

Variability in mesophyll conductance between barley genotypes, and effects on transpiration efficiency and carbon isotope discrimination

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ABSTRACT

Leaf internal, or mesophyll, conductance to CO₂ (g_m) is a significant and variable limitation of photosynthesis that also affects leaf transpiration efficiency (TE). Genotypic variation in g_m and the effect of g_m on TE were assessed in six barley genotypes (four *Hordeum vulgare* and two *H. bulbosum*). Significant variation in g_m was found between genotypes, and was correlated with photosynthetic rate. The genotype with the highest g_m also had the highest TE and the lowest carbon isotope discrimination as recorded in leaf tissue (Δ_p). These results suggest g_m has unexplored potential to provide TE improvement within crop breeding programmes.

Key-words: *Hordeum vulgare*; leaf internal conductance; photosynthesis; stomatal conductance; water use efficiency.

INTRODUCTION

Climate change models predict that many important arable cropping regions of the world will experience increased frequency and severity of drought, on top of the predicted increases in temperature. Consequently, there is an increased need for improvements in the efficiency with which crop plants convert applied water into harvestable production, or water-use efficiency (WUE). In the recent past there have been considerable gains in WUE made, notably in cereals, by selecting for plants with high transpiration efficiency (TE) using stable carbon isotope discrimination (Farquhar & Richards 1984; Acevedo 1993; Rebetzke et al. 2002; Richards et al. 2002). TE is a leaf-level index – the ratio of photosynthesis to transpiration rates – and is an important component of WUE.

Variation in TE has been explored using equations derived from the relationship between photosynthetic rate (A), the leaf intercellular CO₂ partial pressure (C_i), and transpiration rate (E) (Farquhar & Richards 1984; Hubick & Farquhar 1989)

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$$TE = \frac{A}{E} = \frac{C_a \left(1 - \frac{C_i}{C_a}\right)}{1.6(e_i - e_a)}, \quad (1)$$

where C_a is ambient CO₂ partial pressure, and e_i and e_a are intercellular and ambient vapour pressures, respectively. Farquhar *et al.* (1989) proposed that longer-term, whole-plant water-use efficiency also includes respiratory loss of carbon (by leaves in the dark, and non-photosynthetic tissues in the light and the dark) and water loss not associated with carbon gain (such as transpiration in the dark or by organs other than leaves). However, Cernusak *et al.* (2008) recently confirmed that whole-plant WUE was strongly correlated with C_i/C_a across a range of tropical species from a number of functional plant types.

Carbon isotope discrimination during C₃ photosynthesis ($\Delta^{13}C$) has been used since the 1980s (Farquhar, O'leary & Berry 1982) as an indicator of TE and WUE, because $\Delta^{13}C$ often shares a dependence on C_i/C_a with TE. This dependence may be expressed (Hubick, Farquhar & Shorter 1986; Farquhar *et al.* 1989) as:

$$\Delta^{13}C = a + (b - a) \frac{C_i}{C_a} - d, \quad (2)$$

and

$$TE = \frac{(1 - \phi)C_a(b - d - \Delta^{13}C)}{1.6(e_i - e_a)(b - a)}, \quad (3)$$

where a is diffusional fraction through the stomata (4.4‰), b is net fractionation during carboxylation and d is a term combining fractionation during photorespiration, dark respiration, and dissolution and diffusion from the gaseous phase to the chloroplasts.

However, combining respiration fractionations and fractionations associated with CO₂ diffusion through the mesophyll into the term d assumes these effects are either constant or negligible. A more complete description of $\Delta^{13}C$ is given by (modified from Farquhar & Richards 1984):

$$\Delta^{13}C = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + (b_s + a_1) \frac{C_i - C_c}{C_a} + b \frac{C_c}{C_a} - f \frac{\Gamma^*}{C_a} - e' \frac{R_d}{A + R_d} \frac{C_c - \Gamma^*}{C_a} \quad (4)$$

where a_b (2.9‰) and a_1 (0.7‰) are fractionations associated with diffusion through the leaf boundary layer and leaf water, respectively; b_s is fractionation as CO_2 moves into solution (1.1‰ at 25 °C); C_s and C_c are the CO_2 concentrations at the leaf surface and at the sites of carboxylation, respectively; e' and f are fractionations associated with mitochondrial respiration in the light (corrected for source CO_2 $\delta^{13}\text{C}$) and photorespiration, respectively; and Γ^* is the CO_2 compensation point in the absence of mitochondrial respiration (R_d). Note fractionation during carboxylation is now weighted by the ratio of the CO_2 concentrations in chloroplasts to the ambient air (C_c/C_a), rather than C_i/C_a . This distinction is important if $\Delta^{13}\text{C}$ is used as a surrogate for TE (or WUE), and if C_c/C_a does not scale with C_i/C_a .

Recent work suggests that there is a significant, variable, resistance to diffusion of CO_2 between the intercellular air spaces and the chloroplasts (leaf internal conductance or g_m), meaning that C_c is significantly less than C_i (Evans & von Caemmerer 1996; Warren & Dreyer 2006; Flexas *et al.* 2008). There is some evidence that g_m and g_s (stomatal conductance to water vapour) are correlated (e.g. Evans 1999), so that C_c/C_a may scale with C_i/C_a , and this will be explored further in this paper. The ideal crop plant in areas with limited water availability would have low g_s to reduce water loss, but maintain high rates of carbon fixation (hence high productivity). One way to maintain high A when C_i is low is to increase g_m , so that C_c remains high (Flexas *et al.* 2010).

There is currently limited mechanistic understanding of variability in g_m . Leaf anatomy, and particularly the surface area of chloroplasts exposed to the intercellular spaces, is correlated with g_m in leaves from different species (Evans 1999) and from the same species grown under differing environmental conditions (Perez-Martin *et al.* 2009). Light-saturated photosynthetic rate is correlated with g_m in some studies (e.g. Evans & von Caemmerer 1996; Evans 1999), but not in all (Warren & Adams 2006). Recent studies have highlighted the dynamic nature of g_m by showing rapid responses to light and CO_2 concentration (Flexas *et al.* 2007). It is possible that these dynamic responses may be due to the activity of aquaporins in chloroplastic membranes (Uehlein *et al.* 2008).

The degree to which g_m influences TE and WUE is currently not well explored experimentally. Genetic variability in g_m has previously been reported in a number of species, including wheat, where g_m varied between 0.20 and 0.43 $\text{mol m}^{-2} \text{s}^{-1}$ among five cultivars (Evans & Vellen 1996), European chestnut provenances (Lauteri *et al.* 1997), between *Populus* populations from different latitudes (Sool-anayakanahally *et al.* 2009), and between *Phaseolus vulgaris* genotypes (Flowers *et al.* 2007) although only after exposure to high ozone concentration. Evans *et al.* (1994) found that tobacco transgenic plants, antisense to components of the

Rubisco small subunit, had lower g_m than wild-type plants when grown under the same light conditions.

