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RESEARCH PAPER

Photosynthetic flexibility in maize exposed to salinity and shade

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Abstract

C_4 photosynthesis involves a close collaboration of the C_3 and C_4 metabolic cycles across the mesophyll and bundle-sheath cells. This study investigated the coordination of C_4 photosynthesis in maize plants subjected to two salinity (50 and 100 mM NaCl) treatments and one shade (20% of full sunlight) treatment. Photosynthetic efficiency was probed by combining leaf gas-exchange measurements with carbon isotope discrimination and assaying the key carboxylases [ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phosphoenolpyruvate carboxylase (PEPC)] and decarboxylases [nicotinamide adenine dinucleotide phosphate malic enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PEP-CK)] operating in maize leaves. Generally, salinity inhibited plant growth and photosynthesis to a lesser extent than shade. Salinity reduced photosynthesis primarily by reducing stomatal conductance and secondarily by equally reducing Rubisco and PEPC activities; the decarboxylases were inhibited more than the carboxylases. Salinity increased photosynthetic carbon isotope discrimination (Δ_p) and reduced leaf dry-matter carbon isotope composition ($^{13}\delta$) due to changes in p_i/p_a (intercellular to ambient CO_2 partial pressure), while CO_2 leakiness out of the bundle sheath (ϕ) was similar to that in control plants. Acclimation to shade was underpinned by a greater downregulation of PEPC relative to Rubisco activity, and a lesser inhibition of NADP-ME (primary decarboxylase) relative to PEP-CK (secondary decarboxylase). Shade reduced Δ_p and ϕ without significantly affecting leaf $^{13}\delta$ or p_i/p_a relative to control plants. Accordingly, shade perturbed the balance between the C_3 and C_4 cycles during photosynthesis in maize, and demonstrated the flexible partitioning of C_4 acid decarboxylation activity between NADP-ME and PEP-CK in response to the environment. This study highlights the need to improve our understanding of the links between leaf $^{13}\delta$ and photosynthetic Δ_p, and the role of the secondary decarboxylase PEP-CK in NADP-ME plants such as maize.

Key words: C_4 photosynthesis, carbon isotope discrimination, leakiness, NADP-ME, PEP-CK, PEPC, Rubisco.

Introduction

C_4 photosynthesis evolved as a spatial and biochemical adaptation to remedy the inefficiency of C_3 photosynthesis under conditions of high temperature, low CO_2, and water stress, all of which exacerbate photorespiration (Ludwig, 2013). The propensity of photorespiration is determined by the extent of oxygenation carried out by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Andrews *et al.*, 1973; Cleland *et al.*, 1998). For C_3 plants, the current O_2 concentration in the atmosphere (210 mmol mol^{-1}) severely inhibits the carboxylation of ribulose-1,5-bisphosphate by Rubisco. Together, these environmental conditions are hypothesized to be the drivers for the independent evolution of plants operating a CO_2 concentration mechanism (CCM; Sage *et al.*, 2012). The distinguishing features of the CCM in most C_4

Abbreviations: BSC, bundle-sheath cell; CABP, carboxyarabinitol bisphosphate; CCM, CO_2 concentration mechanism; MC, mesophyll cell; NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PEPC, phosphoenolpyruvate carboxylase; PEP-CK, phosphoenolpyruvate carboxykinase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

