

# Theoretical reconsiderations when estimating the mesophyll conductance to CO<sub>2</sub> diffusion in leaves of C<sub>3</sub> plants by analysis of combined gas exchange and chlorophyll fluorescence measurements

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## ABSTRACT

Existing methods to estimate the mesophyll conductance to CO<sub>2</sub> diffusion ( $g_m$ ) are often based on combined gas exchange and chlorophyll fluorescence measurements. However, estimations of average  $g_m$  by these methods are often unreliable either because the range of usable data is too narrow or because the estimations are very sensitive to measurement errors. We describe three method variants to estimate  $g_m$ , for which a wider range of data are usable. They use curve-fitting techniques, which minimise the sum of squared model deviations from the data for  $A$  (CO<sub>2</sub> assimilation rate) or for  $J$  (linear electron transport rate). Like the existing approaches, they are all based on common physiological principles assuming that electron transport limits  $A$ . The proposed variants were far less sensitive than the existing approaches to ‘measurement noise’ either created randomly in the generated data set or inevitably existing in real data sets. Yet, the estimates of  $g_m$  from the three variants differed by approximately 15%. Moreover, for each variant, a stoichiometric uncertainty in linear electron transport-limited photosynthesis can cause another 15% difference. Any estimation of  $g_m$  using gas exchange and chlorophyll fluorescence measurements should be considered with caution, especially when  $g_m$  is high.

**Key-words:** CO<sub>2</sub> transfer; diffusion resistance; internal conductance; photosynthesis.

## INTRODUCTION

Net photosynthetic rate ( $A$ ) in leaves of C<sub>3</sub> species strongly depends on the CO<sub>2</sub> level. For CO<sub>2</sub> fixation to occur in a leaf, CO<sub>2</sub> from atmosphere must diffuse into the intercellular airspace of the leaf and then into the site of the carboxylation in the chloroplasts. Methods to estimate the intercellular CO<sub>2</sub> partial pressure ( $C_i$ ) have been well established, based on gas exchange (GE) measurements, whereas those to estimate the CO<sub>2</sub> partial pressure at Rubisco

carboxylation sites ( $C_c$ ) are less certain. From Fick’s first law of diffusion, the relation between  $A$ ,  $C_i$  and  $C_c$  is expressed as

$$A = g_m (C_i - C_c), \quad (1)$$

where  $g_m$  is the mesophyll diffusion conductance. Therefore,  $g_m$  affects the drawdown from  $C_i$  to  $C_c$ , which can be used to analyse photosynthetic limitation by mesophyll diffusion (e.g. Niinemets *et al.* 2009a).

Various methods have been developed to estimate  $g_m$  (for reviews, see Warren 2006; Flexas *et al.* 2008; Pons *et al.* 2009). Of these methods, the chlorophyll fluorescence (CF)-based methods (Harley *et al.* 1992) have been widely used. In these methods, the rate of linear electron ( $e^-$ ) transport (LET) in support of CO<sub>2</sub> reduction and photorespiration is estimated from the CF signals for the photosystem II (PSII) operating efficiency  $\Delta F/F'_m$  (Genty, Briantais & Baker 1989), where  $F'_m$  is fluorescence yield during a saturating pulse of light, and  $\Delta F$  is the difference between  $F'_m$  and the steady-state fluorescence yield. When the rate of LET,  $J$ , is known,  $g_m$  can be estimated from Eqn 1 in combination with the model of Farquhar, von Caemmerer & Berry (1980) (the FvCB model) for the  $e^-$  transport-limited  $A$  of C<sub>3</sub> photosynthesis:

$$A = \frac{C_c - \Gamma_*}{4(C_c + 2\Gamma_*)} J - R_d, \quad (2)$$

where  $\Gamma_*$  is the  $C_c$ -based CO<sub>2</sub> compensation point in the absence of day respiration ( $R_d$ ) – the respiratory CO<sub>2</sub> release in the light other than by photorespiration.

The FvCB model employs a different equation to describe the Rubisco-limited  $A$ ; so the use of Eqn 2 to estimate  $g_m$  can, strictly speaking, be applied only to the  $e^-$  transport-limited range. However, the variable  $J$  method, one of the two well-known methods to estimate  $g_m$  (Harley *et al.* 1992), was suggested to be applied to the Rubisco-limited range (Long & Bernacchi 2003), where  $J$  is varying, implicitly assuming that the required down-regulation of LET by Rubisco limitations can be fully mirrored by  $\Delta F/F'_m$  signals. In this method,  $C_c$  in Eqn 2 is replaced with

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$(C_i - A/g_m)$ , and rearranging the terms gives (Di Marco *et al.* 1990; Harley *et al.* 1992)

$$g_m = \frac{A}{C_i - \frac{\Gamma_*[J/4 + 2(A + R_d)]}{J/4 - (A + R_d)}} \quad (3)$$

By using Eqn 3,  $g_m$  can be calculated at each  $C_i$  if  $R_d$  and  $\Gamma_*$  are known. So, this method can be used to examine whether  $g_m$  varies with the  $C_i$  level for a wide range of conditions. Using this method, Flexas *et al.* (2007) found that  $g_m$  varied with  $C_i$  and light levels, resembling  $\text{CO}_2$  and light responses of stomatal conductance.

The other method described by Harley *et al.* (1992), based on the analysis of Bongi & Loreto (1989), is the constant J method. For  $g_m$  to be estimated, a test value of  $g_m$  is used in the equation obtained from combining Eqns 1 and 2 and solving for J:

$$J = (A + R_d) \frac{4(C_i - A/g_m + 2\Gamma_*)}{C_i - A/g_m - \Gamma_*} \quad (4)$$

This is carried out for all points within the range of an  $A-C_i$  curve, usually the  $e^-$  transport-limited range in which the CF data indicate that J is constant. The calculated J values are then used for estimating its variance. The test value of  $g_m$  that gives the minimum variance is the best estimate of  $g_m$ . In view of the recent reports (Flexas *et al.* 2007; Yin *et al.* 2009b) on the  $\text{CO}_2$ -dependent variation,  $g_m$  estimated by this method should be considered as the average value over the  $C_i$  range used. Clearly, the constant J method is based on a number of GE measurements, whereas the CF measurements are not explicitly used to estimate  $g_m$ . So, it can be argued that the constant J method is not a real CF-based method. Values of  $g_m$  estimated by the constant J method sometimes disagreed significantly with those estimated by the variable J method (Bunce 2008).