Mesophyll conductance has been technically challenging to measure and, until recently, has been assumed to contribute either a negligible or constant limitation to photosynthetic rate. Traditionally, g_m has been measured using combined leaf gas exchange and fluorometry, or combined leaf gas exchange and online stable carbon isotope discrimination, or applying curve-fitting procedures to photosynthetic CO_2 response curves. The advantages and disadvantages of each of these techniques were reviewed by Warren (2006) and Pons *et al.* (2009). Recent advances in laser absorption spectrometry have significantly improved the temporal resolution with which carbon isotope discrimination measurements can be made, and theoretical work has placed constraints on parameter assumptions (Lanigan *et al.* 2009).

Here we quantify the influence of g_m on TE among six barley genotypes, four genotypes of *Hordeum vulgare* (L.), including two named cultivars, and two wild (i.e. uncultivated) genotypes of *H. bulbosum* (L.), a barley that is adapted to extremely arid environments.

MATERIALS AND METHODS

To characterize the differences in gas exchange properties of the six genotypes, a number of measurements and calculations were performed. Firstly, g_m was measured under daytime growth conditions for each plant using coupled leaf gas exchange and online carbon isotope discrimination. Secondly, the diurnal courses of carbon and water exchange were measured for three replicate plants of each genotype. Thirdly, photosynthetic CO_2 response curves were measured for five replicate leaves from one of the genotypes, and the effects of variable g_m and g_s on TE explored using theoretical equations. Finally, the carbon isotope composition of leaves from each plant was measured to compare with measured TE.

Genotypes and growth conditions

Barley seeds (for cultivars 'Dash' and 'Omaka' and diploid and tetraploid lines of 'Golden Promise', described in the further discussion) were sown, or immature tillers transplanted (for diploid and tetraploid lines of *H. bulbosum*, described in the further discussion), in 5 L pots containing a bark-based potting mix and grown for 6 weeks. Plants were thinned to one per pot and transferred to a controlled-environment growth cabinet 12 d prior to the start of measurements. Pots were well watered throughout. The cabinet was maintained at 20 °C, 70% relative humidity (RH), 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) during the 16 h light period, and 15 °C, 70% RH during the 8-h dark period. The genotypes were (six replicate plants each): (1) *H. bulbosum*, a wild (uncultivated) barley that is adapted to extremely dry environments. We tested two types: 'Cb2920/4' a diploid (HB2) and 'Cb2920/4' a tetraploid (HB4); (2) *H. vulgare*, 'Golden Promise', a gamma

ray-induced semi-dwarf mutant of the cultivar 'Maythorpe' that has a high salt tolerance (Forster 2001). Again we tested two types: a diploid (GP2) and a tetraploid (GP4); (3) *H. vulgare* 'Omaka' and 'Dash', two commercially grown cultivars with contrasting morphologies. 'Omaka' is a forage barley, and 'Dash' is a high-yielding grain-feed barley for dry land conditions. All plants were in the vegetative phase during the experiment.

Tetraploidy of 'Golden Promise' was confirmed using flow cytometry. An expanding leaf was removed from each plant and placed in a Petri dish. A leaf of lawn daisy (*Bellis perennis* L.) was also placed in the Petri dish to act as an internal reference. DNA was extracted and dyed following the procedure of Otto (1990). Extracted samples were filtered and passed through a Partec PAII flow cytometer. Two frequency peaks were recorded for each sample, one representing the *B. perennis* reference and the other representing the barley. The position of the barley peak was expressed relative to the reference peak to give a ploidy ratio. This procedure was conducted for all candidate tetraploid plants as well as six reference diploid plants. The candidate tetraploids that had exactly twice the ploidy ratio of the diploid references were selected as true 28-chromosome tetraploids.

Leaf gas exchange

A photosynthesis system (Li6400, Li-Cor, Lincoln, NE, USA) quantified gas exchange parameters (photosynthetic rate, A ; respiration rate, R ; stomatal conductance to water vapour, g_s ; and transpiration rate, E) of youngest fully expanded leaves under growth conditions (i.e. PAR = 650 $\mu\text{mol m}^{-2} \text{s}^{-1}$, T_a = 26 °C in light, 19 °C in dark, RH = 60–70%, $[\text{CO}_2]$ = 370–420 ppm). Measurements were recorded every 5 min over a 24 h period for three plants of each genotype.

Photosynthetic response to CO_2 concentration between 0 and 2000 ppm was measured for five replicate plants of 'Dash' with the standard 2 × 3 cm leaf chamber and the blue-red light source set at 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Response curves were measured starting at ambient $[\text{CO}_2]$, decreasing to 0 ppm, then increasing from ambient to 2000 ppm. The response curves are presented in terms of CO_2 partial pressures. All measurements were made in Lincoln, New Zealand, at 30 m above sea level, and atmospheric pressure varied between 101 and 104 kPa. The five curves were averaged to generate a 'baseline' curve, from which values of stomatal conductance to CO_2 (g_{sc} , from diurnal gas exchange measurements) and g_m (from online ^{13}C discrimination measurements) for three contrasting genotypes ('Dash', GP4 and HB4) were applied to construct the responses of A to C_a and C_c using $C_c = C_i - (A/g_m)$ and $C_a = C_i + (A/g_{\text{totc}})$, where g_{totc} is the combined stomatal and boundary layer conductances to CO_2 . The one-sided boundary layer conductance to CO_2 was assumed to be 1.74 mol $\text{CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for all three constructed curves, a value appropriate for leaf gas exchange chambers.

The photosynthetic limitation analysis approach presented by Farquhar & Sharkey (1982) was applied to explore the stomatal and mesophyll limitations to photosynthesis, and the influence of these limitations on TE. The limitations on A imposed by g_{sc} and g_m were assessed by comparing the values of A assuming g_{sc} and g_m were infinite with those as measured during diurnal leaf gas exchange (for g_s) or using the online ^{13}C discrimination technique (for g_m , as described in the further discussion). Light-saturated rates of net photosynthesis were estimated using assumed values of g_{sc} and g_m at $C_a = 380 \mu\text{mol mol}^{-1}$ (A_n), assuming g_m was infinite and g_s as measured (A_{mi} , at $C_c = C_i$), or assuming infinite g_s and measured g_m (A_{sl} , at $C_i = 380 \mu\text{mol mol}^{-1}$). The relative limitations of photosynthesis due to mesophyll resistances (L_m) and stomatal resistances (L_s) were calculated (Warren *et al.* 2003):

$$L_m = \frac{A_{mi} - A_n}{A_{mi}} \quad (5)$$

$$L_s = \frac{A_{sl} - A_n}{A_{sl}} \quad (6)$$

Transpiration rate (E) was then estimated assuming leaf temperature is 23 °C, atmospheric pressure is 100 kPa (so that intercellular water mole fraction is 28.2 mmol mol^{-1}), while the ambient air water mole fraction is 21.0 mmol mol^{-1} for all constructed curves. TE is then simply A_n divided by E .