plants include the operation of two metabolic cycles (C_3 and C_4) 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 HCO_3^- fixation to phosphoenolpyruvate is catalysed by phosphoenolpyruvate carboxylase (PEPC) to produce oxaloacetate, which is subsequently converted into C_4 acids within the MCs (Jenkins *et al.*, 1987). These organic acids then diffuse into neighbouring BSCs where decarboxylation of C_4 acids releases CO_2. A high CO_2 concentration within the semi-gas-tight BSCs suppresses photorespiration and enhances the capacity for CO_2 fixation by Rubisco. C_4 photosynthesis has three biochemical subtypes depending on the C_4 decarboxylase enzyme: nicotinamide adenine dinucleotide phosphate malic enzyme (NADP-ME), NAD malic enzyme, and phosphoenolpyruvate carboxykinase (PEP-CK) (Gutierrez *et al.*, 1974; Kanai and Edwards, 1999). C_4 plants have been classified into one of the three subtypes based on the dominant C_4 acid decarboxylation enzyme. Specialized leaf anatomy, biochemistry, and physiology are associated with each of the C_4 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 C_4 cycle may exist depending on species or environmental conditions (Leegood and Walker, 2003; Furbank, 2011). In maize, an NADP-ME C_4 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 C_4 grasses (Gutierrez *et al.*, 1974). Therefore, the presence of alternative decarboxylase 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 C_4 plants, there is an additional energetic cost associated with the operation and overcycling of the CCM. Minimally, an extra two ATP molecules per CO_2 fixed are required for the regeneration of PEP from pyruvate. During C_4 photosynthesis, the C_4 cycle operates faster than the C_3 (Calvin) cycle in order to raise the BSC CO_2 concentration and saturate the carboxylation reaction of Rubisco. Inevitably, a fraction of this CO_2 is not fixed by Rubisco and ultimately leaks back from the BSCs to the MCs. This fraction is termed leakiness (ϕ) and entails additional energy costs associated with the overcycling of the C_4 cycle (Farquhar, 1983; Furbank *et al.*, 1990). Consequently, the efficiency of C_4 photosynthesis requires the tight regulation of CO_2 supply with Rubisco activity within the BSCs in order to minimize leakiness and associated energy costs. This is often the case, given that leakiness varies within a narrow range and averages about 20% for a wide range of C_4 plants and environments (Henderson *et al.*, 1992; von Caemmerer *et al.*, 1997*a*; Cousins *et al.*, 2008). Bundle-sheath leakiness can be estimated by concurrently measuring leaf gas exchange with carbon isotope discrimination (Evans *et al.*, 1986). 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; Fravolinil *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 C_4, 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 C_4 plants (Henderson *et al.*, 1992), and leakiness was unchanged under long-term exposure to low light (Bellasio and Griffiths, 2013*a*). 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 C_4 leaves (Farquhar and Cernusak, 2012). Leakiness depends on a number of anatomical (e.g. CO_2 diffusion path length, chloroplast position in the BSC, BSC wall conductance) and biochemical (e.g. activities of the carboxylases and decarboxylases during C_4 photosynthesis) factors (Henderson *et al.*, 1992; von Caemmerer and Furbank, 2003). In contrast to manipulations using transgenic C_4 plants (von Caemmerer *et al.*, 1997*b*; 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 C_4 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 C_4 acid decarboxylases in response to these environmental variables. Salinity and shade were chosen because they impact on photosynthesis through contrasting effects on leaf CO_2 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_{sat}) and stomatal conductance (g_s) were taken between 10:00 and 14:00 at ambient CO_2 (400 µl l^{-1}), a leaf temperature of 26 °C, and a photosynthetic photon flux density of 1800 µmol m^{-2} s^{-1}. Each leaf was allowed to reach a steady state of CO_2 uptake in the LI-6400XT leaf chamber before measurements were taken.

Photosynthetic responses to intercellular CO_2 concentration (A/C_i curves) were measured at 10 CO_2 steps using similar conditions as described above. The A/C_i curves were fitted using a C_4 photosynthesis model (von Caemmerer, 2000) to estimate maximal PEPC (*in vivo* V_{pmax}) and Rubisco (*in vivo* V_{cmax}) activities. V_{cmax} and V_{pmax} were varied simultaneously until the best fit with the gas-exchange data was obtained.

