

# Using a biochemical C<sub>4</sub> photosynthesis model and combined gas exchange and chlorophyll fluorescence measurements to estimate bundle-sheath conductance of maize leaves differing in age and nitrogen content

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## ABSTRACT

**Bundle-sheath conductance ( $g_{bs}$ ) affects CO<sub>2</sub> leakiness, and, therefore, the efficiency of the CO<sub>2</sub>-concentrating mechanism (CCM) in C<sub>4</sub> photosynthesis. Whether and how  $g_{bs}$  varies with leaf age and nitrogen status is virtually unknown. We used a C<sub>4</sub>-photosynthesis model to estimate  $g_{bs}$  based on combined measurements of gas exchange and chlorophyll fluorescence on fully expanded leaves of three different ages of maize (*Zea mays* L.) plants grown under two contrasting nitrogen levels. Nitrogen was replenished weekly to maintain leaf nitrogen content (LNC) at a similar level across the three leaf ages. The estimated  $g_{bs}$  values on leaf-area basis ranged from 1.4 to 10.3 mmol m<sup>-2</sup> s<sup>-1</sup> and were affected more by LNC than by leaf age, although  $g_{bs}$  tended to decrease as leaves became older. When converted to resistance ( $r_{bs} = 1/g_{bs}$ ),  $r_{bs}$  decreased monotonically with LNC. The correlation was presumably associated with nitrogen effects on leaf anatomy such as on wall thickness of bundle-sheath cells. Despite higher  $g_{bs}$ , meaning less efficient CCM, the calculated loss due to photorespiration was still low for high-nitrogen leaves. Under the condition of ambient CO<sub>2</sub> and saturating irradiance, photorespiratory loss accounted for 3–5% of fixed carbon for the high-nitrogen, versus 1–2% for the low-nitrogen, leaves.**

*Key-words:* diffusive resistance; leaf age; leaf nitrogen; leakiness; modelling.

## INTRODUCTION

The high photosynthetic capacity and, therefore, biomass productivity of C<sub>4</sub>, as compared with C<sub>3</sub>, plants (see review of El-Sharkawy 2009) can be attributed to the CO<sub>2</sub>-concentrating mechanism (CCM) that creates a high

CO<sub>2</sub> concentration in the vicinity of Rubisco. This mechanism effectively suppresses the oxygenase activity of Rubisco and hence photorespiration in C<sub>4</sub> photosynthesis. C<sub>4</sub> photosynthesis requires a number of biochemical, physical and structural adaptations, especially the specialization of the mesophyll and bundle-sheath (BS) cells (Hatch 1987; Leegood 2002).

In C<sub>4</sub> photosynthesis, CO<sub>2</sub> is first fixed via phosphoenolpyruvate (PEP) carboxylase (PEPc) in mesophyll cells into C<sub>4</sub> acids, which are then transported to BS cells where the C<sub>4</sub> acids are decarboxylated and the released CO<sub>2</sub> refixed by Rubisco in the C<sub>3</sub> pathway of the Benson–Calvin cycle. An efficient CCM would require that: (1) PEPc has higher CO<sub>2</sub> affinity and carboxylation capacity than Rubisco; and (2) the rate of CO<sub>2</sub> leakage from BS cells back to mesophyll cells is low. This CO<sub>2</sub> leakage is dependent on both the BS conductance for CO<sub>2</sub> and the CO<sub>2</sub> concentration gradient between mesophyll and BS cells. The concentration of CO<sub>2</sub> requires an input of energy, and this occurs in the mesophyll cells. Two ATPs are used by pyruvate phosphate dikinase for the regeneration of one PEP from pyruvate, resulting in the requirement of two ATPs for every CO<sub>2</sub> that passes through the C<sub>4</sub> cycle (Hatch 1987). A CO<sub>2</sub> leakage represents an inefficiency in the CO<sub>2</sub> concentrating processes and increases the quantum requirement in C<sub>4</sub> photosynthesis (Farquhar 1983; Furbank, Jenkins & Hatch 1990). Therefore, leakiness ( $\phi$ ), defined as the rate of CO<sub>2</sub> leakage as a fraction of the rate of PEP carboxylation ( $V_p$ ), is an important C<sub>4</sub> photosynthesis parameter. Based on various methods, the older literature estimated  $\phi$  to be in the range of 0.08 to 0.70 [reviewed by Jenkins (1997) and von Caemmerer & Furbank (2003)]. However, more reliable methods such as the online measurements of carbon isotopic discrimination (Henderson, von Caemmerer & Farquhar 1992; Saliendra *et al.* 1996) and radio-labelling (Hatch, Agostino & Jenkins 1995) suggest that  $\phi$  is between 0.08 and 0.30. These more recent estimates of  $\phi$ , made upon a variety of C<sub>4</sub> species, are believed to be more accurate

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than the earlier estimates (von Caemmerer & Furbank 2003).

Leakiness is not a constant parameter and is known to be determined by environmental variables (Henderson, von Caemmerer & Farquhar 1992; Saliendra *et al.* 1996):  $\phi$  increases with decreasing irradiance (Henderson *et al.* 1992; Kromdijk *et al.* 2008; Tazoe *et al.* 2008; Kromdijk, Griffiths & Schepers 2010) and with increasing CO<sub>2</sub> level (Henderson *et al.* 1992). BS conductance to CO<sub>2</sub> leakage ( $g_{bs}$ ), or its inverse – resistance ( $r_{bs}$ ), is considered the major physical factor determining the value of  $\phi$ . Using a constant  $g_{bs}$ , responses of  $\phi$  to both irradiance and CO<sub>2</sub> levels can be correctly described by a biochemical C<sub>4</sub> photosynthesis model (von Caemmerer & Furbank 1999).

Values of  $g_{bs}$  can be estimated from measurements of photosynthesis made when the C<sub>4</sub> cycle is not functional. So,  $g_{bs}$  has been estimated by measuring photosynthetic rates under varying CO<sub>2</sub> concentrations in isolated BS cells (Furbank, Jenkins & Hatch 1989), in excised leaves fed with an inhibitor of the C<sub>4</sub> cycle (Jenkins, Furbank & Hatch 1989; Brown & Byrd 1993; Brown 1997) or using a PEPc mutant that results in a defective C<sub>4</sub> cycle (Kiirats *et al.* 2002). Early estimates of  $g_{bs}$  ranged from 0.6 to 2.5 mmol m<sup>-2</sup> s<sup>-1</sup> on a leaf area basis, corresponding to an  $r_{bs}$  of 400 to 1670 m<sup>2</sup> s mol<sup>-1</sup> (Jenkins *et al.* 1989; Brown & Byrd 1993). More recent estimates by Kiirats *et al.* (2002) gave lower values of  $r_{bs}$  (72.4 to 180.8 m<sup>2</sup> s mol<sup>-1</sup>). Significantly, Kiirats *et al.* (2002) showed that their estimates of  $r_{bs}$  using the PEPc mutant were similar to those obtained from feeding the PEPc inhibitor to wild-type plants. All these methods, however, are based on the assumption that Rubisco kinetic constants, gas diffusion resistance and BS cell structure are not altered by the PEPc inhibitor or mutation, and there is no guarantee that this major assumption is always met. Therefore, He & Edwards (1996) used a model-based approach to estimate  $r_{bs}$  using the measurements of O<sub>2</sub> inhibition of CO<sub>2</sub> fixation in maize (*Zea mays* L.) leaves of different ages (Dai, Ku & Edwards 1993, 1995); their estimates of  $r_{bs}$  were very low, ranging between 15 and 90 m<sup>2</sup> s mol<sup>-1</sup>.

The model used by He & Edwards (1996) assumes that the PEPc and Rubisco activities limit, respectively, the C<sub>4</sub> and C<sub>3</sub> cycles. For C<sub>3</sub> photosynthesis, however, leaf net photosynthesis rate ( $A$ ) has been modelled as being limited either by enzyme (Rubisco) activity or by RuBP regeneration, with a limitation in RuBP regeneration rate often being attributed to a limitation by the photosynthetic electron (e<sup>-</sup>) transport rate (Farquhar, von Caemmerer & Berry 1980). Similarly, for C<sub>4</sub> photosynthesis the regeneration of PEP in the mesophyll and the regeneration of RuBP in the BS cells both require ATP, and therefore  $A$  in C<sub>4</sub> photosynthesis has been modelled to be enzyme-limited or e<sup>-</sup> transport-limited (von Caemmerer & Furbank 1999). If a more complete model is used, the modelling approach of He & Edwards (1996) can be used to more accurately estimate  $g_{bs}$  and other C<sub>4</sub> parameters using a wider range of data.

Overall, biochemical C<sub>3</sub> photosynthesis models, and the responses of model parameters to environmental factors,

have been well studied. Using these models, it is possible to estimate a range of important parameters for C<sub>3</sub> photosynthesis from the response of assimilation rate, which can be usefully measured in parallel with the quantum yield of photosystem (PS) II e<sup>-</sup> transport, to CO<sub>2</sub> concentration and light intensity (e.g. Yin *et al.* 2009). Relatively less is known about comparable parameters for C<sub>4</sub> photosynthesis. It is known that, like Rubisco activity, PEPc activity varies with leaf age, nitrogen (N) content and light environment during the growth of plants (e.g. Hunt, Weber & Gates 1985; Wong, Cowan & Farquhar 1985; Sage, Percy & Seemann 1987; Tazoe, Noguchi & Terashima 2006). Little is known, however, about the influence of leaf age and growth conditions on those diffusion processes that are specific to C<sub>4</sub> photosynthesis, such as  $g_{bs}$  (von Caemmerer & Furbank 1999). There have been two reports describing the effect of leaf age on  $g_{bs}$  or  $r_{bs}$ . He & Edwards (1996) showed that  $r_{bs}$  was lower in young and senescing leaves than in mature leaves, whereas Kiirats *et al.* (2002), working on *Amaranthus edulis*, showed that  $r_{bs}$  increased monotonically from 30% expanded young leaves to 100% expanded leaves at grain-filling stage, despite a lower photosynthetic capacity of the fully expanded leaves compared with the young leaves. A few reports have described the effects of leaf N levels on  $\phi$  using carbon isotope discrimination measurements, but the results were not consistent either between species (Fravolini, Williams & Thompson 2002) or reports (Ranjith *et al.* 1995; Meinzer & Zhu 1998). We are not aware of any report of the quantitative relationship between  $g_{bs}$  *per se* and leaf N. Moreover, it is unclear whether the effect of leaf aging is primarily due to age, or if it is due to the decrease in leaf N content (LNC) that also occurs as leaves age.

In this study we used a model approach to estimate  $g_{bs}$  from combined gas exchange (GE) and chlorophyll fluorescence (CF) measurements of maize leaves of different ages and grown under two contrasting levels of N supply. To minimize the confounding effects of leaf age and leaf N, N was continuously added to plants so that LNC was maintained at a similar level across different leaf ages.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Maize plants (experimental line '2-02R10074') were grown under well-watered conditions in a glasshouse compartment of Wageningen, in 10 L pots with nitrogen-poor soil. The 60% of the photosynthetically active radiation incident on the glasshouse was transmitted to plant level. During daytime, supplemental light from 600 W HPS Hortilux Schröder lamps (Monster, South Holland, The Netherlands; 0.4 lamps m<sup>-2</sup>) was switched on automatically as soon as the global solar radiation incident on the glasshouse dropped below 400 W m<sup>-2</sup>, and then switched off if it exceeded 500 W m<sup>-2</sup>. Photoperiod was maintained at 12 h d<sup>-1</sup>. The CO<sub>2</sub> level was about 370 μmol mol<sup>-1</sup>, the relative humidity was 60–80%, and glasshouse temperature was 25 ± 3 °C during the day (for 12 h) and 18 °C during the night.