Estimation of mesophyll conductance

An online, stable isotope, tunable diode laser absorption spectrometer (TDL) coupled to a photosynthesis system fitted with a custom-built leaf chamber (Barbour *et al.* 2007) was used to measure the photosynthetic carbon isotope discrimination (Δ_{obs}) under the conditions described earlier. The TDL measures concentrations of $^{13}\text{CO}_2$ and $^{12}\text{CO}_2$, and these values are converted to the familiar $\delta^{13}\text{C}$ notation with a precision of $\pm 0.1\%$ (standard deviation of 12 repeat analyses of an unknown cylinder). Δ_{obs} was calculated following Evans *et al.* (1986):

$$\Delta_{\text{obs}} = \frac{\xi(\delta_o - \delta_c)}{1 + \delta_o - \xi(\delta_o - \delta_c)}, \quad (7)$$

where:

$$\xi = \frac{c_e}{c_e - c_o}, \quad (8)$$

and c_e and δ_c are concentrations and isotope compositions of CO_2 entering the leaf chamber, and c_o and δ_o are concentrations and isotope compositions of CO_2 leaving the leaf chamber. All air streams were passed through a nafion drying tube prior to entering the TDL, so values presented are all at zero vapour concentration. g_m was estimated from the difference between calculated carbon isotope

discrimination assuming infinite g_m (Δ_i), and that measured by the coupled system (modified from Evans *et al.* 1986):

$$g_m = \frac{\left(b - a_i - b_s - \frac{e'R_d}{A + R_d}\right)A}{C_a(\Delta_i - \Delta_{\text{obs}})}, \quad (9)$$

and

$$\Delta_i = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + b \frac{C_i}{C_a} - f \frac{\Gamma^*}{C_a} - e' \frac{R_d}{A + R_d} \frac{C_i - \Gamma^*}{C_a} \quad (10)$$

To parameterize the equations, we assume $a_b = 2.9\text{‰}$, $a = 4.4\text{‰}$, $b = 28\text{‰}$, $f = 11.6\text{‰}$ (Lanigan *et al.* 2009), $e' = -0.5\text{‰}$ [taking into account both isotope effects of -3‰ (Bickford *et al.* 2009) and the ^{13}C disequilibrium of $+2.5\text{‰}$ between growth and measurement CO_2 (Wingate *et al.* 2007; Tazoe *et al.* 2009)].

Measurements were made using the coupled Li-6400/TGA100A system at growth irradiance, temperature and air saturation deficit ($650 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, $T_a = 26^\circ\text{C}$, $D = 0.7 \text{ kPa}$), but $[\text{CO}_2]$ was controlled within the leaf chamber to be $400 \mu\text{mol mol}^{-1}$. Each leaf remained in the chamber for at least 30 min, allowing the leaf to adjust to the chamber conditions (10 min) before gas exchange and online discrimination measurements were made. g_m was calculated using Eqns 9 and 10 for each coupled measurement, and seven sequential measurements were used to calculate mean and standard error of relevant parameters for each leaf.

Stable isotope analysis of leaf water and organic material

The youngest, fully expanded leaves were collected for analysis of nitrogen concentration and stable carbon isotope composition between 7 and 8 h after the start of the light period on the completion of the gas exchange measurements. Analysis of leaf material was completed at the Center for Stable Isotope Biogeochemistry at the University of California, Berkeley. Carbon isotope compositions are converted to isotope discriminations assuming $\delta^{13}\text{C}$ of cabinet air is -8‰ .

Statistical analysis

A Monte Carlo analysis was performed to assess the variability in g_m caused by instrument noise at high and low g_m , using Eqns 7–10. The values for assumed and measured parameters, with associated instrument variability, are shown in Table 1. Monte Carlo simulations were carried out using the statistical computing environment 'R' (Version 2.9.2; R Development Core Team 2009). Random variables with means and standard deviations derived from observed leaf gas exchange parameters and online discrimination were used in the simulations. One million simulations of each high and low g_m were carried out. Occasionally the simulation produces nonsensical values (depending on the values of the random variables generated for the parameter values). All generated values of g_m less than 0 and values greater than 0.5 and 3 (for low and high g_m , respectively)

Variables (assigned values)			
Parameter	Value (high g_m)	Value (low g_m)	
b (‰)	28	28	
e' (‰)	-0.5	-0.5	
f (‰)	11.6	11.6	
R_d ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	0.81	0.86	
Γ^* ($\mu\text{mol mol}^{-1}$)	39.2	40.5	
Variables (measured)			
Parameter	Value (high g_m)	Value (low g_m)	SD over 15 s
A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	28.0	4.7	0.5
C_a ($\mu\text{mol mol}^{-1}$)	399.5	399.2	0.2
C_s ($\mu\text{mol mol}^{-1}$)	383.6	396.7	0.2
C_i ($\mu\text{mol mol}^{-1}$)	323	274	1
c_c (ppm)	513.95	440.78	0.05
c_o (ppm)	420.85	418.52	0.05
δ_c (‰)	-5.13	-5.21	0.1
δ_o (‰)	-1.60	-4.52	0.1
Monte Carlo estimates of g_m ($\text{mol m}^{-2} \text{s}^{-1}$)			
	High g_m	Low g_m	
Mean	0.765	0.054	
2.5%	0.460	0.026	
97.5%	1.924	0.251	

Table 1. Assumed and measured values for parameters used to calculate mesophyll conductance in a Monte Carlo analysis. Also shown are 95% confidence intervals of g_m for typical low and high g_m measurements

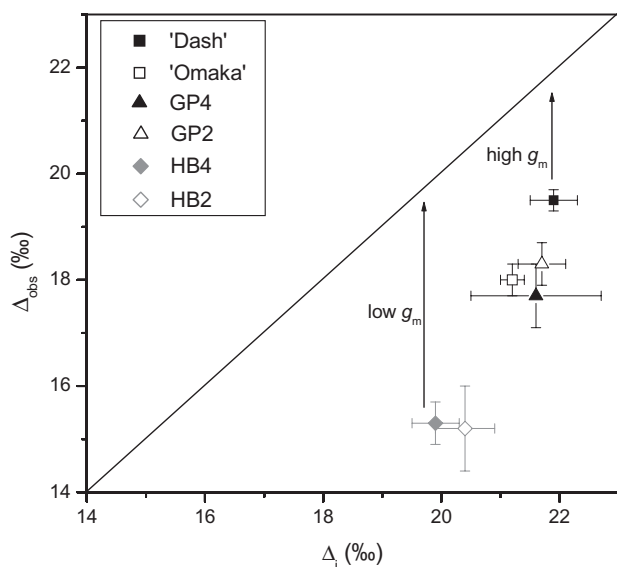


Figure 1. Relationship between predicted discrimination assuming infinite mesophyll conductance (Δ_i) and measured discrimination (Δ_{obs}). The arrows indicate the difference from the 1:1 line, and the length of the arrows is negatively related to mesophyll conductance. Values are mean \pm standard error, $n = 6$ leaves from six plants.

were discarded. About 0.2 and 2% of simulations fell below zero and above the chosen upper limit. Statistics presented are based on these reduced data sets.

Variability between measurements was assessed using analysis of variance in GenStat, and linear relationships between related parameters using least-squares linear regression in Origin.