Photosynthetic carbon isotope discrimination

Bundle-sheath leakiness was determined by measuring real-time $^{13}CO_2/^{12}CO_2$ carbon isotope discrimination using a gas exchange system (LI-6400XT: LI-COR) attached to a tunable diode-laser (model TGA100; Campbell Scientific, Logan, UT, USA), under similar conditions to the spot gas exchange measurements. Photosynthetic discrimination against ^{13}C (Δ_p) was calculated using the following equations (Evans *et al.*, 1986):

$$\Delta_p = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \quad (1)$$

$$\xi = \frac{C_e}{C_e - C_o} \quad (2)$$

where δ_e, δ_o, C_e, and C_o are the $\delta^{13}C$ (δ) and CO_2 mol fraction (C) of the air entering (e) and leaving (o) the leaf chamber and were measured with the tunable diode-laser. In this study, ξ ranged between 5 and 11. Leakiness (ϕ) was calculated using the model of Farquhar (1983) as modified by Pengelly *et al.* (2010, 2012). The formulae used are described briefly below.

$$\phi = \frac{\left(\frac{1-t}{1+t}\right)\Delta - \frac{a'}{1+t} - (a_i - b_4')\frac{A}{g_m C_a} - \left(b_4' - a'\right)\frac{C_i}{C_a}}{(b_3' - s)\left(\frac{C_i}{C_a} - \frac{A}{C_a g_m}\right)} \quad (3)$$

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

$$t = \frac{(1 + a')E}{2g^t_{ac}} \quad (4)$$

where E is the transpiration rate and g^t_{ac} the total conductance to CO_2 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:

$$a' = \frac{a_b(C_a - C_{ls}) + a(C_{ls} - C_i)}{C_a - C_i} \quad (5)$$

where C_a, C_i, and C_{ls} are the ambient, intercellular, and leaf surface CO_2 partial pressures, a_b (2.9‰) is the fractionation occurring through 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 CO_2 out of the bundle sheath, and a_i is the fractionation factor associated with the dissolution of CO_2 and diffusion through water. Here, we assume that s=a_i.

$$b_3' = b_3 - e\left(\frac{R_d}{A + R_d} - \frac{0.5R_d}{A + 0.5R_d}\right) - f\frac{0.5V_0}{V_c} \quad (6)$$

and

$$b_4' = b_4 - e\frac{0.5R_d}{(A + 0.5R_d)} \quad (7)$$

where b_3 is the fractionation by Rubisco (30‰), b_4 is the combined fractionation of the conversion of CO_2 to HCO_3^- and PEP carboxylation (–5.74‰ at 25 °C), f is the fraction associated with photosrespiration, and V_o and V_c are the rates of oxygenation and carboxylation, respectively. The fractionation factor e associated with respiration was calculated from the difference between $\delta^{13}C$ in the CO_2 cylinder (–40.5‰) used during experiments and that in the atmosphere under growth conditions (–8‰; Tazoe *et al.*, 2008). A and R_d denote the CO_2 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. In this study, leaf gas exchange was measured at high light, and hence V_o=0 (i.e. $f\frac{0.5V_0}{V_c}$=0) (Pengelly *et al.*, 2010, 2012; Ubierna *et al.*, 2013).

Rubisco content and soluble protein determination

Following gas-exchange measurements, replicate leaf discs (0.74 cm^2) 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 $NaHCO_3$, 20 mM $MgCl_2$, 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, 100*g* for 1 min and the supernatant used for Rubisco and soluble protein assays. Rubisco content was estimated by the irreversible binding of [^{14}C]carboxyarabinitol 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 [^{14}C]CABP binding assay by the Rubisco *in vitro* k_{cat} (5.5 s^{-1}) determined using a $^{14}CO_2$ 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% polyvinyl polypyrrolidone, 2 mM EDTA, 2 mM $MnCl_2$, and 0.05% Triton X-100. $MgCl_2$ 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 $NaHCO_3$, 1 mM ADP, 2 mM $MnCl_2$, 0.14 mM NADH, and malate dehydrogenase (6 U)] after the addition of PEP to 5mM. The final concentration of 4 mM $MnCl_2$ 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-Protean 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× transfer buffer [20×: 25 mM Bicine, 25 mM Bis/Tris, 1 mM EDTA, 20% (v/v) methanol]. After 1 h transfer at 30 V, the membrane was placed in blocking solution [3% (w/v) skimmed milk powder in TBS, 50 mM Tris/HCl (pH 8), 150 mM NaCl)] for 1 h at room temperature with gentle agitation.