Nitrogen in a nutrient solution containing  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{NH}_4\text{NO}_3$ ,  $\text{K}_2\text{SO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ ,  $\text{FeEDTA}$ ,  $\text{ZnSO}_4$  and other micronutrients (Vos, van der Putten & Birch 2005) was added to pots weekly to maintain LNC at a constant level during growth. Two N levels were applied, with total available N being 0.6 and 2.8 g N per pot, and the resulting average total LNC was about 0.7 and 1.4 g m<sup>-2</sup>, for low-(N1) and high-nitrogen (N2) treatments, respectively, at the time of measurement.

### Gas exchange and chlorophyll fluorescence measurements

We selected leaf 8 or 9, which were measured at three ages (I, II and III, respectively) corresponding to about 1, 2 and 3 weeks after their full emergence. Each set of measurements was replicated four times (one leaf from one replicate plant) and included simultaneous GE and CF measurements at both 21 and 2% O<sub>2</sub> levels.

We used an open GE system Li-Cor 6400 (Li-Cor Inc., Lincoln, NE, USA) with an integrated fluorescence chamber head (2 cm<sup>2</sup>; LI-6400-40). All measurements were made at a leaf temperature of 25 °C and a leaf-to-air vapour pressure difference of 1.0–1.6 kPa. For CO<sub>2</sub> response curves, ambient CO<sub>2</sub> ( $C_a$ ) was increased stepwise: 50, 60, 70, 80, 90, 100, 200, 370, 500, 700 and 1500  $\mu\text{mol mol}^{-1}$ , while keeping incident irradiance ( $I_{\text{inc}}$ ) at 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . For  $I_{\text{inc}}$  response curves, the photon flux density was increased stepwise: 20, 40, 60, 80, 100, 200, 500, 1000, 1500 and 2000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , while keeping  $C_a$  either at 250  $\mu\text{mol mol}^{-1}$  when the O<sub>2</sub> concentration was 21% or 1000  $\mu\text{mol mol}^{-1}$  when the O<sub>2</sub> concentration was 2%; this was done to produce conditions in which photorespiration could (21% O<sub>2</sub>) or could not (2% O<sub>2</sub>) occur. Light and CO<sub>2</sub> responses at the two O<sub>2</sub> levels were measured on the same region of leaf. For the measurements at 2% O<sub>2</sub>, gas from a cylinder containing a mixture of 2% O<sub>2</sub> and 98% of N<sub>2</sub> was humidified and supplied via an overflow tube to the air inlet of the Li-Cor 6400 where CO<sub>2</sub> was blended with the gas, and the IRGA calibration was adjusted for 2% O<sub>2</sub> composition of the gas mixture.

After each increment in irradiance or CO<sub>2</sub> concentration,  $A$  was allowed to reach steady state, and  $F_s$  (the steady-state relative fluorescence yield) was recorded, after which a saturating light-pulse (>8500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 0.8 s) was applied to determine  $F_m'$  (the maximum relative fluorescence yield, produced by completely reducing the Q<sub>A</sub> pool in the leaf). The apparent quantum efficiency of PSII e<sup>-</sup> transport was obtained as  $\Delta F/F_m' = (F_m' - F_s)/F_m'$  (Genty, Briantais & Baker 1989).

All CO<sub>2</sub> exchange data were corrected for leakage of CO<sub>2</sub> into and out of the leaf cuvette, using heat-killed leaves according to Flexas *et al.* (2007).

### Determination of leaf N content

The portion of the leaf used for the said measurements was excised with a punch that produced a disc of 2 cm<sup>2</sup>. The leaf

disc was then weighed after drying at 70 °C to constant weight, and its total N content was analysed using an element analyser based on the Micro-Dumas combustion method.

### Model to estimate bundle sheath conductance on the leaf area basis ( $g_{\text{bs}}$ )

We estimated  $g_{\text{bs}}$  using the model of von Caemmerer & Furbank (1999) with slight modifications. According to von Caemmerer & Furbank (1999), leaf CO<sub>2</sub> assimilation rate ( $A$ ) can be written in terms of three basic mesophyll processes as

$$A = V_p - L - R_m \quad (1)$$

where  $V_p$  is the rate of PEP carboxylation,  $L$  is the rate of CO<sub>2</sub> leakage from the BS to the mesophyll and  $R_m$  is the mitochondrial respiration occurring in the mesophyll. The rate of leakage,  $L$ , is given by

$$L = g_{\text{bs}}(C_c - C_m) \quad (2)$$

where  $C_m$  is the CO<sub>2</sub> level in the mesophyll cell, and  $C_c$  is the CO<sub>2</sub> level at the carboxylation site of Rubisco in the BS.

In the model of von Caemmerer & Furbank (1999), the reference CO<sub>2</sub> level was  $C_m$ , which is generally unknown. However,  $C_m$  can be calculated from the values of CO<sub>2</sub> levels in intercellular air spaces ( $C_i$ ) because the drawdown of  $C_m$  from  $C_i$  depends on mesophyll diffusion conductance  $g_m$  as follows

$$C_m = C_i - A/g_m \quad (3)$$

As in the C<sub>3</sub> photosynthesis model of Farquhar *et al.* (1980),  $A$  can be written in terms of reactions in the BS for the Rubisco carboxylation and oxygenation in the C<sub>3</sub> cycle, which can be limited either by the Rubisco activity or by the e<sup>-</sup> transport:

$$A = \frac{(C_c - \gamma^* O_c) x_1}{C_c + x_2 O_c + x_3} - R_d \quad (4)$$

where  $\gamma^* = 0.5/S_{\text{c/o}}$ , in which  $S_{\text{c/o}}$  is relative CO<sub>2</sub>/O<sub>2</sub> specificity factor of Rubisco;  $O_c$  is the O<sub>2</sub> level at the carboxylation sites of Rubisco in the BS cell;  $R_d$  is the mitochondrial respiration;  $x_1$ ,  $x_2$  and  $x_3$  are intermediate constants or variables. For the enzyme (Rubisco)-limited rate,  $x_1 = V_{\text{cmax}}$  (carboxylation capacity of Rubisco),  $x_2 = K_{\text{mC}}/K_{\text{mO}}$ ,  $x_3 = K_{\text{mC}}$ , where  $K_{\text{mC}}$  and  $K_{\text{mO}}$  are Michaelis-Menten constants of Rubisco for CO<sub>2</sub> and O<sub>2</sub>, respectively. For the e<sup>-</sup> transport-limited rate,  $x_1 = (1-x)J_{\text{atp}}/3$ ,  $x_2 = 7\gamma^*/3$  and  $x_3 = 0$ , where  $x$  is the fraction of electrons or ATP partitioned to the C<sub>4</sub> cycle, and  $J_{\text{atp}}$  is the rate of ATP production driven by e<sup>-</sup> transport.

The O<sub>2</sub> partial pressure in the BS cell ( $O_c$ ) in Eqn 4 is described in terms of the intercellular air space O<sub>2</sub> partial pressure ( $O_i$ ) as

$$O_c = \alpha A / (0.047 g_{bs}) + O_i \quad (5)$$

where  $\alpha$  is the fraction of  $O_2$  evolution occurring in the BS, and 0.047 accounts for the diffusivities for  $O_2$  and  $CO_2$  in water and their respective Henry constants (von Caemmerer & Furbank 1999). Equation 5 implies that the  $O_2$  level is the same in the intercellular air spaces and the mesophyll cells; while this is not strictly true, the difference in  $O_2$  partial pressures is very small compared with  $O_i$ .

As is the case for the carboxylation of the  $C_3$  cycle, the rate of carboxylation for the  $C_4$  cycle ( $V_p$ ) could be limited either by the enzyme activity or by the rate of photosynthetic  $e^-$  transport in the mesophyll cells. It is assumed that  $C_4$  acid decarboxylation is not a limiting process and occurs at the same rate as  $V_p$ . So, for the enzyme-limited case, von Caemmerer & Furbank (1999) used a Michaelis–Menten equation to describe  $V_p$ :

$$V_p = \frac{C_m V_{pmax}}{C_m + K_p} \quad (6)$$

where  $V_{pmax}$  is the carboxylation capacity of PEPC,  $K_p$  is the Michaelis–Menten constant of PEPC for  $CO_2$ . For the  $e^-$  transport-limited rate,  $V_p$  is expressed as

$$V_p = x J_{atp} / 2 \quad (7)$$

As both Rubisco and PEPC reactions can be limited by either enzyme activity or photosynthetic  $e^-$  transport, we include all four combinations of rate limitations instead of only the two combinations used in the formulation of von Caemmerer & Furbank (1999) that assumes that enzyme activity or  $e^-$  transport limits the rate of both  $C_4$  and  $C_3$  cycles simultaneously. Compared with the two-limitation model, the four-limitation model generates smoother  $A-C_i$  and  $A-I_{inc}$  curves that agree better with the measured data. The four-limitation model for  $A$  is

$$A = \min(A_{EE}, A_{ET}, A_{TE}, A_{TT}) \quad (8)$$

where  $A_{EE}$  is the net  $CO_2$ -assimilation rate when both  $C_4$  and  $C_3$  cycles are limited by enzyme activity,  $A_{ET}$  is the net rate when the  $C_4$  cycle is limited by enzyme activity and the  $C_3$  cycle is limited by  $e^-$  transport,  $A_{TE}$  is the rate when the  $C_4$  cycle is limited by  $e^-$  transport and the  $C_3$  cycle is limited by enzyme activity, and  $A_{TT}$  is the rate when both  $C_4$  and  $C_3$  cycles are limited by  $e^-$  transport.

Combining Eqns 1–5 yields a quadratic expression for  $A_{TE}$  or  $A_{TT}$ , depending on whether the  $C_3$ -cycle reaction (Eqn 4) is limited by enzyme activity or  $e^-$  transport. The solution for this quadratic equation is given in Appendix A, where  $V_p$  is given by Eqn 7. Combining Eqns 1–6 yields a cubic expression for  $A_{EE}$  or  $A_{ET}$ . The solution for this cubic equation is given in Appendix B.

### Model parameterization

Based on these solutions and Eqn 8,  $g_{bs}$  can be estimated by curve fitting to the assimilation data measured over a range

of  $C_i$  and  $I_{inc}$  levels, by minimizing the difference between the calculated and measured  $A$ . To facilitate this estimation, we assumed a linear relationship between  $V_{pmax}$  or  $V_{cmax}$  and LNC above a base leaf-N content ( $n_b$ ); a relationship of this kind is generally observed (e.g. Sage *et al.* 1987; Meinzer & Zhu 1998; Tazoe *et al.* 2006), and we will later discuss the potential influence of this assumption.  $n_b$  and the slopes of the linearity ( $\chi_{vpmax}$  and  $\chi_{vcmax}$  for  $V_{pmax}$  and  $V_{cmax}$ , respectively) were estimated together with  $g_{bs}$ . Because a common value for each of the parameters  $\chi_{vpmax}$ ,  $\chi_{vcmax}$  and  $n_b$  was assumed, whereas different estimates of  $g_{bs}$  were allowed for different leaf ages and N contents, we used a dummy variable approach (see Yin *et al.* 2009) in the curve-fitting procedure.