RESULTS

Estimates of variability in g_m

The Monte Carlo analysis revealed that instrument noise contributes significant variability in calculated g_m , with most of the variability contributed by measurement of Δ_{obs} . For the two examples, at high g_m (median = $0.765 \text{ mol m}^{-2} \text{ s}^{-1}$) the calculated value of g_m fell between 0.460 and $1.924 \text{ mol m}^{-2} \text{ s}^{-1}$ (95th percentiles), whereas at low g_m (median = $0.054 \text{ mol m}^{-2} \text{ s}^{-1}$) the calculated value of g_m fell between 0.026 and $0.251 \text{ mol m}^{-2} \text{ s}^{-1}$ (95th percentiles). There are highly significant differences in g_m , even when instrument noise is taken into account (Wilcoxon test $W > 10^6$, $P < 0.001$).

Online carbon isotope discrimination and mesophyll conductance

Significant differences were found between genotypes in the measured online carbon isotope discrimination (Δ_{obs}) under leaf chamber conditions close to growth environmental conditions: $650 \mu\text{mol m}^{-2} \text{ s}^{-1}$ PAR, T_a of 26°C and leaf-to-air vapour pressure difference of 0.7 kPa. 'Dash' had the highest

Δ_{obs} , at 19.5‰, whereas the two *H. bulbosum* genotypes had much lower Δ_{obs} of about 15.3‰. The predicted discrimination if g_m were assumed to be infinite (Δ_i) was not significantly different between *H. vulgare* genotypes, and was only slightly lower for the two *H. bulbosum* genotypes. That is, C_i/C_a was very similar between genotypes. As shown in Fig. 1, $\Delta_i - \Delta_{\text{obs}}$ was smallest for 'Dash' and largest for the two *H. bulbosum* genotypes, implying that g_m is highest for 'Dash' and smallest for *H. bulbosum*. Figure 2 confirms that the greatest contrast in g_m was between *H. vulgare* and *H. bulbosum* genotypes, with the latter having g_m less than half of the *H. vulgare* genotypes. However, significant differences in g_m ($P = 0.043$) were found between the two named *H. vulgare* cultivars, with 'Omaka' having lower g_m than 'Dash'.

Mesophyll conductance was positively related to both photosynthetic rate and stomatal conductance to CO_2 (g_{sc}) across all genotypes (Fig. 3). However, the relationship was stronger between A and g_m than between g_{sc} and g_m , with 76% of variability in g_m explained by A compared with 58% for g_{sc} . g_m was only significantly related to A within an individual genotype for the diploid *H. bulbosum*, and g_m was not related to g_{sc} within any individual genotype.

TE and its components

Diurnal gas exchange revealed significant differences in A , g_s , E and TE among genotypes (Fig. 4). 'Dash' had the lowest g_s of the *H. vulgare* genotypes but all *H. vulgare* genotypes had similar photosynthetic rates. Both *H. bulbosum* genotypes had lower A and g_s during the day than any of the *H. vulgare* genotypes. 'Dash' had the highest midday TE of the *H. vulgare* genotypes, due to low g_s and E , but high A (Fig. 4).

L_m and L_s , and influence on TE

Taking the measured values of g_{sc} and g_m for three contrasting genotypes ('Dash', GP4 and HB4), but an artificially

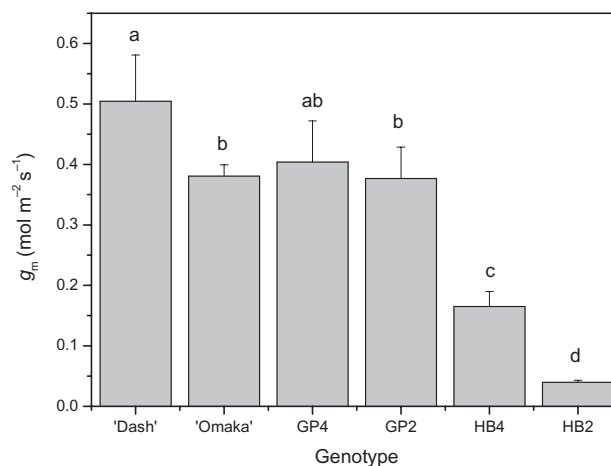


Figure 2. Average mesophyll conductance for six barley genotypes measured using online $\Delta^{13}\text{C}$ under growth conditions. Values are means \pm standard errors, $n = 6$. Letters indicate significant differences, $P < 0.05$.

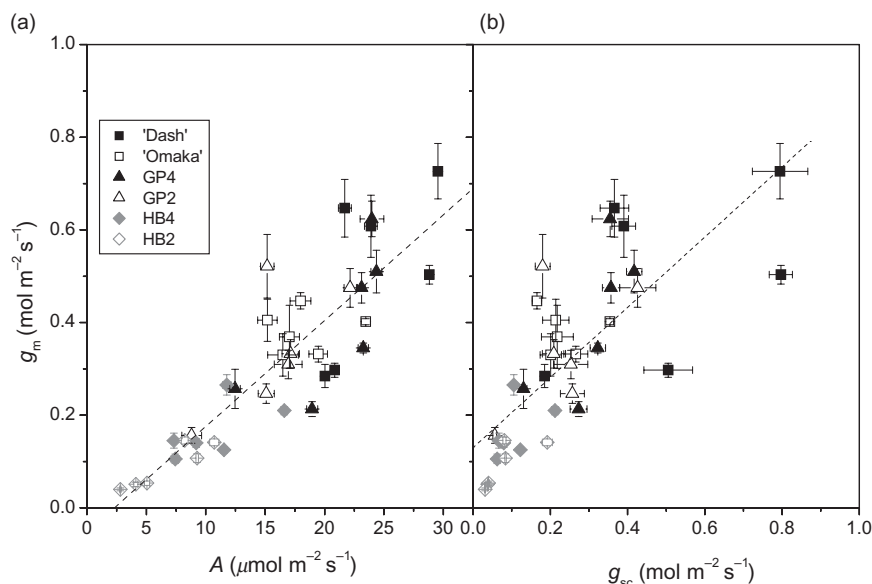


Figure 3. Relationships between mesophyll conductance and (a) photosynthetic rate and (b) stomatal conductance to CO_2 for six plants of each of six barley genotypes measured under standard conditions of $650 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) and T_a of 26°C and C_a of $400 \mu\text{mol mol}^{-1}$. The lines are least squares regression across all genotypes: (a) $g_m = -0.05 + 0.023A$ $r^2 = 0.76$, $P < 0.0001$; (b) $g_m = 0.130 + 0.758 g_{sc}$, $r^2 = 0.58$, $P < 0.0001$.

equivalent A/C_i response curve, allows an assessment of the relative limitations on photosynthetic rates imposed by the stomata (L_s) and the mesophyll (L_m) without some of the complications arising from differences in photosynthetic biochemistry between leaves. Figure 5 shows that, as could be expected, high g_{sc} resulted in a lower value for L_s than when g_{sc} was lower (compare panel B with A and C in Fig. 5), whereas high g_m resulted in lower L_m than when g_m was lower (compare panels A and B with panel C). The response curves mimicking GP4 had the highest A_n at C_a of $380 \mu\text{mol mol}^{-1}$, and those mimicking HB4 the lowest ($20.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ compared with $14.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ for GP4 and HB4, respectively, with 'Dash' at $19.0 \mu\text{mol m}^{-2} \text{s}^{-1}$). However, the high g_{sc} for GP4 resulted in the lowest TE of

the three examples. This is consistent with the results obtained during diurnal gas exchange measurements.