Assessing whether  $g_m$  indeed varies with  $\text{CO}_2$  and light levels, as reported by, for example, Flexas *et al.* (2007), still needs further studies, as highlighted in several recent papers on  $g_m$  (e.g. Pons *et al.* 2009). It is, nevertheless, important to reliably estimate the average  $g_m$  over certain ranges of conditions in order to examine, for example, how  $g_m$  responds to environmental events such as water, salinity and nutrient stress. A method to estimate the 'average'  $g_m$ , exploring the curvature of an  $A-C_i$  curve, was introduced by Ethier & Livingston (2004), which was followed by a similar method by Sharkey *et al.* (2007). These methods fit the data of an  $A-C_i$  curve to estimate a number of parameters (including  $g_m$ ) in the full FvCB model (also see Niinemets *et al.* 2005; Miao *et al.* 2009), so no CF information is used and there is a risk that  $g_m$  and other parameters do not have good statistical properties when they are simultaneously estimated only from GE data (Dubois *et al.* 2007). One way to estimate an average  $g_m$  based on CF measurements is to use the variable J method to calculate  $g_m$  values at several  $C_i$  and to choose the arithmetic average of  $g_m$  across these  $C_i$  (e.g. Loreto *et al.* 1992;

Niinemets *et al.* 2005), although the method is typically based on single-point data. However, measurement 'noise' in GE and CF data easily affects the estimation of  $C_i$ -specific  $g_m$  made by the variable J method: any negative or unbelievably high  $g_m$  value at particular  $C_i$  can have a strong impact on the arithmetic average. Therefore, Harley *et al.* (1992) calculated  $dC_c/dA = 12\Gamma_*J/[J - 4(A + R_d)]$  and set an empirical restriction that any measurements in which  $dC_c/dA$  is  $<10$  or  $>50$  are unreliable. This restriction may eliminate the use of the method at high  $C_i$  levels, where  $dC_c/dA$  is often  $>50$ , or at low  $C_i$  levels, where  $dC_c/dA$  is often  $<10$  (see fig. 7 of Harley *et al.* 1992 and figs 3 & 4 of Flexas *et al.* 2007). Harley *et al.* conceded, however, that there are data points outside this acceptable range that also give a realistic value for  $g_m$ .

Epron *et al.* (1995) and Evans & von Caemmerer (1996) described a variant of the CF-based method to directly estimate an average  $g_m$ . By using J estimated from CF measurements,  $C_c$  is calculated from rearranging Eqn 2 as

$$C_c = \frac{\Gamma_*[J/4 + 2(A + R_d)]}{J/4 - (A + R_d)} \quad (5)$$

According to Eqn 1, the inverse of the slope for the plot of  $(C_i - C_c)$  against A, with forcing the regression line through the origin, gives the estimate of  $g_m$ . As the data upon which  $g_m$  is estimated are values of  $(C_i - C_c)$ , we shall call this the  $C_iC_c$  variant. This variant, as will be shown later, is highly sensitive to errors in measured A or J.

Clearly, the above-existing  $g_m$ -estimation approaches are all based on Eqn 2. Equation 2 assumes that it is the insufficient supply of NADPH that causes  $e^-$  transport limitation (Farquhar & von Caemmerer 1982). Chloroplasts may exhibit some plasticity, so that NADPH and ATP co-limit the photosynthetic efficiency (e.g. Allen 2003; Avenson *et al.* 2005). However, there are uncertainties regarding the efficiency of ATP production in thylakoid reactions. Other cellular processes may also produce or consume ATP, which can contribute to the balance of NADPH and ATP as required for carbon reduction and photorespiration. Any impact of these uncertainties on the estimation of  $g_m$  has not received sufficient attention in the literature.

The objective of this paper is twofold. Firstly, three alternative ways to estimate an average  $g_m$  from combined GE and CF measurements will be described. Like the existing approaches, they are based on the similar physiological principles, are rooted in the same equations (i.e. Eqns 1 & 2) and use the same type of input parameters and data. So we will consider them not as really new methods but as 'variants' of the CF-based method. Merits of these variants, compared with the standard existing approaches, will be assessed by using data of  $e^-$  transport-limited range, within which Eqn 2 of the FvCB model is valid. Secondly, the impact of stoichiometric uncertainties for linear  $e^-$  transport-limited photosynthesis on the estimated  $g_m$  will be examined.

## MODEL AND THEORY

### The modified J variant

This variant is similar to the constant J method. However, instead of assessing the variance as used in the constant J method, Eqn 4 is used as the model for non-linear curve fitting, minimising the sum of squared model deviations from the data for  $J$  based on CF measurements. Thus, unlike the standard constant J method, this variant absolutely requires the CF data, in addition to GE data for  $A$ . We shall call it the modified J variant, also in recognition that the data to be fitted are  $J$ , the flux of LET.

Among others Pons *et al.* (2009) discussed both variable and constant J methods, proposing least-squares fitting procedures with which  $g_m$  can be solved iteratively with another parameter. This extended procedure is very similar to our modified J variant, but differences, albeit not fundamental, exist. Unlike the standard or extended constant J methods, there is no restriction for our modified J method that it should be only applied to the portion of an  $A$ - $C_i$  curve, where  $J$  is constant; for example, it can be applied to the low-light range, where  $e^-$  transport limits photosynthesis, while  $J$  is varying. Conversely, unlike the extended variable J method, where  $g_m$  is suggested to be estimated from fitting data over the range of  $C_i$  with varying  $J$ , our modified J method can also be applied to the data range where  $J$  is constant.

### The NRH-A variant

In line with the derivation of von Caemmerer & Evans (1991) for the Rubisco-limited FvCB model, replacing  $C_c$  in Eqn 2 with  $(C_i - A/g_m)$  and then solving for  $A$  give

$$A = 0.5 \left\{ \frac{J/4 - R_d + g_m(C_i + 2\Gamma_*) - \sqrt{[J/4 - R_d + g_m(C_i + 2\Gamma_*)]^2 - 4g_m[(C_i + 2\Gamma_*)J/4 - R_d(C_i + 2\Gamma_*)]}}{4g_m[(C_i + 2\Gamma_*)J/4 - R_d(C_i + 2\Gamma_*)]} \right\}. \quad (6)$$

Mathematically, Eqn 6 is the inverse function of Eqn 4. Equation 6 provides a model to estimate  $g_m$  by curve fitting, using a non-linear least squares regression that minimises the difference between measured and estimated  $A$ . Ethier & Livingston (2004) and Ethier *et al.* (2006) used Eqn 6 together with its equivalent for the Rubisco-limited range, in which  $g_m$  and  $J$  and  $V_{cmax}$  (the Rubisco carboxylation capacity) are estimated only from data of an  $A$ - $C_i$  curve (also see Niinemets *et al.* 2005, 2009b), so no CF measurements were used in their method. Here,  $J$  at each  $C_i$  is derived from CF measurements as input to Eqn 6. As Eqn 6 has a non-rectangular hyperbolic form and the data to be fitted are  $A$ , this procedure is termed the NRH-A variant.

### The RH-A variant

As for the NRH-A variant,  $C_c$  in Eqn 2 is replaced with  $(C_i - A/g_m)$ , but it does not follow in solving for  $A$ . Thus,  $A$  is given as

$$A = \frac{C_i - A/g_m - \Gamma_*}{4(C_i - A/g_m + 2\Gamma_*)} J - R_d. \quad (7)$$

Although Eqn 7 is circular,  $g_m$  can be estimated by minimising the sum of squared model deviations from the data for  $A$ , using observed data for  $A$  *per se* as input to the left part of the model in curve fitting. Dubois *et al.* (2007) and Sharkey *et al.* (2007) discussed the use of Eqn 7, together with its equivalent for the Rubisco-limited range to estimate up to five parameters of the FvCB model, including  $g_m$  (also see Miao *et al.* 2009), from only GE data, that is, an  $A$ - $C_i$  curve. Here,  $J$  in Eqn 7 comes from CF measurements. As Eqn 7 maintains the rectangular hyperbolic form of the original FvCB model and the data to be fitted are  $A$ , it is called the RH-A variant.