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 raised against PEPC 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 by 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 antiserum 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 biomass, leaf water potential, and nitrogen and carbon isotope composition

Before harvest, leaf water potential (Ψ_L) 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 ($^{13}\delta$), ground leaf samples were combusted in a Carlo Erba elemental analyser (Model 1108) and the CO_2 was analysed by mass spectrometry. Isotopic composition (δ) was calculated as $[(R_{sample} - R_{standard})/R_{standard}]\times1000$, where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ 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 areas basis, leaf N concentration tended to be lower in the shaded plants relative to the control (Table 1).

Leaf photosynthesis

Leaf water potential (Ψ_L) 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 CO_2 (A_{sat}) decreased in maize plants exposed to the higher salinity treatment only (Fig. 1C, Table 1), whereas g_s decreased in plants exposed to both salinity levels (Fig. 1D, Table 1). Plants exposed to shade underwent larger decreases in photosynthesis and g_s (Fig. 1C, D, Table 1). A common linear relationship related A_{sat} to g_s (r^2=0.73) in all the maize plants regardless of the treatment (Fig. 2C).

A/C_i curves were fitted using the C_4 photosynthesis model (von Caemmerer, 2000) to estimate *in vivo* V_{cmax} and V_{pmax}. Both parameters decreased in the shaded plants relative to the control, while there was a small but non-significant reduction in V_{cmax} in the higher salinity treatment. The ratio V_{pmax}/V_{cmax} (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 $^{13}CO_2/^{12}CO_2$ discrimination and leaf gas exchange showed that photosynthetic discrimination (Δ_p) varied linearly with p_i/p_a for plants in the control and

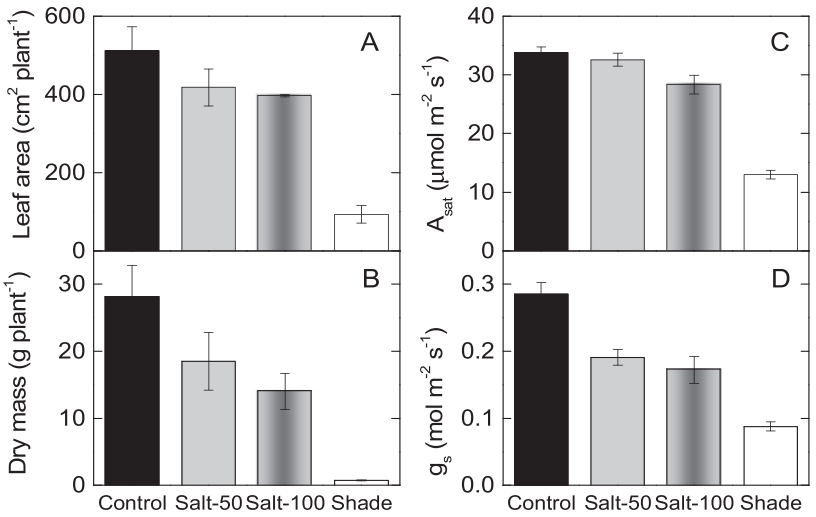

Fig. 1. Growth of maize plants exposed to salinity and shade. Total leaf area (A), plant dry mass (B), light-saturated photosynthetic rates in ambient air, A_{sat} (C), and stomatal conductance, g_s (D), of maize plants grown in full sunlight and irrigated with water (control), 50 mM NaCl (Salt-50), or 100 mM NaCl (Salt-100), or grown in 20% sunlight (shade).