Integrated carbon isotope discrimination

Significant differences were found between genotypes for carbon isotope discrimination as recorded in leaf tissue (Δ_p). 'Dash' had the lowest Δ_p , at 18.5‰, whereas GP4 had the highest at 20.0‰. Δ_p was negatively related to average midday TE (Fig. 6, $P = 0.04$).

DISCUSSION

Variability in g_m between genotypes

Significant differences in mesophyll conductance were observed between genotypes, with the *H. bulbosum* genotypes having significantly lower g_m than *H. vulgare* genotypes. Significant differences in g_m were also found between genotypes within each species, with the tetraploid *H. bulbosum* (HB4) having higher g_m than the diploid (HB2), and 'Dash' the highest g_m among the *H. vulgare* genotypes. As described earlier, a crop plant with a high maximum carboxylation rate and g_m but low g_{sc} will have a high TE. Of the genotypes assessed, 'Dash' fits this description most closely.

Although g_m measurements were made on leaves in conditions as close as possible to the growth environment, and to the conditions under which the diurnal gas exchange measurements were made (so that measured g_m could be used to interpret differences in TE between genotypes), there were inconsistencies between diurnal gas exchange and online ^{13}C discrimination measurements. The stomatal conductance and photosynthetic rate of 'Dash' during online ^{13}C measurements were considerably higher than during diurnal gas exchange measurements. Given the positive relationship between A and g_m across genotypes, this may mean that g_m

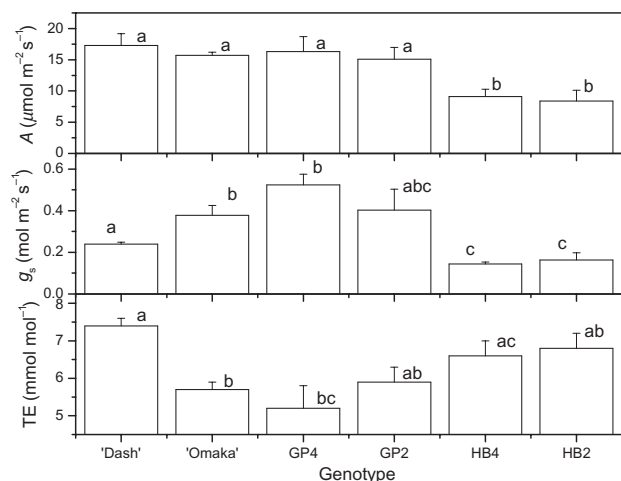


Figure 4. Midday photosynthetic rate (A), stomatal conductance (g_s) and transpiration efficiency (TE) for six barley genotypes. Values are mean \pm standard error, $n = 3$. Significant differences ($P < 0.05$) between genotypes are indicated with letters.

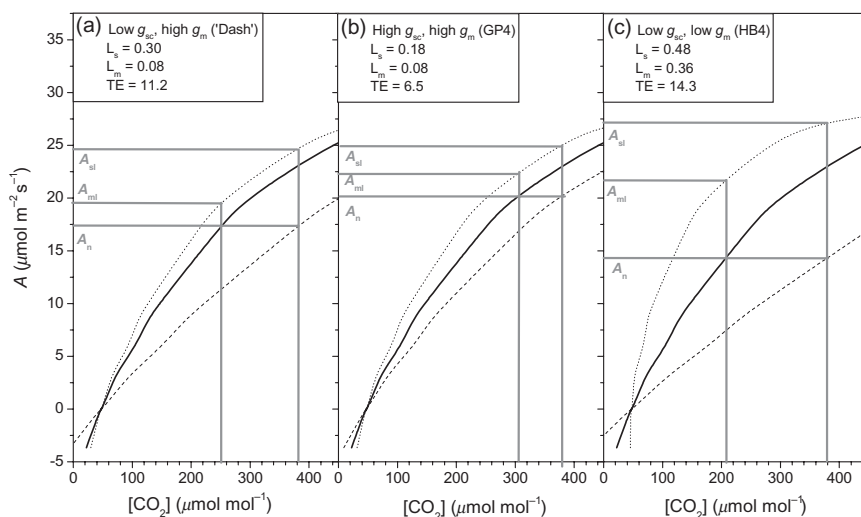


Figure 5. Constructed photosynthetic (A) response curves to CO_2 partial pressure. The A/C_i curve (solid line) is the same in all three panels, and the A/C_a (dashed line) and A/C_c (dotted line) curves were constructed using values for g_{sc} and g_m from three contrasting cultivars. In (a), g_{sc} and g_m were assumed to be 0.14 and 0.50 $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively (as measured for 'Dash'); in (b) g_{sc} and g_m were assumed to be 0.33 and 0.40 $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively (as measured for GP4); in (c) g_{sc} and g_m were assumed to be 0.09 and 0.16 $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively (as measured for HB4). Rates of photosynthesis were estimated assuming g_m and g_{sc} as measured at 380 $\mu\text{mol mol}^{-1}$ (A_n), assuming infinite g_m and g_{sc} as measured (A_{mi}), and assuming g_m as measured and infinite g_{sc} (A_{si}). Eqns 2 and 3 were applied to calculate stomatal (L_s) and mesophyll (L_m) limitations to photosynthesis for each example. Also presented is estimated transpiration efficiency (TE, using assumptions described in the Methods section).

for 'Dash' was rather lower during the diurnal gas exchange measurements than during online ^{13}C discrimination measurements. However, the low g_s , but high A (i.e. low C_i), of 'Dash' leaves during the diurnal measurements suggests that

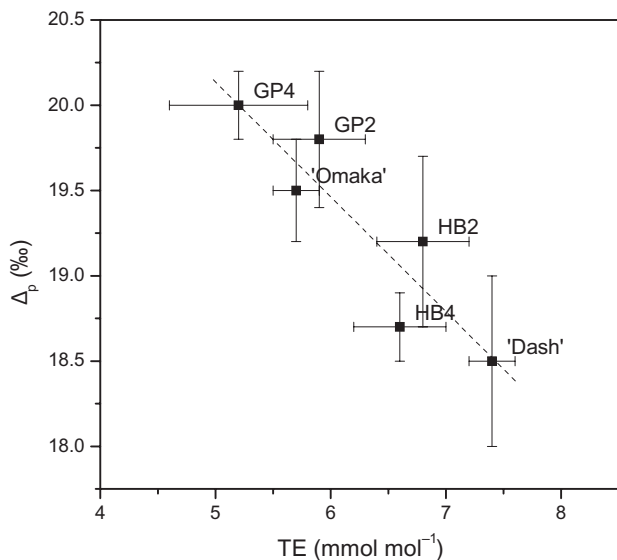


Figure 6. Relationship between average midday transpiration efficiency and photosynthetic carbon isotope discrimination as recorded in whole-leaf tissue for six barley genotypes. The line is a least squares linear regression: $\Delta_p = 23.5 - 0.7\text{TE}$, $r^2 = 0.83$, $P = 0.01$.

the ranking of g_m between *H. vulgare* genotypes was broadly similar during the two measurement conditions. This is also supported by the lowest value of carbon isotope discrimination in whole-leaf tissue for 'Dash'.