### Implications of stoichiometric uncertainties of LET for the estimation of $g_m$

In chloroplasts, NADPH and ATP supply may be balanced to co-limit photosynthetic efficiencies (e.g. Allen 2003; Yin, van Oijen & Schapendonk 2004; Avenson *et al.* 2005; Yin, Harbinson & Struik 2006), for example, via alternative  $e^-$  transfer pathways. However, the efficiency of ATP production is uncertain at present because neither the number of  $H^+$  required to produce one ATP (i.e.  $H^+/ATP$  ratio) nor the extent of the Q-cycle activity (that affects the  $H^+/e^-$  ratio) is known with certainty (von Caemmerer 2000). Yin *et al.* (2004, 2006) described a generalised model to analyse these uncertainties. Various forms of the model for  $e^-$  transport-limited photosynthesis can be obtained from the generalised model. Equation 2 is the special case, in which the supply of NADPH is limiting, so that a deficit of ATP (if  $H^+/ATP = 3$ ) can be removed either from a partial Q-cycle activity or from some ATP import if the Q-cycle does not operate at all (then  $H^+/e^- = 2$ ).

Given the fact that the LET alone combined with the full operation of the Q-cycle can generate the exact NADPH/ATP ratio of 2:3 required by the carbon reduction cycle, it is now generally believed that  $H^+/ATP = 4$  (Steigmiller, Turina & Gräber 2008) and that the full Q-cycle ( $H^+/e^- = 3$ ) is required (e.g. von Caemmerer 2000; Sacksteder *et al.* 2000; Laisk *et al.* 2006). According to this contemporary stoichiometry without cyclic  $e^-$  transport around photosystem I, the new equation for the  $e^-$  transport-limited  $A$  can be derived from the generalised model as (also, see von Caemmerer 2000)

$$A = \frac{C_c - \Gamma_*}{4(C_c + 7\Gamma_*/3)} J - R_d. \quad (8)$$

So, the coefficient 4 remains, but the coefficient 2 in Eqn 2 becomes 7/3 for Eqn 8 basically because oxygenation consumes slightly more ATP than carboxylation does (i.e. 3.5 versus 3, Farquhar & von Caemmerer 1982). The variable  $J$  in Eqn 8 refers to the required rate of LET associated with ATP production. The equation will change if other

stoichiometries or uncertainties (e.g. H<sup>+</sup> leakage) are applied (see Yin, Harbinson & Struik 2009a). These changes will affect the estimation of  $g_m$ , as the CF-revealed rate of e<sup>-</sup> transport through PSII can be associated with either NADPH or ATP production.

## MATERIALS AND METHODS

The modified J, NRH-A and RH-A variants were compared with the three existing approaches in estimating  $g_m$ . Required input parameters for all these approaches are the same, that is,  $R_d$  and relative CO<sub>2</sub>/O<sub>2</sub> specificity of Rubisco ( $S_{c/o}$ ) that is required to calculate  $\Gamma_*$  ( $= 0.5O/S_{c/o}$ , where  $O$  is O<sub>2</sub> partial pressure).

We first created an ideal data set of  $A-C_i$  curves of the e<sup>-</sup> transport-limited range according to Eqns 1 and 2. This is a necessary step, as real data can be afflicted by finite precision because of instrument 'noise', questionable measurement accuracy (e.g. due to leaf-cuvette leakage at high and low CO<sub>2</sub> levels) and various biological reasons that might cause data not to fit Eqn 2 (e.g. the limitation set by triose phosphate utilisation at high CO<sub>2</sub> levels or by Rubisco activity at low CO<sub>2</sub> levels). Using real data only, one cannot tell if differences between methods are a consequence of how the methods cope with finite precision or non-conformance of the data to Eqn 2. By varying  $g_m$  from 0.05 to 0.65 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> (a wide range of reported  $g_m$  values, Loreto *et al.* 1992; note that we keep the unit of  $g_m$  from that paper because it is still popularly used although it is not the SI unit) with intervals of 0.05 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>, 13 ideal  $A-C_i$  curves at 21% O<sub>2</sub> were generated, assuming that  $J = 180 \mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ ,  $R_d = 1.5 \mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$  and  $S_{c/o} = 3.12 \text{mbar } \mu\text{bar}^{-1}$ . A constant value for  $J$  was applied to these  $A-C_i$  curves because the FvCB model predicts that  $J$  depends only on irradiance at a given temperature for a given leaf. Then random noise (i.e.  $\pm 10\%$ ) in data of either  $J$  or  $C_i$  or  $A$  was superimposed to the ideal curves by using the function RAND in MS Excel (Microsoft Corporation) that generates evenly distributed, random, real numbers within the set range. Each curve had 30 points, sufficient to enable a roughly even distribution of the 'noise'. As  $g_m$  was known for each curve, the generated curves allowed to examine how the estimated  $g_m$  by each method were affected by  $\pm 10\%$  'noise' of measurements in either  $J$  or  $C_i$  or  $A$ , or the three combined. Sensitivity to the random 'noise' was assessed by the relative error of estimation (REE, %):

$$\text{REE} = \frac{\hat{g}_m - g_{m,\text{set}}}{g_{m,\text{set}}} \cdot 100, \quad (9)$$

where  $\hat{g}_m$  is the estimated  $g_m$  by an approach and  $g_{m,\text{set}}$  is the preset value of  $g_m$  used to generate the ideal curve. The mean of absolute values of REE was used to judge an overall error of estimation by each approach.

The second step was to use real data. Data on  $\Delta F/F'_m$  from CF measurements need to be converted into the rate of LET, that is,  $J$  as required for Eqn 2. We stress that  $J$

should not be simply calculated as the product of  $\Delta F/F'_m$  and the photosynthetic-pigment absorbed irradiance that is partitioned to PSII, in order to account for any alternative e<sup>-</sup> transport and measurement uncertainties (e.g. Warren 2006). Thus, data of GE and CF, both obtained under real non-photorespiratory conditions, are often used to make a calibration curve (e.g. Harley *et al.* 1992; Warren 2006; Flexas *et al.* 2007). Because the alternative e<sup>-</sup> transport does not occur at a constant fraction of total e<sup>-</sup> fluxes across various levels of incident light ( $I_{\text{inc}}$ ) or  $C_i$  over the Rubisco-limited range, such calibration should be made by using only the data over the e<sup>-</sup> transport-limited range (low  $I_{\text{inc}}$  or/and high  $C_i$ ) (Yin *et al.* 2009b), where only the basal component of the alternative e<sup>-</sup> transport exists and can be proportional to the flux of LET.