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)*

Shade plants were grown in 20% sunlight and irrigated with normal water. Values are treatment means of three replicates±standard error. 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, and values followed by the same letter are not significantly different at the 5% level (P<0.05). ND, not determined

Parameter	Control	Shade	Salt-50	Salt-100	Model *P value*
Plant and leaf traits					
Total leaf area (cm^2 plant^{-1})	512±61*b*	94±23*a*	418±47*b*	397±4*b*	0.0012
Plant dry mass (g plant^{-1})	28.2±4.6*c*	0.75±0.05*a*	18.5±4.3*bc*	14.0±2.7*b*	0.0031
Leaf mass per area (g m^{-2})	71±5*b*	26±2*a*	69±6*b*	59±15*b*	0.0343
Leaf water potential, Ψ_L (MPa)	0.32±0.07*a*	ND	0.53±0.10*a*	1.08±0.09*b*	0.0000
Leaf N content (mg g^{-1})	36±2*b*	38±0*b*	33±2*ab*	29±1*a*	0.0054
Leaf N content (mmol m^{-2})	184±10*a*	95±25*a*	132±24*a*	124±28*a*	0.1027
Leaf C isotope composition, $^{13}\delta$ (‰)	−14.68±0.24*a*	−14.38±0.19*a*	−15.43±0.05*b*	−15.85±0.07*b*	0.0006
Leaf gas exchange					
Photosynthesis, A_{sat} (μmol m^{-2} s^{-1})	33.7±1.0*c*	13.0±0.7*a*	32.6±1.1*c*	28.3±1.6*b*	0.0000
Stomatal conductance, g_s (mol m^{-2} s^{-1})	0.285±0.017*c*	0.088±0.007*a*	0.191±0.012*b*	0.172±0.020*b*	0.0000
in vivo V_{cmax} (μmol m^{-2} s^{-1})	40±10*b*	19±6*a*	40±1*b*	33±1*b*	0.0125
in vivo V_{pmax} (μmol m^{-2} s^{-1})	104±6*b*	45±5*a*	94±1*b*	94±1*b*	0.0012
V_{pmax}/V_{cmax}	2.6±0.2*a*	2.5±0.4*a*	2.3±0.1*a*	2.8±0.1*a*	0.4682
Photosynthetic Δ_p (‰)	3.66±0.13*b*	2.46±0.36*a*	3.90±0.04*b*	4.23±0.11*b*	0.0040
Leakiness, ϕ	0.26±0.02*ab*	0.13±0.04*a*	0.24±0.02*ab*	0.31±0.01*b*	0.0125
Photosynthetic enzymes					
Rubisco content (g m^{-2})	0.31±0.04*b*	0.14±0.03*a*	0.23±0.01*ab*	0.24±0.03*ab*	0.0141
Soluble proteins (g m^{-2})	4.2±0.2*b*	2.5±0.2*a*	3.2±0.2*ab*	3.9±0.4*b*	0.0057
Rubisco activity (μmol m^{-2} s^{-1})	27.5±1.4*c*	11.5±2.0*a*	18.1±0.8*b*	19.5±2.1*b*	0.0034
PEPC activity (μmol m^{-2} s^{-1})	107±7*d*	21±4*a*	52±4*b*	72±4*c*	0.0000
PEPC/Rubisco	3.9±0.10*c*	1.8±0.08*a*	2.9±0.13*b*	3.3±0.15*b*	0.0000
NADP-ME activity (μmol m^{-2} s^{-1})	53±8*b*	32±5*a*	18±0.1*a*	19±3*a*	0.0073
PEP-CK activity (μmol m^{-2} s^{-1})	12.4±1.6*c*	3.0±0.4*a*	7.6±0.7*b*	8.2±1.2*b*	0.0021

salinity treatments, yielding a common bundle-sheath ϕ value of 0.25 according to the carbon discrimination model for C_4 plants (Farquhar, 1983). Thus, salinity changed p_i/p_a without affecting ϕ. In contrast, shaded plants had lower Δ_p, p_i/p_a, and ϕ 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 Δ_p and leaf dry-matter $^{13}\delta$ changed proportionately for the control and salt-treated