The observed differences in g_m between genotypes could result from variation in a number of leaf traits, including the surface area of chloroplasts exposed to the intercellular spaces (S_c), the mesophyll cell wall thickness, and the permeability of plasma and chloroplastic membranes. Evans *et al.* (2009) estimate that the cell wall may account for 25–50% or more of the total mesophyll resistance to CO_2 , but that changes in one component of the conduction pathway may be compensated for by other components. Future studies of genotypic variability in g_m will include measurement of cell wall thickness, specific leaf density and S_c using microscopic techniques (e.g. Evans & Vellen 1996), to assess the importance of these traits on g_m . Membrane permeability to CO_2 is challenging to measure *in situ*, and may require a modelling approach using improved permeability measurements of isolated membranes (Evans *et al.* 2009). Although not explored in this study, it seems clear that g_m is a dynamic leaf trait. A number of recent studies have found that g_m increases in response to increased irradiance and decreased CO_2 concentration (Flexas *et al.* 2007; but see Tazoe *et al.* 2009), and decreases in response to drought (Flexas *et al.* 2008 and references therein). These recent studies extend the often-observed positive relationships between g_s , A and g_m at ambient C_a across different leaves to within a single leaf (e.g. Loreto, Tsonev & Centritto 2009).

Influence of g_m on TE

g_m has a strong influence on TE. Increasing g_m will increase A without changing E , so that TE goes up. To demonstrate the effect of g_m on TE at a given C_a , we calculate TE for a range of values of g_{sc} and g_m , given a common photosynthetic response to chloroplastic CO_2 concentration (A/C_c response) and leaf boundary layer conductance. The A/C_c curve shown in Fig. 5 was used, and g_{sc} varied from 0.05 to 0.55 $mol\ m^{-2}\ s^{-1}$, with g_m of 0.1, 0.2 or 0.5 $mol\ m^{-2}\ s^{-1}$, and one-sided leaf boundary layer conductance to CO_2 of 2.03 $mol\ m^{-2}\ s^{-1}$ (that is, leaf boundary layer conductance to H_2O of 2.78 $mol\ m^{-2}\ s^{-1}$, a value typical of gas exchange within leaf chambers). The photosynthetic rate at C_a of 380 $\mu mol\ mol^{-1}$ was estimated from each constructed A/C_a curve, and transpiration rate and TE calculated assuming a leaf temperature of 23 °C and a leaf-to-air vapour pressure difference of 0.72 kPa. Figure 7 shows that, at a given g_m , increasing g_{sc} increases A and decreases TE, whereas at a given g_{sc} increasing g_m increases both A and TE. For example, if g_{sc} is 0.3 $mol\ m^{-2}\ s^{-1}$, doubling g_m from 0.1 to 0.2 $mol\ m^{-2}\ s^{-1}$ increases A by 3.5 $\mu mol\ m^{-2}\ s^{-1}$, which increases TE by 1.2 $mmol\ mol^{-1}$ or 24%. Stated another way, and demonstrated in Fig. 7, the same TE may be achieved at a higher g_{sc} if g_m is increased, because of increased A .

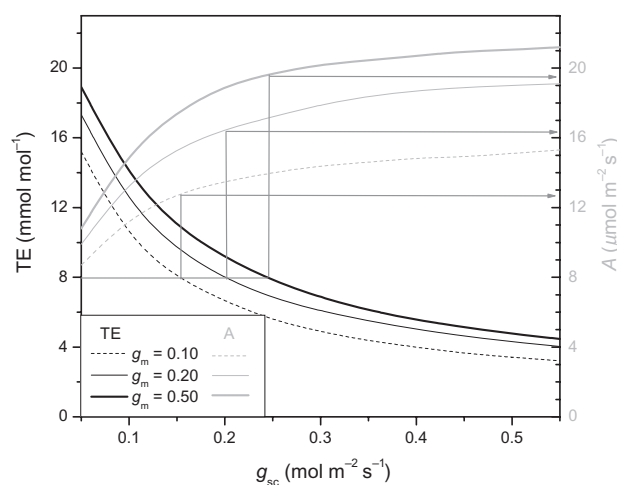


Figure 7. Demonstration of the influence of mesophyll conductance (g_m) on transpiration efficiency (TE; black lines) and photosynthetic rate (A ; light grey lines) for a given photosynthetic biochemistry. To generate these relationships, a single A/C_c curve was used and g_{sc} and g_m varied independently in order to calculate photosynthetic responses to C_i and C_a . Photosynthetic rate at $C_a = 380\ \mu mol\ mol^{-1}$ was estimated from the A/C_a curve, and transpiration rate calculated assuming constant (one-sided) leaf boundary layer conductance to CO_2 of 1.74 $mol\ m^{-2}\ s^{-1}$. With the given A/C_c response, a leaf can produce a TE of 8 $mmol\ mol^{-1}$ (thin grey lines) with g_{sc} of between 0.15 and 0.25 $mol\ m^{-2}\ s^{-1}$ combined with A of between 12.7 and 19.5 $\mu mol\ m^{-2}\ s^{-1}$, depending on g_m .

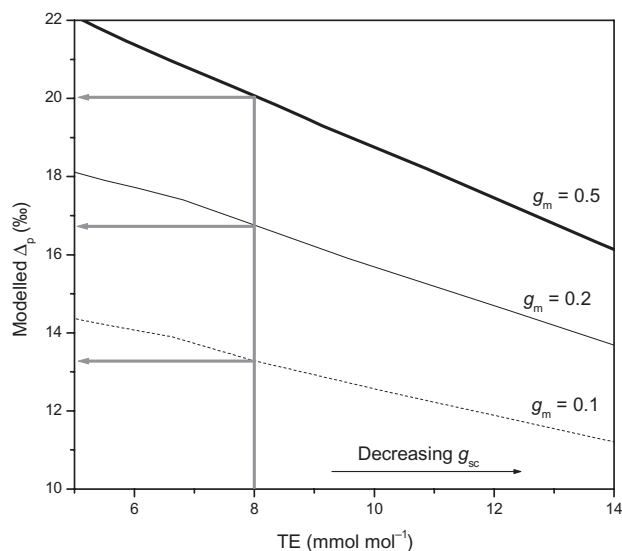


Figure 8. Demonstration of the influence of mesophyll conductance (g_m) on photosynthetic carbon isotope discrimination (Δ_p ; assuming no post-photosynthetic fractionation). Δ_p was estimated from Eqn 8 (a simplified version of the full model, assuming fractionation during diffusion through the stomata is 4.4‰ and fractionation during carboxylation is 28‰) for all combinations of g_{sc} and g_m calculated in Fig. 8. Note that at TE = 8 $mmol\ mol^{-1}$, modelled Δ_p increases by almost 7‰ as g_m increases from 0.1 to 0.5 $mol\ m^{-2}\ s^{-1}$.