The real data sets we used came from two sources. The first was from an experiment in which  $A$  and  $\Delta F/F'_m$  were measured at 25 °C on 3–4 replicate flag leaves at two stages of wheat (*Triticum aestivum* L.) plants grown under two nitrogen (N) supply levels (Yin *et al.* 2009b). Estimates for parameters  $R_d$  and  $S_{c/o}$  and data for  $J$  were taken from that same paper. In brief,  $R_d$  was estimated as the intercept of linear regression of  $A$  against  $I_{\text{inc}}\Delta F/F'_m/4$  by using data collected under limiting light levels. The slope of this regression ( $s$ ), which lumps leaf photosynthetic absorbance, excitation partitioning to PSII and fractions of alternative e<sup>-</sup> transport together (Yin *et al.* 2009b), was derived from data under non-photorespiratory condition (i.e. 2% O<sub>2</sub> combined with a high CO<sub>2</sub> level). The estimated  $s$  was then used to calculate  $J (= sI_{\text{inc}}\Delta F/F'_m)$ .  $S_{c/o}$  at 25 °C was estimated as 3.13 mbar  $\mu\text{bar}^{-1}$ , based on the difference in the slope of the linear part of an  $A-C_i$  curve between 2% and 21% O<sub>2</sub> levels. The second data set came from Cheng, Fuchigami & Breen (2001) for leaves of 'Fuji' apple (*Malus domestica* Borkh.). The apple trees had been grown under six N regimes. The  $C_i$  response curves of  $A$  and  $\Delta F/F'_m$  were obtained at 25 °C, 21% O<sub>2</sub> and an  $I_{\text{inc}}$  of 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . For this data set,  $R_d$  was taken the same, as assumed by Cheng *et al.* (2001). A similar  $S_{c/o}$  at 25 °C (3.12 mbar  $\mu\text{bar}^{-1}$ ) was taken from Yin *et al.* (2009a) for this data set. Parameter  $s$  required to calculate  $J$  for each N level was derived from related parameters (Yin *et al.* 2009a), according to the expression for parameter  $s$  (Yin *et al.* 2009b). For both data sets, only the data at high  $C_i$  of the  $A-C_i$  curves (Cheng *et al.* 2001) or combined with low light levels of the  $A-I_{\text{inc}}$  curves (Yin *et al.* 2009b) were used for any confounding effect of the varying fraction of alternative e<sup>-</sup> transport over the Rubisco-limited range to be avoided.

For the average  $g_m$  to be estimated, their respective procedures as described in the 'Introduction' were applied to constant and variable J methods, whereas curve fitting was performed for the  $C_iC_c$ , modified J, NRH-A and RH-A variants. Although using data for the e<sup>-</sup> transport-limited range does not reflect the common practice with the variable J method, it is nevertheless useful to assess this method as well within the theoretical framework for the validity of Eqn 2 of the FvCB model. The curve fitting was carried out by using the GAUSS method in the PROC NLIN of the

SAS package (SAS Institute Inc., NC, USA). The SAS codes are given in 'Supporting Information'.

## RESULTS

Using the ideal data set without imposing  $\pm 10\%$  'noise', we first run the SAS curve-fitting programmes. The estimated  $g_m$  for this ideal data set by each variant was the same as the preset values ( $g_{m,set}$ ), indicating the validity of the fitting procedure followed.

### Estimation of $g_m$ from the generated A-C<sub>i</sub> curves

A typical pattern of  $\pm 10\%$  random 'noise' in  $J$ ,  $C_i$  and  $A$  is given in Fig. S1. The 10% noise, similar to the error percentages used by Harley *et al.* (1992), may be higher than errors common to GE or CF measurements on a single leaf but resembles variations of measured data among replicated leaves. The estimated  $g_m$  values by various approaches, in response to the 'noise' in  $J$  or  $C_i$  or  $A$ , or the three combined, are listed in Table S1.

For the variable J method, when the empirical restriction (i.e. that  $10 < dC_i/dA < 50$ ) is applied, 50–73% of data points can no longer be used, even if no 'noise' is imposed to the ideal data set (note that  $dC_i/dA$  is independent of  $C_i$ , so the percentage of data removal by the restriction in Table S1 for the case where the 'noise' was imposed on  $C_i$  is equivalent to the percentage of data removal for the ideal data). Thus, the  $g_m$  estimated by the variable J method applying the restriction differed substantially from the estimates obtained without the restriction (Table S1). For the latter case, the estimated  $g_m$  values were very often negative and extremely variable (Table 1), so they will not be discussed further. However, applying the empirical restriction did not guarantee that a good estimation of  $g_m$  was always obtained. In many cases where  $dC_i/dA$  was well between 10

and 50 and  $C_i$  were well close to the commonly used values (e.g. 300  $\mu\text{bar}$ ), Eqn 3 still gave an unrealistic  $g_m$  estimate (negative or unbelievably high or too far from  $g_{m,set}$ ), so the arithmetic average  $g_m$  can still have a strange value like  $-6.7$  (Table S1). For these reasons, the estimates by the variable J method had very high absolute REE (Table 1) and are not compared with other approaches in Fig. 1 for the pattern of REE as a function of  $g_{m,set}$ . Figure 1 shows that all approaches tended to overestimate  $g_m$  because generated noise resulted in slightly more data points above than below the ideal curve (e.g. Fig. S1).

The  $C_iC_c$  variant was also very sensitive to the random 'noise'. This sensitivity was via the calculation of  $C_c$  by Eqn 5, as reasonable values of  $g_m$  were obtained if the 'noise' was only imposed on  $C_i$  (Table S1); note that any error in  $C_i$  does not affect the calculation of  $C_c$  (see Eqn 5). When the 'noise' was imposed on variables other than  $C_i$ , an infinite or extremely low  $g_m$  was obtained (Table S1). So the absolute REE was calculated (Table 1), and the variant was compared with other approaches (Fig. 1b) only for the case where the 'noise' was imposed on  $C_i$ . Although the mean absolute REE was lowest in this case for the  $C_iC_c$  variant (Table 1), the pattern of REE in response to  $g_{m,set}$  differed from that for other approaches (Fig. 1b).

As the data for  $J$  are not explicitly used to estimate  $g_m$  by the constant J method, any random 'noise' for  $J$  alone does not have any relevance on this method, so no result is presented in Table 1 and Fig. 1a for the method if  $J$  was the only variable imposed with the random 'noise'. For other cases, the  $g_m$  estimated by this method was affected by the 'noise', as the variance all reached a minimum at a tested  $g_m$  that is not equal to the  $g_{m,set}$  used to generate the ideal curve. The estimated  $g_m$  was close to  $g_{m,set}$  when the latter is below 0.3 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>; beyond the value, the estimated  $g_m$  can deviate considerably from  $g_{m,set}$ , with a high REE (Fig. 1).

Good estimates of  $g_m$  were obtained by using the three proposed variants (Table S1) including the modified J

Variable <sup>a</sup>	Constant J method	Variable J method		$C_iC_c^f$	Modified J	NRH-A	RH-A
		Without <sup>b</sup>	With <sup>c</sup>				
$J$	– <sup>e</sup>	200.8	142.8	–	6.2	6.1	5.0
$C_i$	12.6	60.8	10.6	5.9	9.1	6.4	7.3
$A$	31.0	70.2	24.6	–	16.1	6.8	11.3
$J + C_i + A$	48.2	444.6	156.6	–	26.7	15.7	16.0
Overall <sup>d</sup>	30.6	194.1	83.7	–	14.5	8.8	9.9

<sup>a</sup>The variable to which a  $\pm 10\%$  random 'noise' was imposed.

<sup>b</sup>Arithmetic average  $g_m$  estimated by variable J method without the restriction that  $10 < dC_i/dA < 50$ .