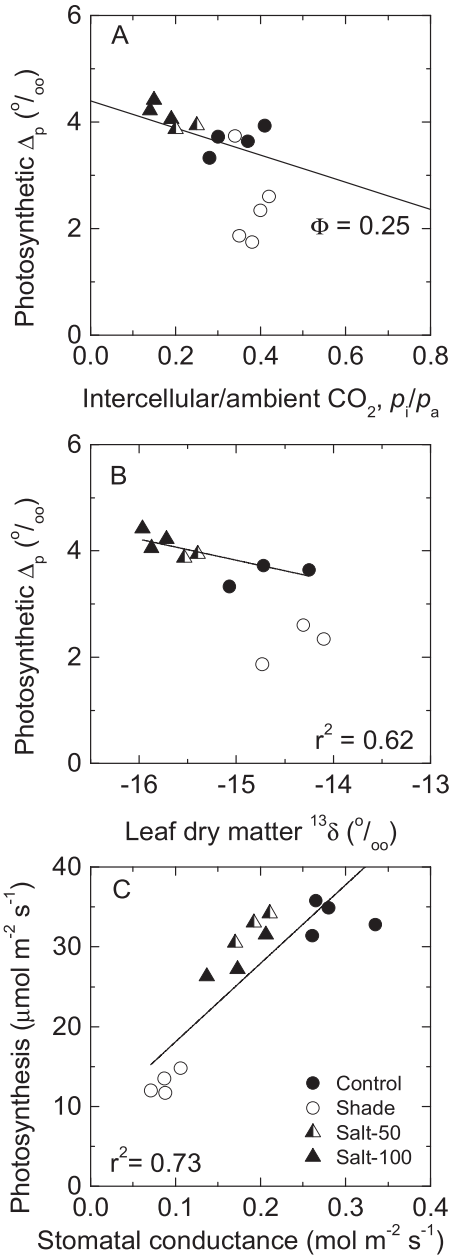

Fig. 2. Leaf gas exchange and carbon isotope discrimination in maize plants exposed to salinity and shade. Photosynthetic carbon isotope discrimination, Δ_p measured during the gas exchange of maize leaves as a function of intercellular to ambient CO_2 ratio (A) or leaf dry matter carbon isotope composition, $^{13}\delta$ (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 (ϕ) 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).

plants (Fig. 2B). In contrast, the shade plants fell outside the common relationship because their photosynthetic Δ_p decreased but not their leaf $^{13}\delta$ relative to the control plants (Fig. 2B).

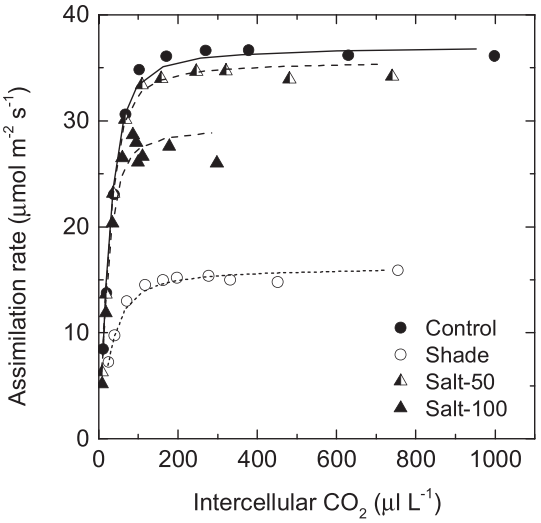

Fig. 3. A/C_i response curves for leaves of maize exposed to salinity and shade. Responses of assimilation rates to intercellular CO_2 (A/C_i curves) measured at a light intensity of 1800 μmol m^{-2} s^{-1} and a leaf temperature of 26 °C. Data points are the average of two replicates. Lines are the 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).