We assumed that  $R_d$  does not vary with  $I_{inc}$  or with  $CO_2$  levels. By analogy to the approach proposed by Yin *et al.* (2009, 2011), the required input parameter  $R_d$  was estimated as the intercept of linear regression of  $A$  measured under limiting irradiances against  $I_{inc}(\Delta F/F_m')/3$ . The slope of the same linear regression ( $s'$ ), but using only the data at 2%  $O_2$  combined with high  $CO_2$ , will give the calibration factor to calculate the required variable  $J_{atp}$ . This calibration factors lumps a number of hard-to-measure parameters (see Yin *et al.* 2009 for the  $C_3$  analogy), including absorptance by leaf photosynthetic pigments, the fraction of absorbed irradiance partitioned to PSII, fractions of basal cyclic and pseudocyclic  $e^-$  transport, stoichiometric constants with regard to the Q-cycle activity and proton requirement for ATP synthesis, and  $1-x$ . We assumed that these unmeasured parameters were constant within the strictly  $e^-$  transport limited range. As for  $C_3$  photosynthesis (Yin *et al.* 2009), this approach implies that the discrepancy arising from the fact that measurements of net  $CO_2$  assimilation represent the integration of all assimilation by the leaf, while the measurements of CF may be biased towards the upper leaf surface, can be accounted for by this calibration procedure. In addition, the presence of physiologically distinct mesophyll and BS cell types in  $C_4$  photosynthesis could mean that the calibration procedure is less reliable for  $C_4$  than for  $C_3$  leaves. However, the good linear relationship between  $\Delta F/F_m'$  and quantum yields of  $CO_2$  fixation that has also been observed for  $C_4$  species (e.g. Edwards & Baker 1993) suggests that our model variable  $J_{atp}$  can be estimated from CF measurements. According to the stoichiometric constants of the  $e^-$  transport-limited form of Eqn 4, the formula for converting CF measurements into  $J_{atp}$  should be expressed as

$$J_{atp} = s' I_{inc} (\Delta F/F_m') / (1-x) \quad (9)$$

Other required input parameters were derived from literature (Table 1). Of these parameters,  $x$  may be obtained by optimization procedure that maximizes  $e^-$  transport-limited photosynthetic rate (Peisker 1988; Kromdijk *et al.* 2010). Any change in  $x$  will influence the estimate of leakiness by changing the relative flux of the  $C_3$  and  $C_4$  cycles. However, even the optimal  $x$  is quite invariant (i.e.  $\approx 0.4$ ) over a wide range of conditions (von Caemmerer & Furbank 1999),

**Table 1.** Some model parameters, with their values as derived from literature

	Definition	Value	Source
$K_p$	Michaelis–Menten constant of PEPc for CO <sub>2</sub>	40 $\mu$ bar	Pfeffer & Peisker (1995)
$K_{mC}$	Michaelis–Menten constant of Rubisco for CO <sub>2</sub>	485 $\mu$ bar	Cousins <i>et al.</i> (2010)
$K_{mO}$	Michaelis–Menten constant of Rubisco for O <sub>2</sub>	146 mbar	Cousins <i>et al.</i> (2010)
$S_{c/o}$	Relative CO <sub>2</sub> /O <sub>2</sub> specificity factor of Rubisco	2862	Cousins <i>et al.</i> (2010)
$\alpha$	Fraction of PSII activity in the bundle sheath	0.1	Chapman <i>et al.</i> (1980)
$R_m$	Mitochondrial respiration in the mesophyll	0.5 $R_d$	von Caemmerer & Furbank (1999)
$x$	Fraction of ATP allocated to the C <sub>4</sub> cycle	0.4	von Caemmerer & Furbank (1999)

PEPc, phosphoenolpyruvate carboxylase; PSII, photosystem II.

although the optimal  $x$  drops to low values at low  $I_{inc}$ . We included parameter  $x$  to be simultaneously fitted to obtain an overall value for  $x$ .

The curve fitting was carried out, using the least-squares non-linear regression with the GAUSS method in PROC NLIN of SAS (SAS Institute Inc., Cary, NC, USA), in order to minimize the difference between measured and modelled  $A$ . The SAS codes can be obtained upon request.

## RESULTS

### CO<sub>2</sub> and irradiance responses of net assimilation rate and PSII quantum efficiency

The measured  $A$ - $C_i$  and  $A$ - $I_{inc}$  curves across six age  $\times$  N combinations are shown in Fig. 1. As expected, a younger leaf age and higher N supply resulted in higher  $A$ . The effect of leaf age was larger at lower N supply than at higher N supply, whereas the effect of N supply was smaller for leaf stage I than for later leaf stages, in line with the differences in LNC between low- and high-N treatments at the different stages (Table 2). In line with expectations, there was a generally small effect of low O<sub>2</sub> (2%) on C<sub>4</sub> assimilation rate; the exceptions were obtained at high irradiances in leaf stage I at both levels of N supply and leaf stage II at the higher N supply where the 2% O<sub>2</sub>/1000  $\mu$ mol mol<sup>-1</sup> C<sub>a</sub> resulted in significantly higher assimilation rates ( $P < 0.05$ ), implying that in these leaves there was more photorespiration in the 21% O<sub>2</sub>/250  $\mu$ mol mol<sup>-1</sup> CO<sub>2</sub> atmosphere. There was little O<sub>2</sub> response at low  $C_i$  in  $A$ - $C_i$  curves.

The said effects of leaf age and N on  $A$  were mirrored in data for their effects on the apparent quantum efficiency of PSII e<sup>-</sup> transport  $\Delta F/F_m'$  (Fig. 2). Given the small effect of changed O<sub>2</sub> concentrations on CO<sub>2</sub> fixation, the absence of any O<sub>2</sub> effect on the light response of  $\Delta F/F_m'$  was not surprising.

### Estimation of $R_d$ and $s'$

As described in the Materials and Methods section, we estimated  $R_d$ , using the combined measurements on the light response of  $A$  and  $\Delta F/F_m'$  under limiting irradiance ( $I_{inc} \leq 200 \mu$ mol m<sup>-2</sup> s<sup>-1</sup>), as the intercept of the linear regression of  $A$  against  $I_{inc}(\Delta F/F_m')/3$  (see an example in Fig. 3). For all six leaf age  $\times$  N combinations, the estimated

$R_d$  did not differ significantly between 2 and 21% O<sub>2</sub> levels ( $P > 0.05$ ); so data were pooled to obtain an overall estimate of  $R_d$  for both O<sub>2</sub> levels. The estimated  $R_d$  varied from 1.16 to 1.93  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> across the six leaf age  $\times$  N combinations (Table 2). The estimated  $R_d$  increased from stage I to stage II, and was higher for N2 than for N1, but the difference between the two N levels became smaller later with leaf aging.

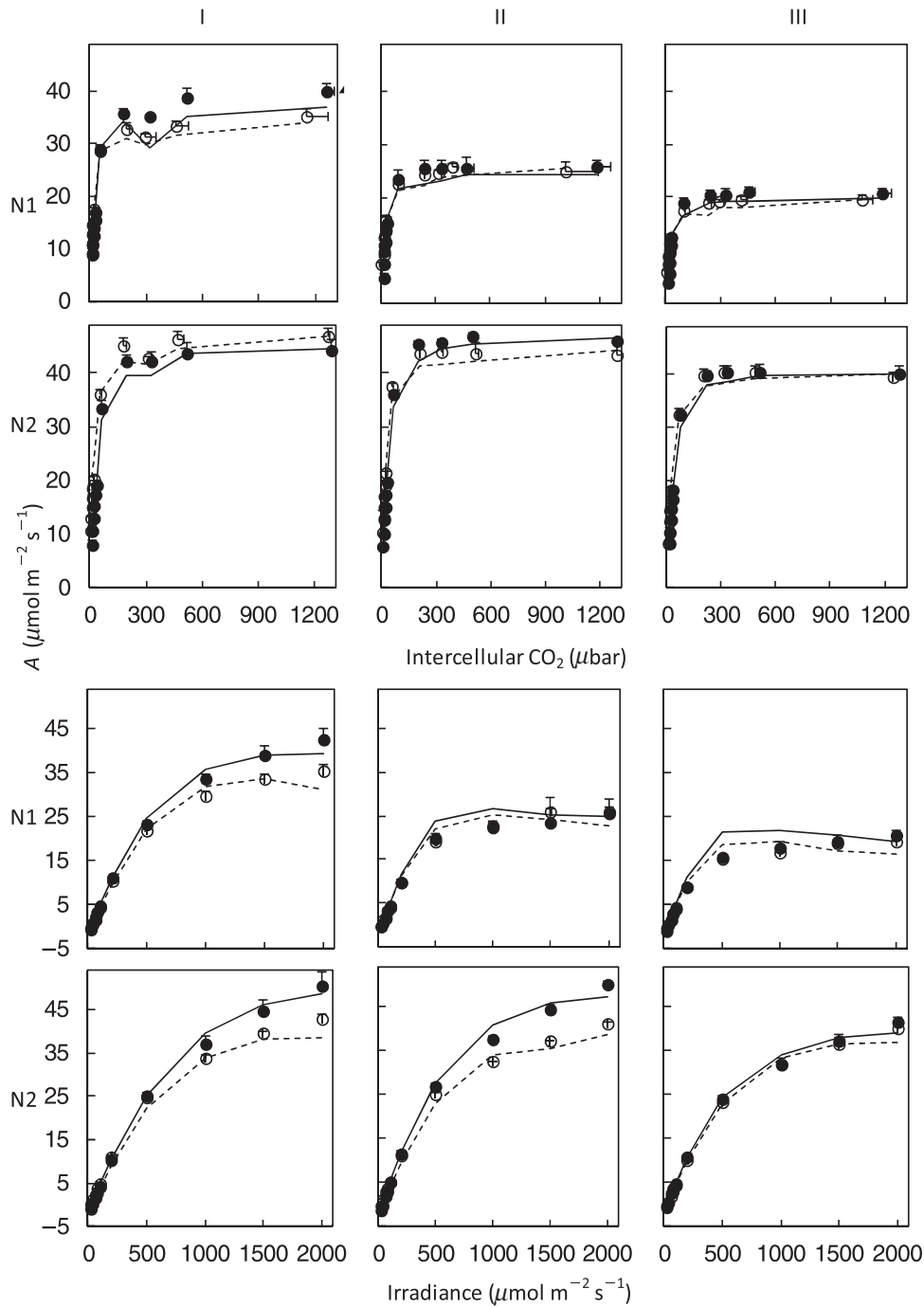
As stated earlier, the calibration factor  $s'$  was calculated as the slope of the linear regression of  $A$  against  $I_{inc}(\Delta F/F_m')/3$  using data of  $A$  and  $\Delta F/F_m'$  collected at 2% O<sub>2</sub> under strictly e<sup>-</sup> transport limited conditions. To ensure that this condition was met, we used only the data from limiting irradiances of the  $I_{inc}$  response (where measured  $A$  was relatively low) and saturating CO<sub>2</sub> of the  $C_i$  response (where measured  $A$  was relatively high); so, linear regression for estimating  $s'$  was based on these data without intermediate points (Fig. 3). There was no systematic trend of the estimated  $s'$  with a change in either leaf N or leaf age. In fact, the estimated  $s'$  were rather conservative, ranging between 0.291 and 0.332 mol ATP (mol e<sup>-</sup>)<sup>-1</sup> (Table 2). As stated earlier,  $s'$  lumps a number of parameters including absorptance. Leaf chlorophyll content determines leaf absorptance (e.g. Tazoe *et al.* 2006) and leaf N strongly influences leaf total chlorophyll content (e.g. Hunt *et al.* 1985; Ghannoum *et al.* 2005); so, LNC is expected to have a positive correlation with  $s'$ . We did not measure chlorophyll content, and are unable to clarify the reasons for the lack of association between LNC and  $s'$  here. Nevertheless, as a check, we also estimated  $s'$  together with  $g_{bs}$  and other parameters, using the full model (Eqn 8) to fit the full data set. The  $s'$  values obtained were virtually the same as those in Table 2 (see example in Fig. 3), indicating the validity of our procedure based on the partial data using a linear regression approach.

A value for  $x$  was obtained by fitting the complete model to the assimilation data and  $x$  was found to be about 0.41 (standard error (s.e.) 0.01), regardless of the variation of any other input and output parameters. Therefore,  $x$  was fixed at 0.4 for the subsequent analysis.