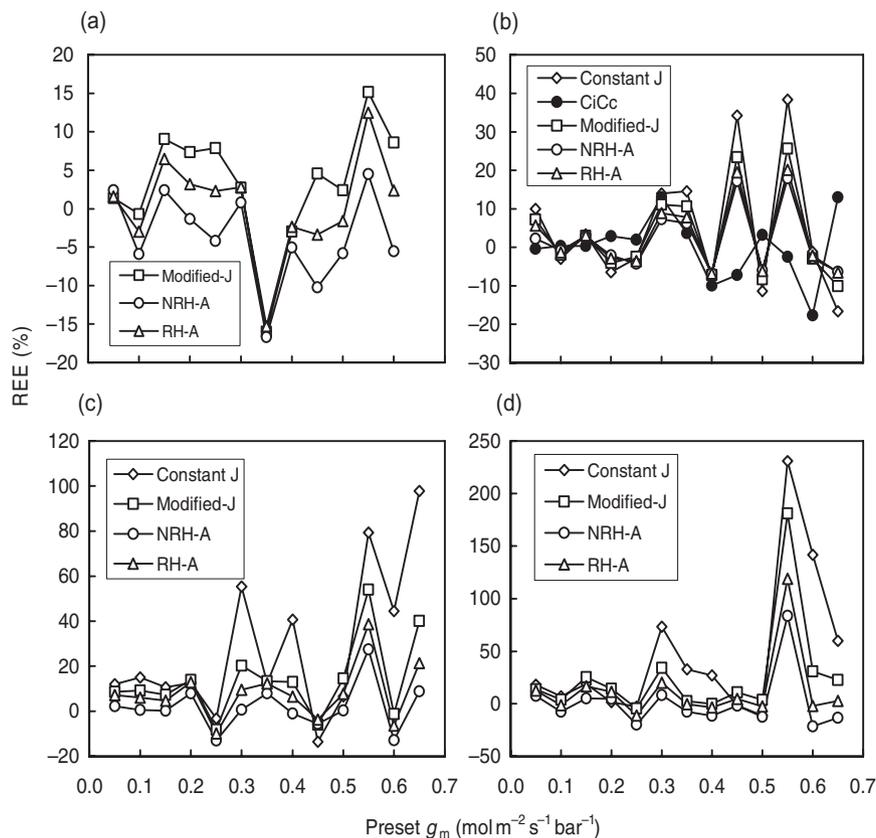
<sup>c</sup>Arithmetic average  $g_m$  estimated by variable J method with the restriction that  $10 < dC_i/dA < 50$ .

<sup>d</sup>An overall mean of the absolute REE for the four scenarios listed above it.

<sup>e</sup>A missing value because a noise in  $J$  has no relevance on the calculation of  $g_m$  by constant J method.

<sup>f</sup>Missing values for this approach because calculated  $g_m$  were often infinite (see the text and Table S1).

**Table 1.** Mean of absolute values of relative error of estimation (REE) (%) (see Eqn 9) for  $g_m$  estimated by different approaches (for values of the estimated  $g_m$ , see Table S1), using the generated data set where a  $\pm 10\%$  random 'noise' was imposed on the ideal values of  $J$  or of  $C_i$  or of  $A$  or of all the three ( $J + C_i + A$ )



**Figure 1.** Response of the estimated  $g_m$  by different approaches to  $\pm 10\%$  random ‘noise’, which was imposed to the ideal values of  $J$  (a) or of  $C_i$  (b) or of  $A$  (c) or of all the three (d), generated according to Eqns 1 and 2. The responsiveness is shown by using relative error of estimation (REE) (%) (see Eqn 9) for different values of pre-set  $g_m$  ranging from 0.05 to 0.65 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>, used to generate ideal  $A$ - $C_i$  curves. For further explanatory notes, see the text. For values of the estimated  $g_m$  by different approaches, see Table S1.

variant for which there was little or no variation of  $J$ , though, because a constant value of  $J$  was used for generating ideal  $A$ - $C_i$  curves. The estimated  $g_m$  were close to  $g_{m,set}$  (having the lower overall absolute REE than that of the existing approaches, Table 1), especially when  $g_{m,set}$  was below 0.3 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> (Fig. 1). At higher values of  $g_{m,set}$ , all became sensitive to the generated random ‘noise’, although the three proposed variants were affected to a lesser extent than the constant  $J$  method (Fig. 1). The uncertainty of the estimates when  $g_{m,set}$  is high is also reflected by high standard errors (SE) of the estimate, which were available for the variants using curve-fitting (Table S1). The highest REE values (84–230%) were obtained for the case when  $g_{m,set}$  was at 0.55 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> and the ‘noise’ was given to all  $J$ ,  $C_i$  and  $A$  (Fig. 1d). To examine whether this extreme REE was caused by abnormal values of the random numbers generated or caused by high ‘noise’ sensitivity *per se* at high  $g_m$ , the same set of random numbers was used for the case of a low  $g_{m,set}$  (i.e. 0.15 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>). The estimated  $g_m$  for such a case was 0.20(SE 0.02), 0.18(SE 0.03) and 0.19(SE 0.03) mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> (with REE being 34%, 21% and 28%) for the modified- $J$ , NRH- $A$  and RH- $A$  variants, respectively. These results indicate that all variants are more sensitive to measurement errors when the real  $g_m$  is high than when it is low. Overall, the mean absolute REE was least for the NRH- $A$  variant, then for the RH- $A$  variant, followed by the modified- $J$  variant, although the lowest mean absolute REE

was calculated for the RH- $A$  variant, when the ‘noise’ was imposed only on  $J$  (Table 1).

### Estimated $g_m$ from real $A$ - $C_i$ curves at 21% O<sub>2</sub>

For the data of Yin *et al.* (2009b) over the e<sup>-</sup> transport-limited range of  $A$ - $C_i$  curves under 21% O<sub>2</sub> condition, the estimated  $g_m$  differed among the four situations (two leaf stages  $\times$  two nitrogen levels) and between the approaches (Table 2). In the case of using the constant  $J$  method, as the calculated variance monotonically decreased with the test value of  $g_m$  for all the four situations, the  $g_m$  estimated by this method should be considered as infinite (so not shown in Table 2). For all other approaches, a finite  $g_m$  was obtained for the wheat data set. However, the constant  $J$  method did give finite  $g_m$  values across N levels for the apple data set of Cheng *et al.* (2001) (Table 3).

For the variable  $J$  method, if the restriction that  $10 < dC_i/dA < 50$  is applied, a large portion of data points cannot be used (Tables 2 & 3) because the obtained  $dC_i/dA$  is above 50. For ‘2WA-N0’ leaves (see Table 2 for the codes) of wheat and for the lowest N level of apple,  $dC_i/dA$  was  $>50$  for all data points, and, thus, no estimate was obtained by the method if the restriction was applied (Tables 2 & 3). The  $g_m$  estimated while using the restriction often differed from the estimate without the restriction. The estimated  $g_m$  without the restriction was highest for the ‘2WA-N0’ leaves of wheat (0.303, Table 2) and the two highest N levels for

**Table 2.** Value of  $g_m$  (mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>, standard error of the estimate in brackets if applicable) estimated by five approaches (see the texts for their description) for flag leaves of wheat plants at flowering (FLW) and 2 weeks after flowering (2WA) grown at low nitrogen (N0) and high nitrogen (N1) levels

Leaves	Leaf N (g m <sup>-2</sup> )	Variable J method			C <sub>i</sub> C <sub>c</sub>	Modified J	NRH-A	RH-A
		Without <sup>a</sup>	With <sup>b</sup>	% excl <sup>c</sup>				
FLW-N0	1.58	0.070	0.070	16.7	0.053 (0.009)	0.105 (0.012)	0.088 (0.013)	0.101 (0.013)
FLW-N1	2.23	0.103	0.114	41.7	0.065 (0.012)	0.145 (0.022)	0.125 (0.022)	0.138 (0.023)
2WA-N0	0.88	0.303	–	100	0.047 (0.011)	0.096 (0.039)	0.082 (0.034)	0.090 (0.038)
2WA-N1	1.95	0.078	0.105	75.0	0.059 (0.013)	0.121 (0.034)	0.105 (0.031)	0.115 (0.034)

<sup>a</sup>Arithmetic average  $g_m$  estimated by variable J method without the restriction that  $10 < dC_j/dA < 50$ ;

<sup>b</sup>Arithmetic average  $g_m$  estimated by variable J method with the restriction that  $10 < dC_j/dA < 50$ ;

<sup>c</sup>Percentage of data points that were excluded by applying the restriction that  $10 < dC_j/dA < 50$ .