Influence of g_m on Δ_p , and suitability of Δ_p to select for TE

g_m not only affects TE, but also affects photosynthetic carbon isotope discrimination (Δ_p) independently of TE. This means that if g_m is variable between plants, or varies with environmental conditions for an individual plant, there will be no unique relationship between Δ_p and TE. To demonstrate the influence of g_m on the relationship between TE and Δ_p , we calculated Δ_p for each combination of g_{sc} and g_m with a common A/C_c curve as earlier, using a simplified discrimination model:

$$\Delta_i = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + b \frac{C_i}{C_a} - f \frac{\Gamma^*}{C_a} - e' \frac{R_d}{A + R_d} \frac{C_i - \Gamma^*}{C_a} \quad (11)$$

where a and b are assumed to be 4.4 and 28‰, respectively. Eqn 11 assumes leaf boundary layer and respiratory effects are constant (at zero) as g_{sc} and g_m change.

Figure 8 shows that when TE is 8 $mmol\ mol^{-1}$, Δ_p increases from 13.2 to 20.0‰ as g_m increases from 0.1 to 0.5 $mol\ m^{-2}\ s^{-1}$. Such a huge effect suggests that Δ_p may not be a reliable marker for TE. However, a strong negative relationship was found between TE and Δ_p in this study, and between Δ_p and TE or WUE in a large number of previous studies (Brugnoli & Farquhar 2000, and references therein). Previous work in barley has also reported a strong negative relationship between Δ_p and WUE (Hubick & Farquhar

1989), and that the ranking of Δ_p was maintained over a wide range of growth environments in the field (Anyia *et al.* 2007). Understanding of the genetic basis for variability in TE in crop plants is in its infancy (but see Masle, Gilmore & Farquhar 2005), but Handley *et al.* (1994) reported that a region on chromosome 4 controls at least some of the observed variation in Δ_p in barley. The fact that Δ_p has been shown to be a useful indicator of TE (even providing the basis for breeding work in the release of two commercial wheat cultivars; Condon *et al.* 2004) indicates the likelihood of a strong correlation between g_{sc} and g_m , or that both g_{sc} and g_m scale with maximum carboxylation rate. Indeed, it seems likely that selection for both low Δ_p (for improved WUE) and high Δ_p (for improved productivity in irrigated systems) has inadvertently selected for high g_m . g_m has not been included as a selection trait in breeding programmes, but combined selection for low Δ_p and high g_m is likely to accelerate progress in TE improvement. We propose that **g_m has unexplored potential for improving TE and productivity.**

Scaling TE

The significant variability in g_m found here among *H. vulgare* genotypes suggests that breeding for higher g_m has the potential to increase TE. However, ranking in g_m between genotypes in controlled conditions must remain under a range of growth conditions, and at the crop scale in the field, for g_m to be a suitable trait for TE improvement. Further controlled-environment testing at a range of RH and soil water availability is required. A field trial of the contrasting commercial cultivars 'Dash' (high g_m , low g_{sc} and high TE) and 'Omaka' (lower g_m , high g_{sc} and lower TE) would also be valuable.

The translation of increased TE into increased WUE depends on allocation of photosynthate to the harvested plant organ, but also on the prevailing environmental conditions and the developmental stage of the crop canopy. If the leaf is one of many in a closed-canopy situation, the air surrounding the leaf will become cooler and moister as transpiration increases. Cowan & Troughton (1971) have shown theoretically that WUE may actually increase with increasing stomatal conductance if carboxylation capacity is high and the crop boundary layer conductance is low (e.g. in a dense, aerodynamically smooth canopy with high stomatal conductance). It should be noted that the influence of g_m on WUE is largely unaffected by the crop boundary layer conductance, so it seems likely that selecting for high g_m to improve TE will also improve WUE (so long as allocation of carbon to the harvested plant organ is not reduced).

Existing crop exchange and growth models (e.g. Garcia *et al.* 1998) need to be updated to include the new understanding of g_m and TE gained in recent studies. Flexas *et al.* (2007) have demonstrated that V_{cmax} , a basic photosynthetic parameter used in many carbon and water balance models, is underestimated when g_m is finite, and may be significantly underestimated when g_m is low under drought conditions. A modelling study would be valuable to assess the impact of

changes in the balance between stomatal and mesophyll limitations to photosynthesis on TE, WUE and crop growth.

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REFERENCES

- Acevedo E. (1993) Potential of carbon isotope discrimination as a selection criterion in barley breeding. In *Stable Isotopes and Plant Carbon-Water Relations* (eds J.R. Ehleringer, A.E. Hall & G.D. Farquhar), pp. 399–417. Academic Press, San Diego, SC, USA.
- Anyia A.O., Slaski J.J., Nyachiro J.M., Archambault D.J. & Juskiw P. (2007) Relationship of carbon isotope discrimination to water use efficiency and productivity of barley under field and greenhouse conditions. *Journal of Agronomy & Crop Science* **193**, 313–323.
- Barbour M.M., McDowell N.G., Tcherkez G., Bickford C.P. & Hanson D.T. (2007) A new measurement technique reveals rapid post-illumination changes in the carbon isotope composition of leaf-respired CO₂. *Plant, Cell & Environment* **30**, 469–482.
- Bickford C.P., McDowell N.G., Erhardt E.B. & Hanson D.T. (2009) High-frequency field measurements of diurnal carbon isotope discrimination and internal conductance in a semi-arid species, *Juniperus monosperma*. *Plant, Cell & Environment* **32**, 796–810.
- Brugnoli E. & Farquhar G.D. (2000) Photosynthetic fractionation of carbon isotopes. In *Photosynthesis: Physiology and Metabolism* (eds R.C. Leegood, T.D. Sharkey & S. von Caemmerer), pp. 399–434. Kluwer, Norwell, MA, USA.
- Cernusak L.A., Winter K., Aranda J. & Turner B.L. (2008) Conifers, angiosperm trees, and lianas: growth, whole-plant water and nitrogen use efficiency, and stable isotope composition ($\delta^{13}C$ and $\delta^{18}O$) of seedlings grown in a tropical environment. *Plant Physiology* **148**, 642–659.
- Condon A.G., Richards R.A., Rebetzke G.J. & Farquhar G.D. (2004) Breeding for high water-use efficiency. *Journal of Experimental Botany* **55**, 2447–2459.
- Cowan I.I. & Troughton J.H. (1971) The relative role of stomata in transpiration and assimilation. *Planta* **97**, 323–336.
- Evans J.R. (1999) Leaf anatomy enables more equal access to light and CO₂ between chloroplasts. *New Phytologist* **143**, 93–104.
- Evans J.R. & Vellen L. (1996) Wheat cultivars differ in transpiration efficiency and CO₂ diffusion inside their leaves. In *Crop Research in Asia: Achievements and Perspective* (eds R. Ishii & T. Horie), pp. 326–329. Asian Crop Science Association, Fukui, Japan.
- Evans J.R. & von Caemmerer S. (1996) CO₂ diffusion inside leaves. *Plant Physiology* **110**, 339–346.
- Evans J.R., Sharkey T.D., Berry J.A. & Farquhar G.D. (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO₂ diffusion in leaves of higher plants. *Australian Journal of Plant Physiology* **13**, 281–292.
- Evans J.R., von Caemmerer S., Setchell B.A. & Hudson G.S. (1994) The relationship between CO₂ transfer conductance and leaf