### Estimates of $g_{bs}$ and other parameters $g_m$ ,

$\chi_{vpmax}$ ,  $\chi_{vcmax}$ ,  $n_b$

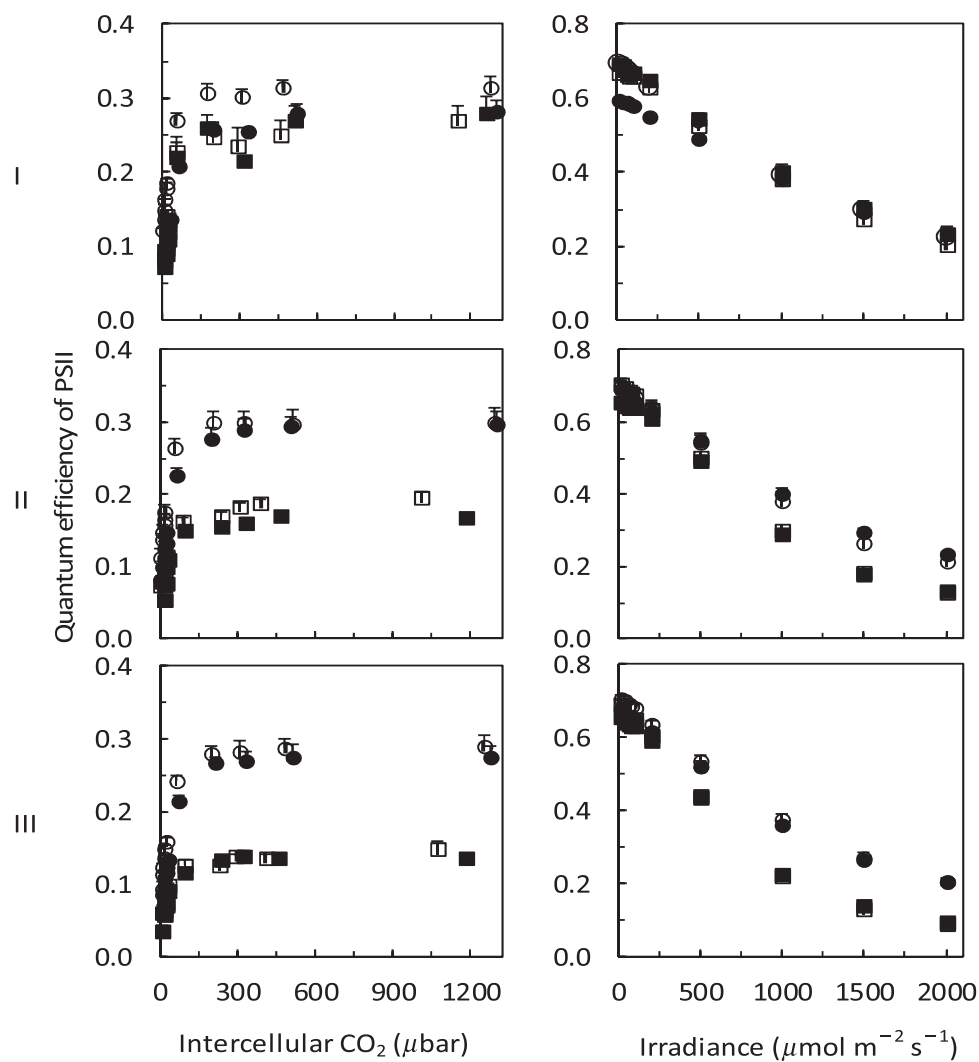
Equations 8 and 9, together with those in Appendices A and B, were arranged so that  $g_{bs}$ ,  $g_m$ ,  $\chi_{vpmax}$ ,  $\chi_{vcmax}$  and  $n_b$  could be



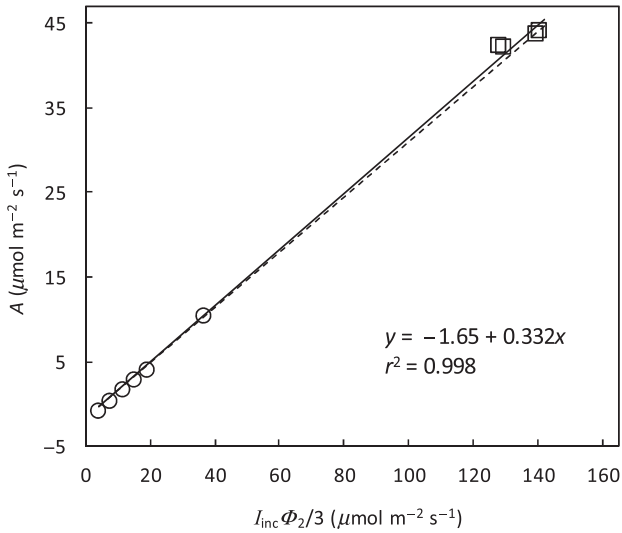
**Figure 1.** Measured (points) and modelled (curves)  $\text{CO}_2$  and irradiance response curves of net  $\text{CO}_2$  assimilation rate ( $A$ ), at 2% (●, full curve) and 21% (○, dashed curve)  $\text{O}_2$ , on leaves at three ages (I, II and III corresponding to 1, 2 and 3 weeks after their full appearance, respectively) from maize plants grown under low-nitrogen (N1) or high-nitrogen (N2) supply. Each point represents the mean (with standard error bar if larger than symbol) of the measurements on four replicated plants. Measurements for the irradiance responses at 21 and 2%  $\text{O}_2$  were conducted at 250 and 1000  $\mu\text{mol mol}^{-1} C_a$ , respectively (see Materials and Methods). Curves were drawn from connecting two nearby values calculated by the model. As the model used the observed  $\Delta F/F_m'$  (see Eqn 9) as input and as these are subject to physiological and measurement noise, the curves are not completely smooth.

**Table 2.** Leaf nitrogen (N) content and estimates (standard error between the following brackets) of model parameters – day respiration  $R_d$ , calibration factor for converting electron flux to ATP flux  $s'$ , mesophyll conductance  $g_m$ , bundle sheath conductance  $g_{bs}$  and bundle sheath resistance  $r_{bs}$  ( $1/g_{bs}$ ) – for leaves at three stages (I, II and III for approximately 1, 2 and 3 weeks after their full appearance) from maize plants grown under the condition of low-nitrogen (N1) and high-nitrogen (N2) supplies

Stage	N-supply	Leaf N ( $\text{g m}^{-2}$ )	$R_d$ ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ )	$s'$ ( $\text{mol mol}^{-1}$ )	$g_m$ ( $\text{mol m}^{-2}\text{s}^{-1}$ )	$g_{bs}$ ( $\text{mmol m}^{-2}\text{s}^{-1}$ )	$r_{bs}$ ( $\text{m}^2\text{s mol}^{-1}$ )
I	N1	0.80 (0.05)	1.16 (0.34)	0.291 (0.006)	1.60 (0.53)	1.84 (0.48)	545
	N2	1.36 (0.04)	1.65 (0.31)	0.332 (0.004)	3.53 (1.15)	10.32 (1.45)	97
II	N1	0.67 (0.09)	1.57 (0.55)	0.328 (0.010)	1.53 (0.75)	1.58 (0.52)	634
	N2	1.48 (0.07)	1.93 (0.41)	0.329 (0.005)	2.50 (0.67)	9.27 (1.64)	108
III	N1	0.59 (0.05)	1.61 (0.45)	0.319 (0.008)	0.90 (0.34)	1.42 (0.35)	704
	N2	1.44 (0.16)	1.80 (0.41)	0.308 (0.004)	2.58 (0.67)	3.43 (0.52)	292



**Figure 2.** Apparent quantum efficiency of PSII ( $\Delta F/F_m'$ ) as a function of intercellular  $\text{CO}_2$  (left panels) or of incident irradiance level (right panels), measured at 21% (open symbols) and 2% (filled symbols)  $\text{O}_2$ , for maize leaves of three stages (I, II and III corresponding to 1, 2 and 3 weeks after their full appearance, respectively) from plants grown under low (squares) and high (circles) nitrogen conditions. Each point represents the mean (with  $\Delta F/F_m'$  standard error bar if larger than symbol) of the measurements on four replicated plants. PSII, photosystem II.

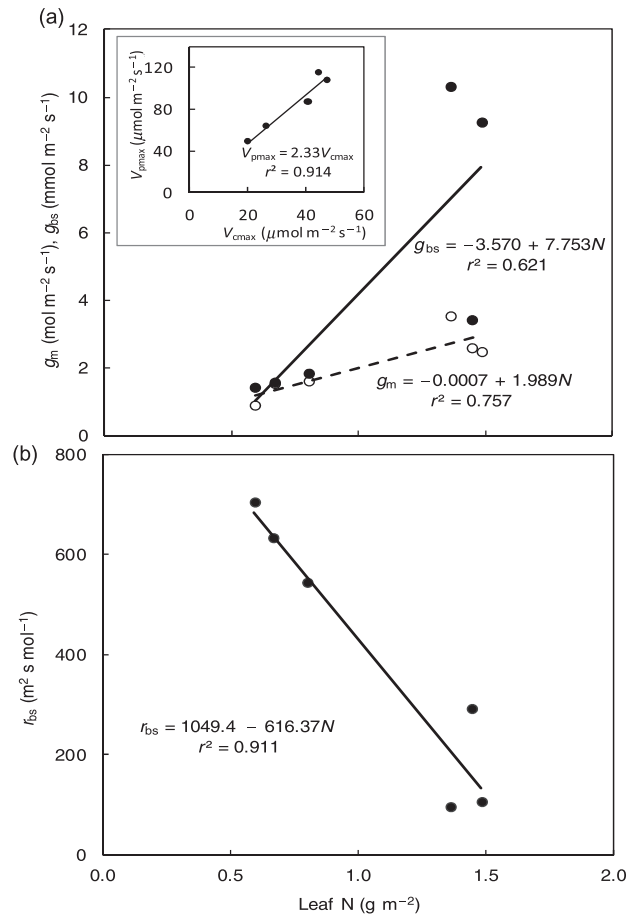


**Figure 3.** Net CO<sub>2</sub> assimilation rate ( $A$ ), measured at 2% O<sub>2</sub> and 1000  $\mu\text{mol mol}^{-1}$  C<sub>a</sub>, on the leaves at stage I from maize plants grown under high-nitrogen supply as a function of  $I_{\text{inc}}\Phi_2/3$ , where  $I_{\text{inc}}$  refers to the incident irradiance level, and  $\Phi_2$  is the photosystem II quantum efficiency of electron transport assumed to be  $\Delta F/F_m'$  from chlorophyll fluorescence measurements. Data points for linear regression (the solid line) are from low light level of the  $A$ - $I_{\text{inc}}$  curve (○) and from high CO<sub>2</sub> levels of the  $A$ -C<sub>i</sub> curve (□). The slope of the regression line is the estimated value for the calibration factor  $s'$ , and the negative intercept is the estimated value for day respiration  $R_d$ . Each data point represents the mean of measurements on leaves from four individual replicated plants. The dashed line was drawn, using the same  $R_d$ , but the estimated value of parameter  $s'$  from non-linear curve fitting based on the full model (see the texts).

estimated from fitting to all data of combined GE and CF measurements on both irradiance and CO<sub>2</sub> response curves at the two O<sub>2</sub> levels. This procedure implicitly assumes that the ratio of PEPc : Rubisco activity is constant across leaf N levels above  $n_b$ . To assess the validity of this assumption for our experiment, we first estimated apparent  $V_{\text{pmax}}$  and  $V_{\text{cmax}}$  from the initial slope and the maximum photosynthetic rate of  $A$ -C<sub>i</sub> curve as  $A$  at low and high C<sub>i</sub> of the curve can be approximated to  $C_i V_{\text{pmax}} / (C_i + K_p) - R_m$  and  $V_{\text{cmax}} - R_d$ , respectively (von Caemmerer & Furbank 1999). The first-order derivative of the first equation was set to equal the slope value of the initial linear part of  $A$ -C<sub>i</sub> curve and the apparent  $V_{\text{pmax}}$  was then solved at  $C_i = 30 \mu\text{mol mol}^{-1}$  (the grand mean C<sub>i</sub> of the linear section). A good proportional relation between apparent  $V_{\text{pmax}}$  and  $V_{\text{cmax}}$  values was obtained and the ratio of the two was ca. 2.33 (see inset of Fig. 4a), justifying our procedure assuming a constant  $V_{\text{pmax}} : V_{\text{cmax}}$  ratio.