Data used for the estimation were from measurements on 3–4 replicate leaves for the e<sup>-</sup> transport-limited range of CO<sub>2</sub> response curve at 21% O<sub>2</sub>, where C<sub>i</sub> is roughly  $\geq 500$   $\mu$ bar (Data of Yin *et al.* 2009b; 12 data points for each estimation).

apple (>0.55, Table 3) because of unrealistically high  $g_m$  obtained at one or more particular data points.

In contrast to unstable and sometimes unrealistic estimations given by the constant J and variable J methods, the order of the estimated  $g_m$  among the four situations in the wheat data set was consistent for the four curve-fitting-based variants (i.e. C<sub>i</sub>C<sub>c</sub>, modified J, NRH-A and RH-A): overall, the higher the leaf N content, the higher the estimated  $g_m$  (Table 2), in line with the reports that  $g_m$  scales with leaf photosynthetic capacity (e.g. Loreto *et al.* 1992, 1994; Evans & von Caemmerer 1996). This consistency was also observed in the apple data set, although, for apple, the highest  $g_m$  was estimated for the second highest leaf N level (Table 3), whereas it was inestimable for this treatment by the C<sub>i</sub>C<sub>c</sub> variant. The absolute value of  $g_m$  estimates for wheat differed among the four variants, with the consistent order: C<sub>i</sub>C<sub>c</sub> < NRH-A < RH-A < modified J (Table 2). This ranking, however, may change when it is applied to other data sets, for example, to the two highest N levels in apple (Table 3).

### Estimated $g_m$ when including more real data

The constant J method requires that J is constant. It cannot be applied to data within the e<sup>-</sup> transport-limited range of an A-I<sub>inc</sub> curve, which may also contain useful information. The variable J method, in principle, can be applied to these situations. However, when applying the variable J method to analyse the data from low I<sub>inc</sub> levels ( $\leq 200$   $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>), there is a high probability that  $g_m$  is negative or that  $dC_j/dA > 50$ .

For the four variants using curve fitting, inclusion of the data from a part of A-I<sub>inc</sub> curves can be implemented easily. The  $g_m$  estimates by modified J, NRH-A and RH-A variants, obtained from the combined data of A-I<sub>inc</sub> and A-C<sub>i</sub> curves measured at both ambient and low O<sub>2</sub> levels (Table 4), were virtually the same as the estimate from the data of A-C<sub>i</sub> curves alone at the ambient O<sub>2</sub> (Table 2), suggesting that  $g_m$  is similar between low light and high CO<sub>2</sub> conditions and between ambient and low O<sub>2</sub> levels. Only the C<sub>i</sub>C<sub>c</sub> variant gave different estimates, again

**Table 3.** Value of  $g_m$  (mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>, standard error of the estimate in brackets if applicable) estimated by six approaches (see the texts for their description) for leaves of apple plants grown under six nitrogen (N) supply levels as indicated by six leaf N contents

Leaf N (g m <sup>-2</sup> )	Constant J method	Variable J method			C <sub>i</sub> C <sub>c</sub>	Modified J	NRH-A	RH-A
		Without <sup>a</sup>	With <sup>b</sup>	% excl <sup>c</sup>				
3.81	0.152	0.592	0.169	40	0.219 (0.098)	0.171 (0.013)	0.171 (0.020)	0.171 (0.016)
3.22	0.199	0.551	0.282	60	Inestimable	0.273 (0.054)	0.287 (0.072)	0.282 (0.068)
2.32	0.307	0.115	0.144	40	0.094 (0.021)	0.160 (0.018)	0.143 (0.021)	0.154 (0.020)
1.99	0.281	0.090	0.101	20	0.071 (0.016)	0.130 (0.012)	0.113 (0.016)	0.124 (0.015)
1.53	0.387	0.067	0.099	60	0.051 (0.012)	0.103 (0.019)	0.086 (0.020)	0.096 (0.020)
1.10	0.242	0.093	–	100	0.030 (0.008)	0.081 (0.111)	0.057 (0.057)	0.068 (0.083)

<sup>a</sup>Arithmetic average  $g_m$  estimated by variable J method without the restriction that  $10 < dC_j/dA < 50$ .

<sup>b</sup>Arithmetic average  $g_m$  estimated by variable J method with the restriction that  $10 < dC_j/dA < 50$ .

<sup>c</sup>Percentage of data points that were excluded by applying the restriction that  $10 < dC_j/dA < 50$ .

Data used for the estimation were from the e<sup>-</sup> transport-limited range of CO<sub>2</sub> response curve at 21% O<sub>2</sub>, where  $\Delta F/F'_m$  is roughly constant (data of Cheng *et al.* 2001; five data points for each estimation).

Leaves	$C_iC_c$	Modified J	NRH-A	RH-A
FLW-N0	0.032 (0.011)	0.107 (0.006)	0.091 (0.007)	0.102 (0.007)
FLW-N1	0.072 (0.049)	0.149 (0.012)	0.131 (0.012)	0.143 (0.013)
2WA-N0	0.025 (0.011)	0.095 (0.018)	0.082 (0.016)	0.090 (0.018)
2WA-N1	0.032 (0.003)	0.119 (0.016)	0.102 (0.015)	0.114 (0.016)

Data used for the estimation were from measurements on three to four replicate leaves for the  $e^-$  transport-limited range of  $CO_2$  response curve at both 21% and 2%  $O_2$ , where  $C_i$  is roughly  $\geq 500 \mu\text{bar}$  and of light response curve at both 21% and 2%  $O_2$ , where incident irradiance is  $\leq 200 \mu\text{mol m}^{-2} \text{s}^{-1}$  (data of Yin *et al.* 2009b; about 60 data points for each estimation).

suggesting that, probably, it is highly sensitive to measurement errors.

### Impact of the biological uncertainty on the estimate of $g_m$

For the influence of the uncertainties associated with stoichiometry of  $H^+$  pumping and ATP synthesis along LET to be shown, the coefficient 2 in Eqns 4–7 for  $C_iC_c$ , modified J, NRH-A and RH-A variants was changed to 7/3, according to the stoichiometry of Eqn 8. The estimated  $g_m$  for all data of wheat (within the  $e^-$  transport-limited range) is given in Table 5. Except for those from the  $C_iC_c$  variant, the estimated  $g_m$  values were consistently higher (approximately 15% on average) than those in Table 4 obtained using the same data.