- anatomy in transgenic tobacco with a reduced content of Rubisco. *Australian Journal of Plant Physiology* **21**, 475–495.
- Evans J.R., Kaldenhoff R., Genty B. & Terashima I. (2009) Resistances along the CO₂ diffusion pathway inside leaves. *Journal of Experimental Botany* **60**, 2235–2248.
- Farquhar G.D. & Richards R.A. (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Australian Journal of Plant Physiology* **11**, 539–552.
- Farquhar G.D. & Sharkey T.D. (1982) Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology* **33**, 317–345.
- Farquhar G.D., O'Leary M.H. & Berry J.A. (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* **9**, 121–137.
- Farquhar G.D., Hubick K.T., Condon A.G. & Richards R.A. (1989) Carbon isotope fractionation and plant water-use efficiency. In *Stable Isotopes in Ecological Research* (eds P.W. Rundel, J.R. Ehleringer & K.A. Nagy), pp. 21–40. Springer-Verlag, New York, USA.
- Flexas J., Diaz-Espejo A., Galmés J., Kaldenhoff R., Medrano H. & Ribas-Carbo M. (2007) Rapid variations of mesophyll conductance in response to changes in CO₂ concentration around leaves. *Plant, Cell & Environment* **30**, 1284–1298.
- Flexas J., Ribas-Carbo M., Diaz-Espejo A., Galmés J. & Medrano H. (2008) Mesophyll conductance to CO₂: current knowledge and future prospects. *Plant, Cell & Environment* **31**, 602–621.
- Flexas J., Galmés J., Gallé A., Gulias J., Pou A., Ribas-Carbo M., Tomás M. & Medrano H. (2010) Improving water use efficiency in grapevines: potential physiological targets for biotechnological improvement. *Australian Journal of Grape and Wine Research* **16**, 106–121.
- Flowers M.D., Fiscus E.L., Burkey K.O., Booker F.L. & Dubois J.-J.B. (2007) Photosynthesis, chlorophyll fluorescence, and yield of snap bean (*Phaseolus vulgaris* L.) genotypes differing in sensitivity to ozone. *Environmental & Experimental Botany* **61**, 190–198.
- Forster B.P. (2001) Mutation genetics of salt-tolerance in barley: An assessment of Golden Promise and other semi-dwarf mutants. *Euphytica* **120**, 317–328.
- García R.L., Long S.L., Wall G.W., Osbourne C.P., Kimball B.A., Nie G.Y., Pinter P.J., Lamorte R.L. & Wechsung F. (1998) Photosynthesis and conductance of spring-wheat leaves: field response to continuous free-air atmospheric CO₂ enrichment. *Plant, Cell & Environment* **21**, 659–669.
- Handley L.L., Nevo E., Raven J.A., Martínez-Carrasco R., Scrimgeour C.M., Pakniyat H. & Forster B.P. (1994) Chromosome 4 controls potential water use efficiency ($\delta^{13}\text{C}$) in barley. *Journal of Experimental Botany* **45**, 1661–1663.
- Hubick K.T. & Farquhar G.D. (1989) Carbon isotope discrimination and the ratio of carbon gain to water lost in barley cultivars. *Plant, Cell & Environment* **12**, 795–804.
- Hubick K.T., Farquhar G.D. & Shorter R. (1986) Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (*Arachis*) germplasm. *Australian Journal of Plant Physiology* **13**, 803–816.
- Lanigan G., Betson N., Griffiths H. & Seibt U. (2009) Carbon isotope fractionation during photorespiration and carboxylation in *Senecio*. *Plant Physiology* **148**, 2013–2020.
- Lauteri M., Scartazza M., Guido M.C. & Brugnoli E. (1997) Genetic variation in photosynthetic capacity, carbon isotope discrimination and mesophyll conductance in provenances of *Castanea sativa* adapted to different environments. *Functional Ecology* **11**, 675–683.
- Loreto F., Tsonev T. & Centritto M. (2009) The impact of blue light on mesophyll conductance. *Journal of Experimental Botany* **60**, 2283–2290.
- Masle J., Gilmore S.R. & Farquhar G.D. (2005) The ERECTA gene regulates plant transpiration efficiency in *Arabidopsis*. *Nature* **436**, 866–870.
- Otto F. (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. *Methods in Cell Biology* **33**, 105–110.
- Perez-Martin A., Flexas J., Ribas-Carbo M., Bota J., Tomás M., Infante J.M. & Diaz-Espejo A. (2009) Interactive effects of soil water deficit and air vapour pressure deficit on mesophyll conductance to CO₂ in *Vitis vinifera* and *Olea europaea*. *Journal of Experimental Botany* **60**, 2391–2405.
- Pons T.L., Flexas J., von Caemmerer S., Evans J.R., Genty B., Ribas-Carbo M. & Brugnoli E. (2009) Estimating mesophyll conductance to CO₂: methodology, potential errors, and recommendations. *Journal of Experimental Botany* **60**, 2217–2234.
- R Development Core Team (2009) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>.
- Rebetzke G.J., Condon A.G., Richards R.A. & Farquhar G.D. (2002) Selection for reduced carbon isotope discrimination increases aerial biomass and grain yield of rainfed bread wheat. *Crop Science* **42**, 739–745.
- Richards R.A., Rebetzke G.J., Condon A.G. & van Herwaarden A.F. (2002) Breeding opportunities for increasing the efficiency of water use and crop yield in temperate cereals. *Crop Science* **42**, 111–121.
- Soolanayakanahally R.Y., Guy R.D., Silim S.N., Drewes E.C. & Schroeder W. (2009) Enhanced assimilation rate and water use efficiency with latitude through increased photosynthetic capacity and internal conductance in balsam poplar (*Populus balsamifera* L.). *Plant, Cell & Environment* **32**, 1821–1832.
- Tazoe Y., von Caemmerer S., Badger M.R. & Evans J.R. (2009) Light and CO₂ do not affect the mesophyll conductance to CO₂ diffusion in wheat leaves. *Journal of Experimental Botany* **60**, 2291–2301.
- Uehlein N., Otto B., Hanson D.T., Fischer M., McDowell N. & Kaldenhoff R. (2008) Function of *Nicotiana tabacum* aquaporins as chloroplast gas pores challenges the concept of membrane CO₂ permeability. *The Plant Cell* **20**, 648–657.
- Warren C.R. (2006) Estimating the internal conductance to CO₂ movement. *Functional Plant Biology* **33**, 431–442.
- Warren C.R. & Adams M.A. (2006) Internal conductance does not scale with photosynthetic capacity: implications for carbon isotope discrimination and the economics of water and N use in photosynthesis. *Plant, Cell & Environment* **29**, 192–201.
- Warren C.R. & Dreyer E. (2006) Temperature response of photosynthesis and internal conductance to CO₂: results from two independent approaches. *Journal of Experimental Botany* **57**, 3057–3067.
- Warren C.R., Ethier G.J., Livingston N.J., Grant N.J., Turpin D.H., Harrison D.L. & Black T.A. (2003) Transfer conductance in second growth Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) canopies. *Plant, Cell & Environment* **26**, 1215–1227.
- Wingate L., Seibt U., Moncrieff J.B., Jarvis P.G. & Lloyd J. (2007) Variations in C-13 discrimination during CO₂ exchange by *Picea sitchensis* branches in the field. *Plant, Cell & Environment* **30**, 600–616.

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