In our procedure, most of the unestimated parameters (most of which are related to the Rubisco and PEPc kinetic constants) were fixed based on values found in the literature (Table 1). Notably,  $g_m$  could not be fixed due to the scarcity of information about  $g_m$  in C<sub>4</sub> plants (see Discussion). When fitted simultaneously with  $g_{\text{bs}}$  and other parameters, unsurprisingly there was an overfitting, with either  $g_m$

or  $\chi_{\text{vpmax}}$  being unestimatable. We performed a sensitivity analysis by changing  $g_m$  from 50 to 0.1 mol m<sup>-2</sup> s<sup>-1</sup> and found that of the parameters to be estimated, only  $\chi_{\text{vpmax}}$  was sensitive to  $g_m$  ( $\chi_{\text{vpmax}}$  increased with decreasing  $g_m$ ), whereas  $g_{\text{bs}}$ ,  $\chi_{\text{vcmax}}$  and  $n_b$  were altered only a little by a change in  $g_m$  (results not shown). Obviously, the exact value of  $g_m$  was hard to obtain and we estimated a value of  $g_m$  for each leaf age-N combination by testing a range of possible values of  $V_{\text{pmax}}$  from 200 to 350  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , at our highest LNC. We obtained 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  as our highest reasonable  $V_{\text{pmax}}$  – this was the value of  $V_{\text{pmax}}$  that gave the best fit to the model. This is also very close to the highest PEPc activity measured at similar temperatures that has been reported (Usuda, Ku & Edwards 1984; Sage *et al.* 1987; Uribelarrea, Crafts-Brandner & Below 2009). The resulting estimates of  $g_m$  and  $g_{\text{bs}}$  are given in Table 2; and the estimates of  $\chi_{\text{vpmax}}$ ,  $\chi_{\text{vcmax}}$  and  $n_b$  were 242.2 (s.e. 95.3)  $\mu\text{mol CO}_2 (\text{g N})^{-1} \text{s}^{-1}$ , 96.0 (10.7)  $\mu\text{mol CO}_2 (\text{g N})^{-1} \text{s}^{-1}$  and 0.242 (0.045) g N m<sup>-2</sup>,



**Figure 4.** The estimates of (a) mesophyll conductance  $g_m$  (○) and bundle sheath conductance  $g_{\text{bs}}$  (●), and (b) bundle sheath resistance  $r_{\text{bs}}$ , as a function of leaf nitrogen content  $N$  in maize plants. The inset in panel (a) shows the relationship between apparent  $V_{\text{pmax}}$  (maximum PEPc carboxylation rate) and apparent  $V_{\text{cmax}}$  (maximum Rubisco carboxylation rate) estimated from the initial slope and maximum photosynthetic rates at high C<sub>i</sub>, respectively, of  $A$ -C<sub>i</sub> curves across the six leaf age  $\times$  nitrogen treatment combinations (see the main text).

respectively, meaning that for leaf N above  $0.242 \text{ g m}^{-2}$  the ratio of total PEPc : Rubisco activity was 2.5, similar to the estimate for the apparent  $V_{\text{pmax}} : V_{\text{cmax}}$  ratio. The corresponding fit was good (Fig. 1), with an overall  $R^2$  of 0.96. The said assumption for a maximum  $V_{\text{pmax}}$ , despite some uncertainty, had little impact on the estimated values of  $g_{\text{bs}}$ , although a change of the assumed highest PEPc activity did have an impact on  $g_{\text{m}}$  (but had little impact on the relative magnitude of  $g_{\text{m}}$  across the six leaf age  $\times$  N combinations).

While nutrient solution was repeatedly added to the soil of pots, there was a small difference in LNC across leaf ages (Table 2). Neither estimated  $g_{\text{bs}}$  nor  $g_{\text{m}}$  was significantly correlated with leaf thickness as indicated by leaf mass: area ratio ( $P > 0.05$ ; results not shown). Both  $g_{\text{bs}}$  and  $g_{\text{m}}$  seemed to be affected more by leaf N than by leaf age, although  $g_{\text{bs}}$  tended to decrease with increasing age (Table 2). The estimated  $g_{\text{m}}$  increased with increasing LNC, whereas the relation between  $g_{\text{bs}}$  and leaf N was less certain although overall it also increased with leaf N (Fig. 4a). If  $g_{\text{bs}}$  is converted to  $r_{\text{bs}}$  (the inverse of  $g_{\text{bs}}$ ), it was found that  $r_{\text{bs}}$  better correlated linearly with LNC within the range of variation in the experiment (Fig. 4b), in contrast with the case of  $g_{\text{m}}$  for which the estimated  $g_{\text{m}}$  better scaled linearly with LNC (Fig. 4a). Further analysis using the two-variable regression confirmed that LNC was more important than leaf age in affecting  $r_{\text{bs}}$ ; when LNC was included as a covariate in the regression model, leaf age was found not to affect  $r_{\text{bs}}$  significantly ( $P > 0.05$ ).

## Sensitivity analysis

Given the wide range of variation in the reported values for Rubisco parameters  $K_{\text{mC}}$ ,  $K_{\text{mO}}$  and  $S_{\text{c/o}}$  (reviewed by Cousins *et al.* 2010) and the possible uncertainty in the values for  $K_{\text{p}}$  and  $\alpha$ , we conducted sensitivity analysis by varying these parameters within their physiologically relevant range. Again,  $V_{\text{pmax}}$  at our highest LNC was  $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , so  $V_{\text{pmax}}$  was not included in the sensitivity analysis. Furthermore, we fixed  $n_{\text{b}}$  at the estimated value of  $0.242 \text{ g N m}^{-2}$  (see above) so that sensitivity analysis would focus on the most relevant parameters, that is,  $g_{\text{bs}}$ ,  $g_{\text{m}}$  and  $\chi_{\text{vcmax}}$ .

When  $V_{\text{pmax}}$  was fixed, the estimated  $g_{\text{m}}$  was unsurprisingly very sensitive to a change in  $K_{\text{p}}$ : it increased its values with increasing  $K_{\text{p}}$ . In contrast,  $g_{\text{bs}}$  and  $\chi_{\text{vcmax}}$  were virtually insensitive to a change in this parameter (Fig. 5a).  $g_{\text{m}}$  did not have a regular pattern in response to a change in Rubisco parameters ( $K_{\text{mC}}$ ,  $K_{\text{mO}}$  and  $S_{\text{c/o}}$ ) and  $\alpha$  (Fig. 5b–e), probably a reflection of not very reliable estimate of  $g_{\text{m}}$  (see Discussion). However, the response of  $g_{\text{bs}}$  and  $\chi_{\text{vcmax}}$  to changes in  $\alpha$ ,  $K_{\text{mC}}$ ,  $K_{\text{mO}}$  and  $S_{\text{c/o}}$  was systematic.  $g_{\text{bs}}$  had the highest sensitivity to  $S_{\text{c/o}}$  and the lowest to  $\alpha$ .

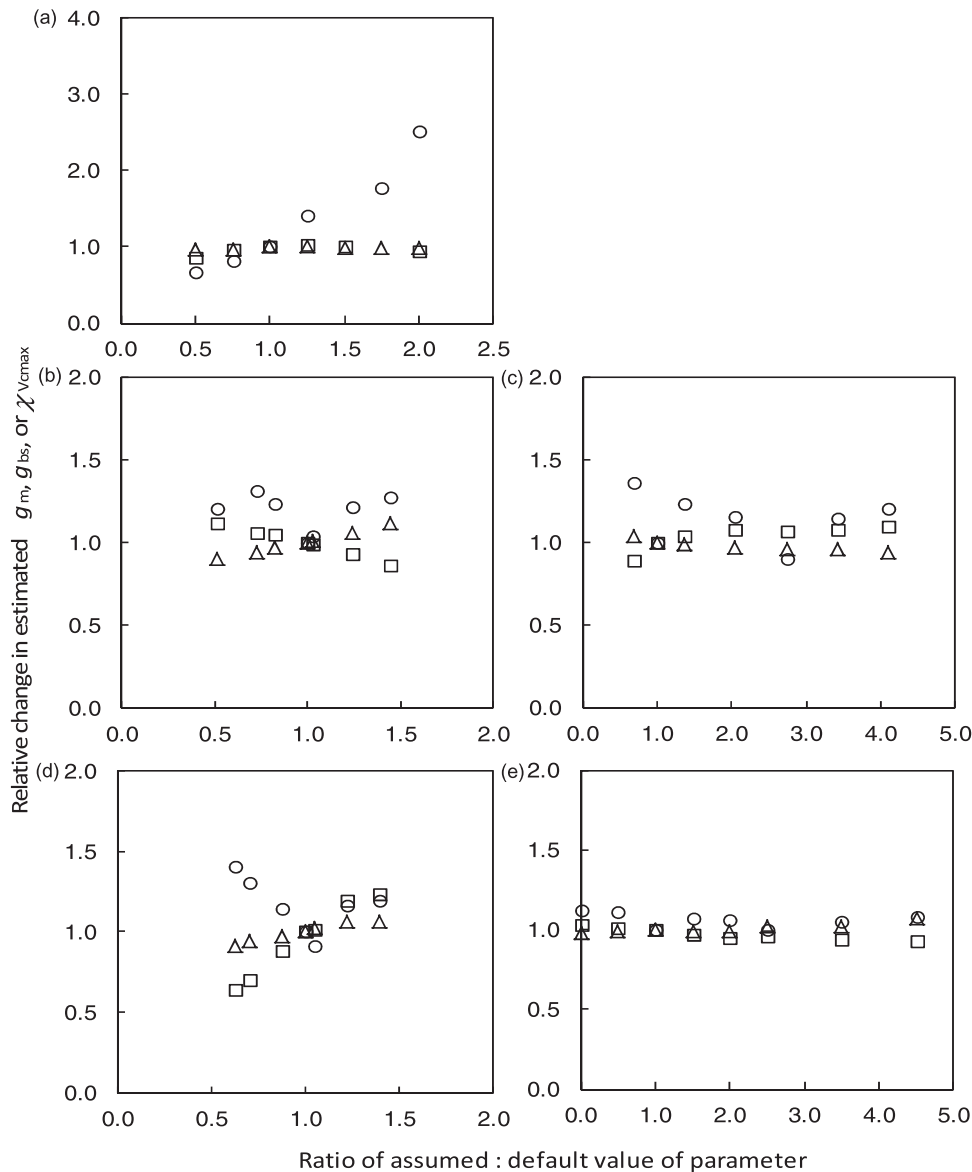
## CO<sub>2</sub> concentration, CO<sub>2</sub> leakiness and photorespiration in the BS cells

When model parameters are known, one can obtain the relevant  $V_{\text{p}}$ , depending on which of the  $A_{\text{EE}}$ ,  $A_{\text{ET}}$ ,  $A_{\text{TE}}$  and

$A_{\text{TT}}$  is limiting  $A$  (see Eqn 8). The rate of leakage ( $L$ ) can be solved from Eqn 1 and the CO<sub>2</sub> concentration in the BS cells ( $C_{\text{c}}$ ) can then be solved from Eqns 2 and 3. Figure 6 shows the response of the calculated  $C_{\text{c}}$  to  $C_{\text{i}}$  and  $I_{\text{inc}}$  for leaf stage I as an example, and the pattern was similar for the other two stages. As a consequence of the lower  $g_{\text{bs}}$  at low compared with high N, the calculated  $C_{\text{c}}$  was higher in low-N than in high-N leaves. In general,  $C_{\text{c}}$  increased with increasing  $C_{\text{i}}$  (Fig. 6a), but the response of  $C_{\text{c}}$  to irradiance was not consistent (Fig. 6b). At low N,  $C_{\text{c}}$  increased with increasing irradiance; whereas at high N,  $C_{\text{c}}$  generally increased with increasing irradiance at 21% O<sub>2</sub>, but decreased at 2% O<sub>2</sub>.  $C_{\text{c}}$  was lower at 2% than 21% O<sub>2</sub> levels for CO<sub>2</sub> response curves, but this was not so evident for the irradiance response curves. Across all data of the response curves for all leaf stages, the calculated  $C_{\text{c}}$  varied from 92 to  $4832 \mu\text{bar}$  (and was mostly higher than the level of  $C_{\text{a}}$  used), and a wide range of  $C_{\text{c}} : C_{\text{m}}$  ratios were calculated (varying from 1.1 to 188.1). The highest  $C_{\text{c}} : C_{\text{m}}$  ratios were obtained for low CO<sub>2</sub> levels of  $C_{\text{i}}$  response curves and the lowest ratios were from high CO<sub>2</sub> levels of  $C_{\text{i}}$  response curves or low irradiances of  $I_{\text{inc}}$  response curves. In the  $I_{\text{inc}}$  response at 2% O<sub>2</sub> and  $1000 \mu\text{mol mol}^{-1} C_{\text{a}}$ ,  $C_{\text{c}}$  was lower than the ambient  $C_{\text{a}}$  for high-N plants (Fig. 6b).

