studies, leakiness decreased in our study as a result of reduced $\Delta p$ with little impact on $p/p_{\nu}$, suggesting two main conclusions. Firstly, reduced leakiness in our maize study was accompanied by a reduced PEPC/Rubisco ratio, highlighting the role of this ratio in particular, and the balance between the activity of the C$_4$ and C$_3$ cycle enzymes in general, for optimizing the efficiency of C$_4$ photosynthesis. Our results in maize make it clear that acclimation to low light reduced PEPC activity and protein expression to a greater extent than those of Rubisco. High light dependence of PEPC gene expression is well documented in C$_4$ plants (Chollet et al., 1996). Secondly, leaf $^{13}$δ and photosynthetic $\Delta p$ in our maize study did not change together under low light, mainly because the former decreased while the latter was only marginally and not significantly affected by shade (Fig. 2). This is in contrast to a large survey of C$_4$ grasses, showing that leaf $^{13}$δ decreased under shade conditions (Buchmann et al., 1996). On the one hand, our results highlight the problems of using leaf $^{13}$δ as a proxy for photosynthetic $\Delta p$, especially when inferring leakiness and C$_4$ regulation. On the other hand, our results point to a stronger dependence of leaf $^{13}$δ on the diffusive components (salinity effects) within the $\Delta p$ equation as opposed to the metabolic factors for which light can have complex effects (Farquhar, 1983; Henderson et al., 1992; von Caemmerer et al., 1997a; Ubierna et al., 2011). Solving the link between leaf $^{13}$δ and photosynthetic $\Delta p$ remains a key challenge for elucidating the underpinnings of carbon isotope discrimination in C$_4$ leaves.

In another contrast with the salinity treatments, shade reduced the activity of the primary decarboxylase NADP-ME less, while strongly suppressing the activity of the secondary decarboxylase PEPC-CK. Taken together, these results constitute rare evidence for decarboxylase flexibility in response to environmental conditions, with salinity and shade having opposite effects on the ratio of PEPC-CK to NADP-ME activity in maize. It is unlikely that the observed changes in NADP-ME and PEPC-CK were due to anaplerotic activities due to their low contribution relative to that of the photosynthetic isoforms (Drincovich et al., 2001). The differential engagement of the decarboxylation pathways enables C$_4$ plants to acclimate to varying conditions of light (Furbank, 2011). For example, it has been shown that the flexible operation of NADP-ME and PEPC-CK decarboxylases in maize allows the bundle sheath to regulate NADPH supply under variable light conditions (Bellasio and Griffiths, 2013b). In the current study, we demonstrated the differential engagement of the primary and secondary decarboxylases under long-term acclimation to low light through the significant reductions of PEPC-CK activity and protein content (Figs 4 and 5).

In summary, we demonstrated that long-term acclimation to low light in maize causes a reduction in BSC leakiness. This reduction was underpinned by a greater downregulation of PEPC activity and content relative to those of Rubisco, and by a flexible partitioning of C$_4$ acid decarboxylation activity between NADP-ME and PEPC-CK.

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