Activity of photosynthetic enzymes

Leaf Rubisco content and Rubisco activity calculated from k_{cat} and the irreversible binding of the transition state analogue [^{14}C]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

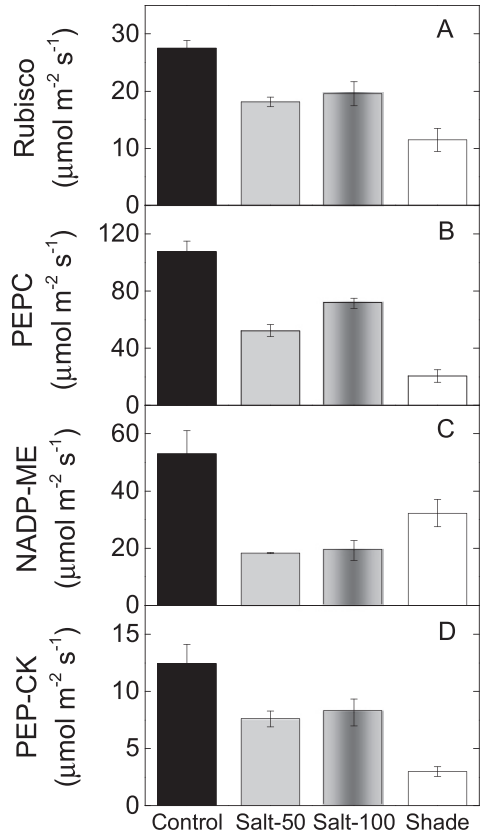

Fig. 4. Activity of carboxylases and decarboxylases in maize plants exposed to salinity and shade. Activity of Rubisco (A), PEPC (B), NADP-ME (C), and PEP-CK (D) for maize plants grown in full sunlight and irrigated with water (control), 50 mM NaCl (Salt-50), or 100 mM NaCl (Salt-100), or grown in 20% sunlight (shade).

and 5). In the control treatment, PEP-CK accounted for 20% of the total C_4 acid decarboxylation activity measured in maize leaves. This proportion increased to 30% in salt-treated plants and declined to 10% in the shaded plants (Table 1). In absolute terms, PEP-CK activity decreased by 40% in salt-treated plants and by 75% in shaded plants (Figs 4D and 5, Table 1).

Discussion

Contrasting impacts of salinity and shade on maize

The main objective of this study was to investigate the regulation of C_4 photosynthesis subjected to environmental manipulations that are known to have contrasting impacts on the processes of CO_2 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 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 C_4 photosynthesis requires close coordination between the C_4 and C_3 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 CO_2 within the BSCs. Importantly, regulating the balance between Rubisco, PEPC, NADP-ME, and other enzymes of the C_3 and C_4 cycles allows the dynamic regulation of C_4 efficiency that other features such as BSC wall conductance or CO_2 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 C_4 plants unable to grow effectively in air (Dever *et al.*, 1997; Cousins *et al.*, 2007). Leakiness of CO_2 from the BSCs as determined from measurements of $^{13}C/^{12}C$ carbon isotope discrimination represents a key surrogate indicator of the coordination between the C_3 and C_4 cycles (Farquhar, 1983). Combining measurements of leakiness with activities of the key enzymes in the C_3 and C_4 cycles can elucidate the regulation and efficiency of C_4 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 g_s but not A_{sat}, while moderate salinity (100 mM NaCl) reduced both g_s and A_{sat}. Hence, reduced photosynthetic rates were largely caused by increased resistance to CO_2 diffusion under both salinity treatments, and this was born out in the lower p_i/p_a ratio and more negative dry-matter $^{13}\delta$ observed in the leaves of salt-treated maize plants (Fig. 2). Reduced stomatal conductance and leaf $^{13}\delta$ in response to salinity is commonly reported in C_3 (Seemann and Critchley, 1985; Brugnoli and Lauteri, 1991) and C_4 (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).