Figure 7 shows the calculated leakiness  $\phi (= L/V_{\text{p}})$  under various  $C_{\text{i}}$  and  $I_{\text{inc}}$  conditions. The calculated  $\phi$  in response to changing  $C_{\text{i}}$  ranged from 0.03 to 0.23, generally decreasing with increasing  $C_{\text{i}}$ , although in some cases the opposite trend was obtained. For the  $I_{\text{inc}}$  response curves, the calculated  $\phi$  was high (approaching 0.9) at strictly limiting irradiances, and then decreased rapidly to stable low values (as low as 0.04). Non-photorespiratory conditions reduced the value of  $\phi$  consistently.

Although the loss of assimilation due to photorespiration is generally low in C<sub>4</sub> species, it was possible to calculate the fraction of assimilation lost,  $f_{\text{pr}} (= 0.5V_{\text{o}}/V_{\text{c}}$ , where  $V_{\text{c}}$  and  $V_{\text{o}}$  are the rates of Rubisco carboxylation and oxygenation, respectively), from the estimated parameters. Values of  $V_{\text{c}}$  and  $V_{\text{o}}$  for each level of  $C_{\text{i}}$  and  $I_{\text{inc}}$  can be solved from the combined equations, as for the C<sub>3</sub> photosynthesis model:  $V_{\text{c}} - 0.5V_{\text{o}} = A + R_{\text{d}}$  and  $V_{\text{o}}/V_{\text{c}} = O_{\text{c}}/(C_{\text{o}}S_{\text{c/o}})$ , where  $O_{\text{c}}$  is solved from Eqn 5. Figure 8 shows an example of the calculated  $f_{\text{pr}}$  in response to  $C_{\text{i}}$  and  $I_{\text{inc}}$  for leaf stage I; the pattern was similar for the other two stages. Not surprisingly, the calculated  $f_{\text{pr}}$  decreased rapidly with increasing  $C_{\text{i}}$  level (Fig. 8). Similarly,  $f_{\text{pr}}$  also decreased with increasing  $I_{\text{inc}}$  at 21% O<sub>2</sub>. For the irradiance response at 2% O<sub>2</sub>, however,  $f_{\text{pr}}$  increased monotonically with an increase of  $I_{\text{inc}}$ , although its value remained very small ( $< 0.013$ ). Overall, high N increased photorespiration, because high N led to a high  $g_{\text{bs}}$ , thus a less efficient CCM, although even under these conditions  $f_{\text{pr}}$  was still low. Even under extreme conditions, that is,  $50 \mu\text{mol mol}^{-1}$  ambient CO<sub>2</sub> or strictly limiting irradiance, where photorespiration is most likely to occur, the estimated maximum  $f_{\text{pr}}$  was only 0.10 to 0.15 (Fig. 8). Under the conditions of saturating irradiance and ambient CO<sub>2</sub> and O<sub>2</sub>, the calculated  $f_{\text{pr}}$  was 0.03–0.05 for the high-N leaves, and 0.01–0.02 for the low-N leaves.



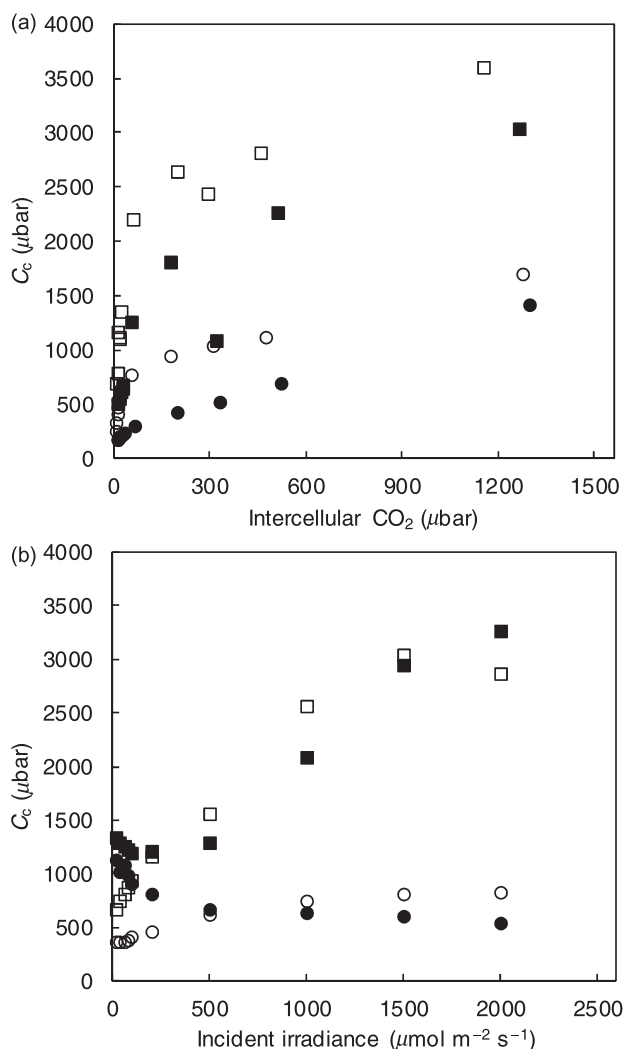
**Figure 5.** Relative changes in the estimated mesophyll conductance  $g_m$  (○), bundle sheath conductance  $g_{bs}$  (□) and the slope of the  $V_{cmax}$ -leaf nitrogen linearity  $\chi_{vmax}$  (△) in response to a change of five model parameters [(a)  $K_p$ , (b)  $K_{mC}$ , (c)  $K_{mO}$ , (d)  $S_{co}$  and (e)  $\alpha$ ] from their default values. The default values of these parameters are given in Table 1. The relative change is defined as the ratio of the estimate at the changed value of a parameter to the estimate at its default value. For  $g_m$  and  $g_{bs}$ , the ratio was calculated from the average of the estimates across the six leaf stage  $\times$  nitrogen treatment combinations at the changed parameter value to the average of the estimates at its default value.

## DISCUSSION

### Estimation of $g_{bs}$

In this study we used a biochemical  $C_4$  photosynthesis model and combined GE and CF measurements to investigate how  $g_{bs}$  varies with leaf age and LNC in maize.  $g_{bs}$  is an important physical parameter affecting the efficiency of CCM in  $C_4$  photosynthesis, and there have been attempts to estimate it for several  $C_4$  species (Furbank *et al.* 1989; Jenkins *et al.* 1989; Brown & Byrd 1993; He & Edwards 1996; Brown 1997; Kiirats *et al.* 2002).

Values of  $g_{bs}$  can potentially be estimated from measurements of photosynthesis under the conditions where the  $C_4$  cycle is not functional, for example, by measuring photosynthetic rates under varying  $CO_2$  concentrations in isolated BS cells (Furbank *et al.* 1989), or in excised leaves fed a chemical inhibitor of the  $C_4$  cycle (Jenkins *et al.* 1989; Brown & Byrd 1993; Brown 1997), or using a PEPc mutant that has a defective  $C_4$  cycle (Kiirats *et al.* 2002). When the  $C_4$  cycle is dysfunctional,  $V_p$  can be set to zero, and one can obtain an equation from combining Eqns 1 and 2:



**Figure 6.** The estimated CO<sub>2</sub> level at the carboxylation sites of Rubisco ( $C_c$ ) as a function of (a) intercellular CO<sub>2</sub> or of (b) incident irradiance level, at 21% (open symbols) and 2% (filled symbols) O<sub>2</sub>, for maize leaves of stage I (1 week after their full appearance) from plants grown under low (squares) and high (circles) nitrogen conditions.

$$A = g_{bs}(C_m - C_c) - R_m \quad (10)$$

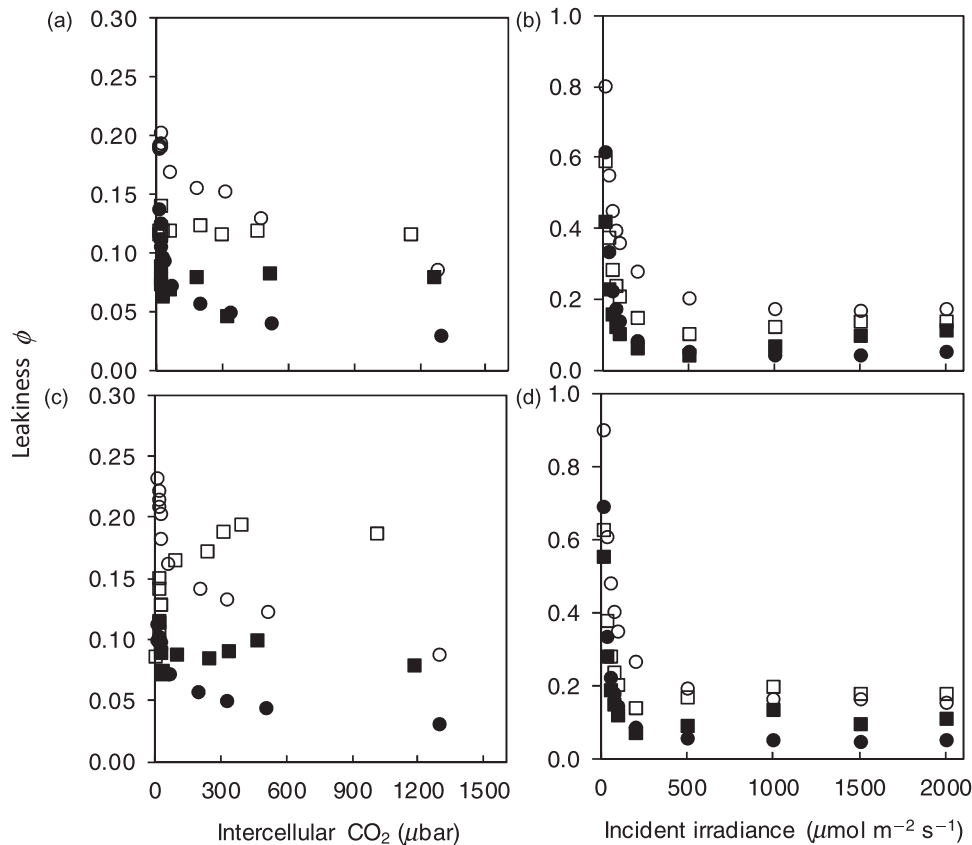
So, the first-order derivative  $dA/dC_m$  would be the estimate of  $g_{bs}$ . Brown & Byrd (1993) followed this approach, by finding the slope value of the linear portion of the relationship between  $A$  and CO<sub>2</sub> level for leaves fed with a chemical inhibitor of the C<sub>4</sub> cycle. Their estimated  $g_{bs}$  ranged from 1.13 to 2.35 mmol m<sup>-2</sup> s<sup>-1</sup> for different C<sub>4</sub> species (*Panicum maximum*, *Panicum miliaceum* and *Sorghum bicolor*), equivalent to  $r_{bs}$  between 425 and 885 m<sup>2</sup> s mol<sup>-1</sup>. However, their analysis suffered a problem that not only  $g_m$  was implied as infinite but also there was no transpiration measurement and thus no data were obtained for  $C_i$ ; consequently, the linear relation established was between  $A$  and  $C_a$ . Obviously, their approach could have overestimated  $r_{bs}$ , as other resistance components (e.g. stomatal resistance)

were not excluded. Furthermore, this linear approach implicitly assumes that  $C_c$  is constant, which is unlikely to be the case (Fig. 6a). To account for a varying  $C_c$ , a quadratic model for  $A$  could be derived by solving  $C_c$  from Eqn 10 and substituting it into the enzyme-limited form of Eqn 4. Based on this quadratic model and its  $dA/dC_m$ , one can see that the inverse of the initial slope of  $A$  versus  $C_m$  should be the total resistance, namely the Rubisco-carboxylation resistance and  $r_{bs}$ , by analogy to the sum of the Rubisco-carboxylation resistance and  $1/g_m$  for C<sub>3</sub>-photosynthesis (see von Caemmerer *et al.* 1994; Yin *et al.* 2009). Using the initial slope of the  $A$ - $C_i$  curve and considering the Rubisco carboxylation resistance, Kiirats *et al.* (2002) gave lower estimates of  $r_{bs}$  from 72.4 to 180.8 m<sup>2</sup> s mol<sup>-1</sup> for *A. edulis*. Although differences between species or other factors could explain the differences in estimates obtained by Brown & Byrd (1993) and Kiirats *et al.* (2002), the more appropriate procedure used by Kiirats *et al.* (2002) contributed to the lower estimate of  $r_{bs}$  in their studies. By re-analysis of the data of Brown & Byrd (1993), He & Edwards (1996) estimated the total resistance to be 97–330 m<sup>2</sup> s mol<sup>-1</sup> based on the initial slope of  $A$ - $C_a$  curves, which is lower than the original estimates of Brown & Byrd (1993) for the total resistance (i.e. from  $C_a$  to  $C_c$ ), rather than for  $r_{bs}$  alone.