## DISCUSSION

While it has long been recognised that  $g_m$  is not infinite, many questions with regard to  $g_m$  are yet to be elucidated (Flexas *et al.* 2008; Warren 2008). One of the issues is developing robust methodologies for estimating  $g_m$  in order, for example, to compare photosynthetic limitations by different levels of an environmental stress. We reveal some problems of existing CF-based methods and propose three alternative variants that overcome these problems. Caveats in these approaches are also highlighted.

### Advantages of the three proposed variants over the existing approaches

The range of data that can be used in the constant J method to estimate an average  $g_m$  is very narrow, although the

Leaves	$C_iC_c$	Modified J	NRH-A	RH-A
FLW-N0	0.032 (0.013)	0.117 (0.008)	0.098 (0.008)	0.111 (0.008)
FLW-N1	0.080 (0.068)	0.169 (0.016)	0.148 (0.016)	0.162 (0.017)
2WA-N0	0.026 (0.013)	0.114 (0.027)	0.097 (0.023)	0.106 (0.027)
2WA-N1	0.033 (0.016)	0.138 (0.022)	0.118 (0.020)	0.131 (0.023)

Data used for the estimation were the same as those in Table 4.

**Table 4.** Value of  $g_m$  ( $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$ , standard error of the estimate in brackets) estimated by four approaches (see the texts for their description) based on Eqn 2 for flag leaves of wheat plants at flowering (FLW) and 2 weeks after flowering (2WA) grown at low nitrogen (N0) and high nitrogen (N1) levels

method has often been reported to give good estimation of  $g_m$  (e.g. Bernacchi *et al.* 2002; Singaas, Ort & Delucia 2003). As CF measurements are not directly used, its estimation of  $g_m$  depends entirely on the GE data. Specifically, the estimation does not depend on the absolute values of  $A$  but on the curvature or slope of  $A$  in response to  $C_i$  (Harley *et al.* 1992). A reduced  $g_m$  will increase the sensitivity of  $A$  to  $C_i$  in the high  $C_i$  region. The method can be reasonably reliable if the data for  $A-C_i$  curves conform Eqn 2, as shown in analysis of the generated data, especially if  $g_m$  is low (Fig. 1). For the real data set in wheat, although CF measurements showed that  $\Delta F/F'_m$  was quite constant across several  $C_i$ , the slope of response of  $A$  to  $C_i$  was approaching zero, indicating the limitation set by triose phosphate utilisation (Yin *et al.* 2009b); as a result, the constant J method yielded an infinite  $g_m$ . Any occurrence of triose phosphate utilisation limitation of  $A$  at high  $C_i$  will make the range of useable data of an  $A-C_i$  curve even narrower for the constant J method. Considering finite  $g_m$  values given by other approaches (Table 2), the constant J method seemed unreliable for the wheat data set. Similarly, fitting approaches of Ethier *et al.* (2006) and Sharkey *et al.* (2007) use GE data alone, relying entirely on the curvature of an  $A-C_i$  curve.

The validity of the variable J method in estimating the average  $g_m$  is plagued by unrealistic values at many data points because of the high sensitivity of this method to measurement errors in  $C_i$ ,  $J$  and  $A$ . If its empirical restriction that  $10 < dC_i/dA < 50$  is applied, many useful data points have to be excluded. On the other hand, there is no guarantee that applying this restriction can always eliminate the unrealistic  $g_m$  values. Our analysis using the generated data set revealed that the restriction that  $10 < dC_i/dA < 50$  may not be an effective 'filter', as unrealistic  $g_m$  values can be calculated even if  $dC_i/dA$  has a value well between 10 and 50. However, we admit that these problems

**Table 5.** Value of  $g_m$  ( $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$ , standard error of the estimate in brackets) estimated by four approaches based on Eqn 8 (see the texts for their description) for flag leaves of wheat plants at flowering (FLW) and 2 weeks after flowering (2WA) grown at low nitrogen (N0) and high nitrogen (N1) levels

may have been exaggerated in our analysis using data of e<sup>-</sup> transport-limited range of A-C<sub>i</sub> curves, compared with the common practice in which the variable J method is used within the moderate C<sub>i</sub> values of approximately 100–350 μbar (e.g. Niinemets *et al.* 2005).

The C<sub>i</sub>C<sub>c</sub> variant, in which C<sub>c</sub> is calculated first from the GE and CF measurements, is also sensitive to measurement errors especially in J and A. Like the variable J method for point-specific g<sub>m</sub> estimation, C<sub>c</sub> calculated by Eqn 5 for each data point is very sensitive to measurement errors in both J and A. At times, the calculated C<sub>c</sub> even became negative, and, in other cases, the obtained (C<sub>i</sub> - C<sub>c</sub>) was negative. As curve fitting for the C<sub>i</sub>C<sub>c</sub> variant uses directly the data of the calculated (C<sub>i</sub> - C<sub>c</sub>), any error in the estimate of C<sub>c</sub> exerts a large effect on the estimation of g<sub>m</sub>. As Eqn 5 does not need C<sub>i</sub> to calculate C<sub>c</sub>, the C<sub>i</sub>C<sub>c</sub> variant is less sensitive to errors in C<sub>i</sub>, as revealed in our analysis of the generated data set (Table S1). For the real data sets, g<sub>m</sub> was not estimated by this variant for the second highest leaf N level of apple (Table 3). The high sensitivity of the C<sub>i</sub>C<sub>c</sub> variant to errors can be further illustrated for the wheat data set by inclusion of data for an A-C<sub>i</sub> curve measured under the 2% O<sub>2</sub> condition, where Γ\* approaches to 0. There were more occasions where the calculated C<sub>c</sub> by Eqn 5 was negative for the low O<sub>2</sub> condition; as a result, the estimated g<sub>m</sub> by the C<sub>i</sub>C<sub>c</sub> variant was reduced by approximately 35% (results not shown).

Three alternative variants (i.e. modified J, NRH-A and RH-A) were described herein, deploying the combined data of GE and CF measurements and using the technique of curve fitting. An uncertainty analysis by varying R<sub>d</sub> and S<sub>c/o</sub> in 10–12 steps from their default value showed that the three variants have a similar sensitivity to errors in input parameter R<sub>d</sub> or S<sub>c/o</sub> (results not shown). They effectively overcome the aforementioned problems of the existing approaches. For example, the modified J, NRH-A and RH-A variants require no empirical restriction as imposed for the variable J method, thereby all providing more objective estimations of g<sub>m</sub>. Moreover, using the modified J, NRH-A and RH-A variants, inclusion of data under low O<sub>2</sub> conditions resulted in virtually no change in the estimated g<sub>m</sub> value (e.g. Table 2 versus Table 4), in line with the result that g<sub>m</sub> is not affected by the O<sub>2</sub> level (Loreto *et al.* 1992; Laisk *et al.* 2006). The possibility for the use of the combined data of both light- and C<sub>i</sub>-responses under both ambient and low O<sub>2</sub> conditions makes modified J, NRH-A and RH-A variants attractive because g<sub>m</sub> can be estimated from a larger data set and thus is less sensitive to measurement errors, as occurred at some data points. However, it should be noted that measurements at the low O<sub>2</sub> level alone do not work for any approach because the estimation of g<sub>m</sub> using Eqn 2 works only in the presence of photorespiration, that is, based on the reassimilation of photorespiratory CO<sub>2</sub> (Laisk *et al.* 2006). Moreover, for the occasions when g<sub>m</sub> is high, our proposed variants are sensitive to errors, albeit to a lesser extent than that of the existing approaches (Fig. 1; Table S1). So when mesophyll diffusion has little limitation to photosynthesis, the estimates have

high standard errors (Table S1) such that it is of little significance in estimating g<sub>m</sub> (in such cases, an assumption that C<sub>c</sub> is equal to C<sub>i</sub> would be inconsequential).