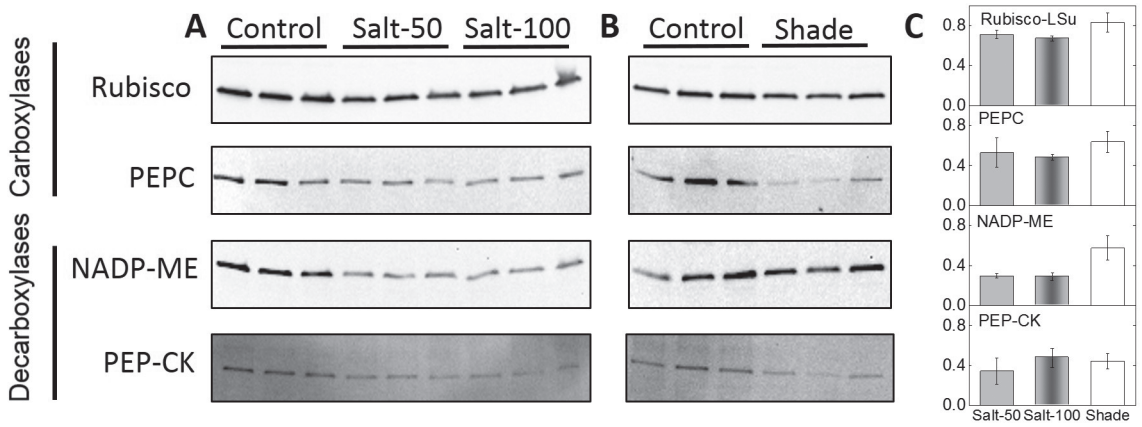

Fig. 5. Immunoblots of carboxylases and decarboxylases in maize plants exposed to salinity and shade. Immunoblots of total leaf proteins probed with antisera raised against the four photosynthetic enzymes Rubisco large subunit, PEPC, NADP-ME, and PEP-CK, as described in Materials and methods. The analysis 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).

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 Δ_p and leaf $^{13}\delta$, which were caused by reduced p_i/p_a (Fig. 2). In line with these results, when the C_4 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 $^{13}\delta$ rather than from photosynthetic Δ_p, correlated positively with the PEPC/Rubisco ratio (Meinzer and Zhu, 1999). Similarly to *Atriplex*, salinity reduced photosynthesis and increased p_i/p_a and ϕ values in sugarcane genotypes. Changes in ϕ 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 ones being the salinity level and the basis for leakiness calculation. Meinzer and Zhu (1999) found that mild salinity mainly affected g_s and had little impacts on ϕ (a similar scenario to the current maize study), and that ϕ 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 Δ_p have not been reconciled yet for C_4 plants. Post-photosynthetic fractionation of $^{13}C/^{12}C$ may be important in C_4 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) decarboxylases. 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_s and secondarily by reducing Rubisco and PEPC activities. The balance between the C_3 and C_4 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 C_3 and C_4 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 Δ_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 C_4 photosynthesis to low light vary depending on whether the condition is transient or a short-term acclimation. Under low light (<200 µmol quanta m^{-2} s^{-1}), ϕ 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, ϕ in maize leaves was unaffected under conditions

of short-term acclimation to low light (Bellasio and Griffiths, 2013*a*, *b*).

In contrast to these studies, leakiness decreased in our study as a result of reduced Δ_p with little impact on p_i/p_a, 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_3 and C_4 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 $^{13}\delta$ and photosynthetic Δ_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 $^{13}\delta$ decreased under shade conditions (Buchmann *et al.*, 1996). On the one hand, our results highlight the problems of using leaf $^{13}\delta$ as a proxy for photosynthetic Δ_p, especially when inferring leakiness and C_4 regulation. On the other hand, our results point to a stronger dependence of leaf $^{13}\delta$ on the diffusive components (salinity effects) within the Δ_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.*, 1997*a*; Ubierna *et al.*, 2011). Solving the link between leaf $^{13}\delta$ and photosynthetic Δ_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 PEP-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 PEP-CK to NADP-ME activity in maize. It is unlikely that the observed changes in NADP-ME and PEP-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 PEP-CK decarboxylases in maize allows the bundle sheath to regulate NADPH supply under variable light conditions (Bellasio and Griffiths, 2013*b*). 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 PEP-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 PEP-CK.

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