All the methods using the PEPc inhibitor or mutation were based on the assumption that Rubisco kinetic constants, gas diffusion resistance and BS cell structure are not altered by the PEPc inhibitor or mutation. He & Edwards (1996) argued that this important assumption could not be verified so they estimated  $r_{bs}$  from GE data obtained from normally functioning maize leaves. The model of C<sub>4</sub> assimilation they used assumed that both the C<sub>4</sub> and C<sub>3</sub> cycles are limited by enzyme activities, and they obtained very low estimates of  $r_{bs}$  between 15 and 90 m<sup>2</sup> s mol<sup>-1</sup> for maize. Although the assimilation rate in the GE data they used could be predominantly limited by enzyme activities, the possibilities that either the C<sub>4</sub> or C<sub>3</sub> cycles, or both, were limited partly by e<sup>-</sup> transport could not be ruled out. The use of a single-limitation model when there was a multiple limitation would underestimate  $r_{bs}$ . We therefore propose a four-limitation approach, Eqn 8, to obtain the best possible estimation of  $g_{bs}$ . The estimates of  $r_{bs}$  using this full-model approach varied from 97 to 704 m<sup>2</sup> s mol<sup>-1</sup> for maize leaves (Table 2), substantially higher than He & Edwards' estimates. Our approach is suited for cases where the  $\Delta F/F_m'$  parameter has also been measured and thus provides data about e<sup>-</sup> transport. As no  $\Delta F/F_m'$  data were available in the data sets used by He & Edwards (1996), we are unable to assess whether their partial model is the only reason for their low estimates of  $r_{bs}$ .

### Use of GE and CF data to estimate diffusive conductances for C<sub>4</sub> photosynthesis

Use of combined GE and CF measurements is a recognized procedure for estimating  $g_m$  in C<sub>3</sub> photosynthesis (Harley *et al.* 1992; Evans & von Caemmerer 1996; Yin & Struik



**Figure 7.** The estimated CO<sub>2</sub> leakiness ( $\phi$ ) as a function of (a,c) intercellular CO<sub>2</sub> or of (b,d) incident irradiance level, at 21% (open symbols) and 2% (filled symbols) O<sub>2</sub>, for maize leaves from plants grown under low (squares) and high (circles) nitrogen conditions. Results are shown for (a,b) leaf stage I (1 week after full appearance) and (c,d) leaf stage II (2 weeks after full appearance); the patterns for stage III (3 weeks after full appearance) were similar to those for stage II, and are thus not given here.

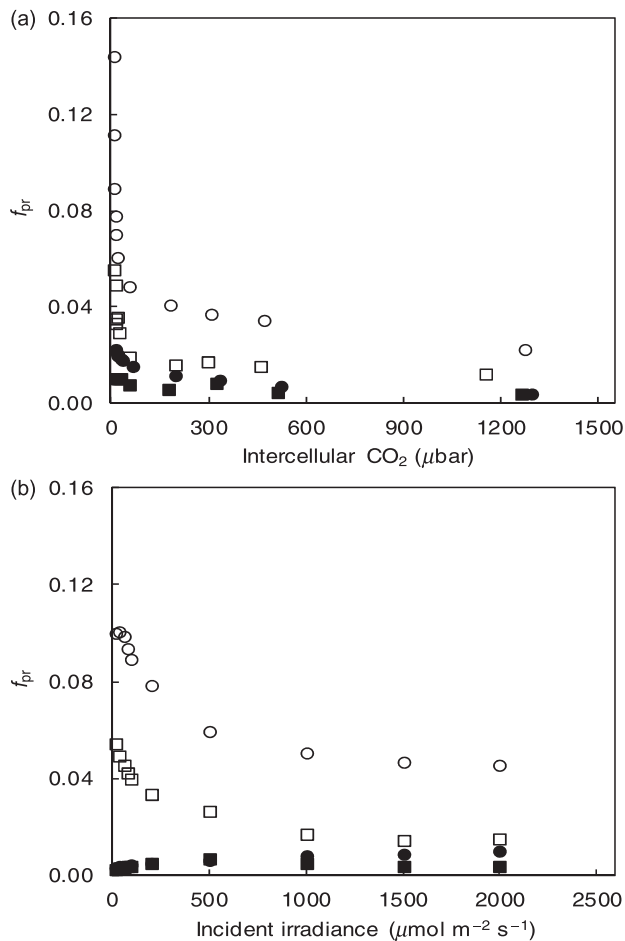
2009). This technique has not been applied for the estimation of  $g_m$  in C<sub>4</sub> photosynthesis (i.e. the conductance for CO<sub>2</sub> diffusion from intercellular air spaces to sites of PEP carboxylation) because the BS and mesophyll cells have different chloroplast populations (Evans & von Caemmerer 1996) which in principle could result in a complex relationship between the  $\Delta F/F_m'$  parameter and the quantum yield of CO<sub>2</sub> fixation. In practice, however, there is generally a good linear relationship for C<sub>4</sub> species between quantum yields of PSII from CF analysis and of CO<sub>2</sub> fixation from GE data over a wide range of conditions (e.g. Krall & Edwards 1990; Edwards & Baker 1993; Oberhuber, Dai & Edwards 1993; Peterson 1994; Laik & Edwards 1998), as found for C<sub>3</sub> species. Therefore, as we did for C<sub>3</sub> species (Yin *et al.* 2009), here we developed a procedure to utilize combined GE and CF data and modelling tools to estimate diffusive conductances ( $g_m$  and  $g_{bs}$ ) for C<sub>4</sub> photosynthesis. This procedure could be enhanced if PEPc and Rubisco activities are measured (see Discussion later).

Our estimates of  $g_m$  are uncertain as they were sensitive to other parameter values (Fig. 5) and conditional on a constant  $V_{pmax} : V_{cmax}$  ratio. However, the  $g_m$  estimates scaled linearly with LNC (Fig. 4a), similar to the behaviour of C<sub>3</sub> leaves in which that  $g_m$  is found to correlate with the

photosynthetic capacity of leaves (e.g. Loreto *et al.* 1992; Evans & von Caemmerer 1996) or with LNC (e.g. Li *et al.* 2009; Yin *et al.* 2009). Our estimates of  $g_m$  for maize varied from 0.9 to 3.5 mol m<sup>-2</sup> s<sup>-1</sup> (Table 2), compared with 0.82 mol m<sup>-2</sup> s<sup>-1</sup> (Pfeffer & Peisker 1995) and 0.75–1.02 mol m<sup>-2</sup> s<sup>-1</sup> (Kromdijk *et al.* 2010) for maize, 0.55–0.92 for the C<sub>4</sub> dicot *Flaveria bidentis* (Pengelly *et al.* 2010). This is higher than the estimated  $g_m$  for C<sub>3</sub> species, which generally lies between 0.05 and 0.5 mol m<sup>-2</sup> s<sup>-1</sup> (Loreto *et al.* 1992; Evans & von Caemmerer 1996). Evans & von Caemmerer (1996) discussed factors contributing to the higher  $g_m$  in C<sub>4</sub> species. Uncertainties in our  $g_m$  estimates for maize mirror the higher uncertainty encountered in estimates for C<sub>3</sub> species when  $g_m$  is high (Harley *et al.* 1992; Yin & Struik 2009). Because  $g_m$  is relatively high (Table 2; Pfeffer & Peisker 1995; Kromdijk *et al.* 2010; Pengelly *et al.* 2010), the assumption of an infinite  $g_m$ , as is often made for C<sub>4</sub> species, for example by Kiirats *et al.* (2002), is less likely to cause a problem.

### The effects of leaf age and N on $g_{bs}$

The effects of leaf age and LNC are often confounded by increases in leaf age being usually accompanied by a



**Figure 8.** The estimated fraction of the loss due to photorespiration  $f_{pr}$  as a function of (a) intercellular  $CO_2$  or of (b) incident irradiance level, at 21% (open symbols) and 2% (filled symbols)  $O_2$ , for maize leaves of stage I (1 week after their full appearance) from plants grown under low (squares) and high (circles) nitrogen conditions.

decrease in LNC. We therefore added nutrient solution weekly to attempt to maintain a constant LNC with leaf aging, a strategy that was successful for the high N treatment and moderately so for the low N treatment (Table 2). There appeared to be a trend of decreasing  $g_{bs}$  with increasing leaf age, similar to the results of Kiirats *et al.* (2002). However, our analysis showed that LNC was more important than leaf age in controlling  $g_{bs}$ .

Our estimation of  $g_{bs}$  was based on the assumption that the  $V_{pmax} : V_{cmax}$  ratio remained constant across LNC above the base N content,  $n_b$ . Reports where Rubisco and PEPc activities have been determined suggest that their ratio can decrease (Hunt *et al.* 1985) or increase (Meinzer & Zhu 1998) with increasing LNC, or can vary depending on the long- or short-term N stresses (Ranjith *et al.* 1995). Other results indicated that N allocation to Rubisco and PEPc (Ghannoum *et al.* 2005; Tazoe *et al.* 2006) changes with N supply levels. Any change in the activity of Rubisco relative to that of PEPc is expected to influence the estimation of  $g_{bs}$ , given that leakiness was found to decrease with

increasing Rubisco : PEPc activity (Saliendra *et al.* 1996) and that reduced Rubisco in transgenic  $C_4$  dicot *F. bidentis* was found to increase leakiness (von Caemmerer *et al.* 1997). We did not measure the activity or content of Rubisco and PEPc, so we are unable to be certain whether the relative Rubisco versus PEPc activity changed with LNC above  $n_b$  in our experiments. However, the good correlation between the initial slope (which is related to  $V_{pmax}$ ) and the maximum photosynthetic rates (which is related to  $V_{cmax}$ ) from the  $A-C_i$  curves across N-treatments and leaf stages (Fig. 4a inset) justified our assumption of a constant  $V_{pmax} : V_{cmax}$  ratio. This is in line with the linear relationship between LNC and Rubisco or PEPc activity (which had a similar  $n_b$  value) found for *Amaranthus retroflexus* (Sage *et al.* 1987) despite an increase in N allocation to Rubisco or PEPc with increasing leaf N.

Overall, our estimated  $g_{bs}$  increased monotonically with an increasing LNC (Fig. 4a), and further analysis showed that  $r_{bs}$ , the inverse of  $g_{bs}$ , correlated better with LNC than did  $g_{bs}$  (Fig. 4b), although our relationships in Fig. 4 were based on two extreme N treatments and more intermediate N levels would be needed to confirm these relationships. The decrease in  $g_{bs}$  or increase in  $r_{bs}$  with decreasing LNC may reflect a plant adaptation mechanism because  $r_{bs}$  would need to increase as photosynthetic capacity decreases in order to reduce  $CO_2$  leakiness that otherwise would increase as a result of decreased photosynthetic capacity at the same  $g_{bs}$  (von Caemmerer & Furbank 2003).

The value of  $g_{bs}$  on the leaf area basis is the product of the conductance across the mesophyll-BS interface ( $g'_{bs}$ ) and the ratio of BS surface area to leaf area ( $S_b$ ) (von Caemmerer & Furbank 1999). In only a few studies (e.g. Brown & Byrd 1993; Pengelly *et al.* 2010) has  $S_b$  been measured. When leaves are fully expanded, as is the case for our measurements,  $S_b$  is fixed (von Caemmerer & Furbank 2003). However,  $S_b$  was probably altered by our N treatments. High LNC has also been reported to increase the surface area of chloroplasts in  $C_3$  plants (Li *et al.* 2009), so it is reasonable to presume that increased N will also result in an increased  $S_b$  via this mechanism for  $C_4$  species. It is likely that in addition to being due to variation in  $S_b$ , the variation we observed in  $g_{bs}$  also originated from variation in  $g'_{bs}$  or its inverse  $r'_{bs}$ . The overall resistance  $r'_{bs}$  can be dissected into various components of resistance across cell wall, plasma and chloroplast membranes, cytoplasm and chloroplast stroma (von Caemmerer & Furbank 2003). The  $CO_2$  permeability of membranes can depend on membrane composition and the number of membranes that need to be crossed for  $CO_2$  to leak from the BS to the mesophyll cell. The value of other resistances is proportional to the ratio of (thickness  $\times$  tortuosity) to (diffusivity  $\times$  effective porosity) of the component (Hall 1971; Evans *et al.* 2009). These individual properties of the equation could be altered by LNC. For example, cell walls are probably thinner under high-N conditions; and a thick BS cell wall is crucial for the CCM effectiveness (Evans & von Caemmerer 1996; Leegood 2002). It is therefore not surprising that there was a negative linear relation between  $r_{bs}$  and LNC (Fig. 4b). Finally, a high LNC may be

associated with a high content of carbonic anhydrase (CA), which facilitates CO<sub>2</sub> diffusion rate (Badger & Price 1994). This is because the BS chloroplasts may contain a small amount of CA (Ludwig *et al.* 1998), although in C<sub>4</sub> plants CA is mainly found in the cytosol alongside PEPc (Evans & von Caemmerer 1996). All these may co-contribute to the reduced  $r_{bs}$  by an increased LNC. Further anatomical and biochemical measurements would help to confirm the components that underpin the change in the estimated  $g_{bs}$ .

### CO<sub>2</sub> concentration, leakiness of CO<sub>2</sub> and photorespiration in the BS cells

As a result of all the combinations of N × age × C<sub>a</sub> × O<sub>2</sub> × I<sub>inc</sub> levels used in our growth conditions and measurements, we estimated a wide spectrum of C<sub>c</sub>,  $\phi$  and  $f_{pr}$  (Figs 6–8). The relationships between these parameters and irradiance sometimes do not display a smooth curvilinear form because e<sup>-</sup> transport rate or J<sub>atp</sub> was calculated from the measured  $\Delta F/F_m'$  (see Eqn 9) rather than being estimated from a smooth non-rectangular hyperbolic function describing irradiance dependency of e<sup>-</sup> transport as in the original model of von Caemmerer & Furbank (1999). For the same reason, the predicted A did not show the smooth curves expected from the hyperbolic irradiance responses (Fig. 1).

Given the stronger influences of measurement noise and of some assumptions (e.g.  $R_m = 0.5R_d$  and constant  $R_d$ ) across low CO<sub>2</sub> or low irradiance levels, the predicted values of C<sub>c</sub>,  $\phi$  and  $f_{pr}$  for these conditions should be considered as tentative. However, the calculated trends in response to C<sub>i</sub> and I<sub>inc</sub> are generally as expected. A notable exception is the monotonically decreasing  $\phi$  with increasing C<sub>i</sub> found in the majority of cases (Fig. 7), while previous modelling analysis suggested the opposite trend (von Caemmerer & Furbank 1999; Kiirats *et al.* 2002). The CO<sub>2</sub> leakage depends on the CO<sub>2</sub> gradient between the BS and mesophyll, and this gradient in turn is dependent on the ratio of PEPc and Rubisco activity (which was estimated to be 2.5 for our data). The original model of von Caemmerer & Furbank (1999) has no feedback regulation from the C<sub>3</sub> cycle to the C<sub>4</sub> cycle (i.e. no curtailment of PEP regeneration when Rubisco is limiting), so that when Rubisco becomes limiting at high CO<sub>2</sub> levels, the model predicts a rapid increase in  $\phi$ . In our analysis, J<sub>atp</sub> was calculated from  $\Delta F/F_m'$  (see Eqn 9). We noticed that V<sub>p</sub> was mostly limited by e<sup>-</sup> transport. As PSII e<sup>-</sup> transport efficiency assessed by CF measurements is highly regulated by CO<sub>2</sub> fixation efficiency (Edwards & Baker 1993; Oberhuber *et al.* 1993; Peterson 1994), the direct use of  $\Delta F/F_m'$  in our formulation results in any feedback regulation from the C<sub>3</sub> cycle to the C<sub>4</sub> cycle being implicitly accounted for.

The predicted declining trend of  $\phi$  with increasing I<sub>inc</sub> (Fig. 7b,d) agrees with previous predictions (von Caemmerer & Furbank 1999). As  $\phi$  defines the efficiency of the CCM and the CCM requires ATP, the progressive increase in  $\phi$  with decreasing I<sub>inc</sub> has been explained by incomplete suppression of photorespiration plus an increasing relative contribution of mitochondrial respiration (Kromdijk *et al.*

2010). Our predicted trends of  $\phi$  and  $f_{pr}$  with I<sub>inc</sub> (Figs 7 & 8) seem to support this proposition. The high value for  $\phi$  at low I<sub>inc</sub> in 2% O<sub>2</sub> (Fig. 7b,d), when photorespiration was suppressed almost completely, means that the CO<sub>2</sub> release from mitochondrial respiration in BS cells might contribute to leakage at low I<sub>inc</sub>. Alternatively, under low light and low O<sub>2</sub> conditions, the activation state of Rubisco may be decreased relative to the C<sub>4</sub>-cycle capacity, causing an increase in leakiness.

Nitrogen affected C<sub>c</sub>,  $\phi$  and  $f_{pr}$ , because higher LNC caused a higher  $g_{bs}$ . High-N leaves had lower C<sub>c</sub> (Fig. 6), in contrast to its effect in C<sub>3</sub> photosynthesis where C<sub>c</sub> is likely higher under high-N supply as a result of higher  $g_m$  (Li *et al.* 2009). Despite the higher  $g_{bs}$ , which means a higher  $\phi$  and less efficient CCM,  $f_{pr}$  was still low for high N leaves – the highest fraction that occurred under extreme conditions (50  $\mu\text{mol mol}^{-1}$  ambient CO<sub>2</sub> or strictly limiting irradiance) was estimated to be 0.10–0.15 (Fig. 8). As a result, there was little detectable effect of O<sub>2</sub> on quantum yield and on the initial slope of A-C<sub>i</sub> curves in our experiment (Fig. 1).

In this paper, we focused on analysing the effect of LNC on  $g_{bs}$  and the related parameters  $\phi$ , C<sub>c</sub> and  $f_{pr}$ . Needless to say, LNC also affects the concentrations of photosynthetic enzymes. It is the interplay of this dual effect of LNC that determines the actual photosynthetic rates of leaves. In line with many reports (e.g. Hunt *et al.* 1985; Wong *et al.* 1985; Meinzer & Zhu 1998; Tazoe *et al.* 2006), our data showed that CO<sub>2</sub> assimilation rate *per se* was increased by the high-N supply (Fig. 1) and with LNC. Therefore, the positive effect of LNC on the absolute fluxes of C<sub>4</sub> and C<sub>3</sub> cycles via V<sub>pmax</sub> and V<sub>cmax</sub> is predominant, relative to the negative effect of LNC on  $r_{bs}$ , in determining the actual C<sub>4</sub> photosynthetic rates of the leaves.

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## APPENDIX A

### The quadratic expression and its solution

Combining Eqns 1–5 in the main text will yield a standard quadratic expression for  $A$  (referring to either  $A_{TE}$  or  $A_{TT}$ , for which the rate of C<sub>4</sub> cycle,  $V_p$ , is limited by e<sup>-</sup> transport)

$$aA^2 + bA + c = 0$$

$$\text{where } A = \left( -b + \sqrt{b^2 - 4ac} \right) / (2a)$$

$$a = x_2 g_m \alpha / 0.047 - g_m - g_{bs}$$

$$b = g_m (C_i g_{bs} + V_p - R_m) + (x_3 + x_2 O_i) g_m g_{bs} + (x_1 \gamma_* + x_2 R_d) g_m \alpha / 0.047 + (g_m + g_{bs})(x_1 - R_d)$$

$$c = -g_m (C_i g_{bs} + V_p - R_m)(x_1 - R_d) + g_m g_{bs} [x_1 \gamma_* O_i + R_d (x_3 + x_2 O_i)]$$

where  $V_p$  is given by Eqn 7.

## APPENDIX B

### The cubic expression and its solution

Combining Eqns 1–6 in the main text will yield a standard cubic expression for  $A$  (referring to either  $A_{EE}$  or  $A_{ET}$ , for which the rate of C<sub>4</sub> cycle,  $V_p$ , is limited by the activity of the enzyme PEPc)

$$A^3 + pA^2 + qA + r = 0$$

The coefficients  $p$ ,  $q$  and  $r$  are complex combinations of model parameters or variables

$$p = m / (g_m + g_{bs} - x_2 g_m \alpha / 0.047)$$

$$q = n / (g_m + g_{bs} - x_2 g_m \alpha / 0.047)$$

$$r = o / (g_m + g_{bs} - x_2 g_m \alpha / 0.047)$$

in which  $m$ ,  $n$  and  $o$  are expressed as

$$m = d - (x_3 + x_2 O_i) g_m g_{bs} + (R_d - x_1)(g_m + g_{bs}) - (x_1 \gamma_* g_m + x_2 R_d g_m - x_2 k / g_{bs}) \alpha / 0.047$$

$$n = f + (x_3 + x_2 O_i) k + d(R_d - x_1) - g_m g_{bs} [x_1 \gamma_* O_i + R_d(x_3 + x_2 O_i)] + (x_1 \gamma_* + x_2 R_d) k \alpha / (0.047 g_{bs})$$

$$o = R_d [f + (x_3 + x_2 O_i) k] - x_1 (f - k \gamma_* O_i)$$

where  $d$ ,  $f$  and  $k$  are expressed as

$$d = g_m [R_m - V_{pmax} - C_i (g_m + 2g_{bs}) - K_p (g_m + g_{bs})]$$

$$f = g_m^2 [C_i V_{pmax} + (C_i + K_p)(g_{bs} C_i - R_m)]$$

$$k = g_m^2 g_{bs} (C_i + K_p)$$

The solution of the above cubic equation is taken from Press *et al.* (1989). Three roots for the equation are:

$$A_1 = -2\sqrt{Q} \cos(\psi/3) - p/3$$

$$A_2 = -2\sqrt{Q} \cos[(\psi + 2\pi)/3] - p/3$$

$$A_3 = -2\sqrt{Q} \cos[(\psi + 4\pi)/3] - p/3$$

where  $Q = (p^2 - 3q)/9$

$$\psi = \arccos(U/\sqrt{Q^3})$$

$$U = (2p^3 - 9pq + 27r)/54$$

We found the root  $A_1$  suitable for calculating either  $A_{EE}$  or  $A_{ET}$  under any combinations of  $C_i$ ,  $I_{inc}$  and  $O_i$ .