### Differences in the estimated g<sub>m</sub> caused by statistical artefacts

The g<sub>m</sub> values estimated by the three proposed variants were different (Tables 2–4 & S1). As stated earlier, Eqn 6 for the NRH-A variant is the inverse function of Eqn 4 for the modified J variant; so, Eqns 4 and 6 represent the same mathematical A-J relationship. But the obtained g<sub>m</sub> from the NRH-A variant was generally lower than that from the modified J variant, on average by approximately 15% (Tables 2–4 & S1). This is in analogy to the situation for a simple model y = bx, where the value of b statistically obtained from regressing y versus x usually differs from the inverse of the slope coefficient obtained from regressing x versus y. So the difference between the estimates by the modified J variant and the NRH-A variant is entirely caused by the artefact of the statistical procedure for fitting the inverse functions. The fitting in the modified J variant, like in the extended constant or variable J methods (Pons *et al.* 2009), is to minimise the squared model deviation from data for J, thereby best matching the principle that the difference between J estimated from GE and J estimated from CF is a function of g<sub>m</sub> (Long & Bernacchi 2003). In both RH-A and NRH-A variants, the fitting is to minimise the squared model deviation from data for A, thereby better describing the values of A along A-C<sub>i</sub> or A-I<sub>inc</sub> curves than the modified J variant does. A further advantage of RH-A and NRH-A variants is that the aforementioned lumped calibration parameter s can be fitted simultaneously with g<sub>m</sub> if the data for calibration were not obtained under the real non-photorespiratory condition. For example, using the same data as for Table 4, the s values estimated with the RH-A variant were 0.363 (SE: 0.004), 0.389 (SE: 0.004), 0.327 (SE: 0.006) and 0.378 (SE: 0.005) for the four groups of wheat leaves, respectively, slightly lower than the values given by Yin *et al.* (2009b), and the simultaneously estimated g<sub>m</sub> became 0.124 (0.016), 0.182 (0.032), 0.113 (0.042) and 0.131 (0.029) mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>, respectively, slightly higher than the estimates by this variant in Table 4. However, the RH-A variant is based on Eqn 7, in which observed A are used as input, and, thus, the estimate of g<sub>m</sub> cannot be used directly for the purpose of predicting A even under the environmental conditions within which g<sub>m</sub> was estimated. In this context, the NRH-A variant is most recommended because its estimated g<sub>m</sub> can be used for predicting A with Eqn 6, provided that g<sub>m</sub> is constant. This assertion is supported by the results that the NRH-A variant had the lowest overall absolute REE for the generated data set (Table 1).

For the existing C<sub>i</sub>C<sub>c</sub> variant, one may regress A against (C<sub>i</sub> - C<sub>c</sub>) so that the slope of the line passing through the origin is the estimate of g<sub>m</sub> according to Eqn 1 (see fig. 3.6 of von Caemmerer 2000). Because A is usually considered as the dependent variable, regressing A against (C<sub>i</sub> - C<sub>c</sub>) is

preferred than the other way around. This alternative way of fitting for the  $C_i C_c$  variant, however, consistently gave an even lower  $g_m$  for both real and generated data sets (results not shown) than the estimate from regressing  $(C_i - C_c)$  versus  $A$ , which already resulted in the lowest  $g_m$  among the four variants using curve fitting for the real data set in wheat (Tables 2 & 4) and for most leaf N levels in apple (Table 3).

### Impact of biological uncertainties on the estimation of $g_m$

In addition to the statistical artefact of curve fitting, there is a biological uncertainty that affects the estimation of  $g_m$  using the combined GE and CF measurements. The sensitivity of the estimated  $g_m$  to the stoichiometry assumption related to linear  $e^-$  transport-limited algorithms received little attention in the literature.

The stoichiometric uncertainties of LET for the  $H^+/e^-$  and  $H^+/ATP$  ratios have an impact on the model of  $e^-$  transport-limited photosynthesis, and various forms of  $e^-$  transport-limited photosynthesis model can be proposed thereof (Yin *et al.* 2004, 2006). Most CF methods of estimating  $g_m$  use Eqn 2 of the original FvCB model based on NADPH requirement. An obvious alternative case is Eqn 8 based on ATP requirement, with the contemporary literature on the  $H^+/e^-$  and  $H^+/ATP$  ratios, in which the coefficient 2 in Eqn 2 is changed to 7/3 because oxygenation consumes slightly more ATP than carboxylation does. Equations 2 and 8 predict the same  $A$  in the absence of photorespiration but differ under the photorespiratory condition, a condition required when using Eqn 2 or 8 to estimate  $g_m$ . The rates of consumption of NADPH and ATP are major factors that determine PSII operating efficiency (Baker 2008). It is unclear whether CF-revealed rate of LET through PSII is more closely associated with NADPH consumption or with ATP consumption under photorespiratory conditions although existing CF-based  $g_m$  estimations implicitly assume that it is more related to NADPH consumption. Similarly unclear is if the slightly higher ATP : NADPH requirement of the photorespiratory cycle, compared with the carbon reduction cycle, can be met by ATP import, for example, from mitochondrial ATP synthesis. Thus, stoichiometric uncertainties along the LET chain with regard to the  $H^+/e^-$  and  $H^+/ATP$  ratios could engender uncertainties in the  $g_m$  estimates by any CF-based approaches. The uncertainties cannot be accounted for by any calibration procedure by using the GE and CF measurements under non-photorespiratory conditions, again, because of a small difference in ATP consumption between oxygenation and carboxylation. We have shown the impact for the case of Eqn 8 on the estimated  $g_m$ , using all data for wheat (Table 5). Extreme stoichiometries such as  $H^+/ATP = 14/3$  (see Allen 2003) will yield a corresponding change in the estimated  $g_m$ , which is not shown here given that doubt has been cast on such a high  $H^+/ATP$  ratio (Laisk *et al.* 2007; Steigmiller *et al.* 2008).

Many (e.g. Warren 2006) drew attention to various uncertain factors (e.g. errors in the estimated  $R_d$  or  $S_{c/o}$ ) influencing the accuracy of the CF-based methods in estimating  $g_m$ . Ethier *et al.* (2006) & Pons *et al.* (2009) briefly indicated that using another  $e^-$  transport-limited form of the FvCB model – that is, the part  $4(C_c + 2\Gamma_*)$  of Eqn 2 is replaced with  $(4.5C_c + 10.5\Gamma_*)$  – the estimated  $g_m$  can be increased by 8–15%. This form of the equation is based on an obsolete  $H^+/e^-$  and  $H^+/ATP$  ratios, and its term  $J$  includes a small amount of alternative non-cyclic  $e^-$  transport in addition to LET (Yin *et al.* 2004, 2009a,b). The response of the estimated  $g_m$  to the uncertainties in relation to contemporary stoichiometry of only LET in thylakoid reactions is directly assessed here for the first time, although the magnitude of the impact is similar to those briefly shown by Ethier *et al.* (2006) and Pons *et al.* (2009).

### Using the data of $e^-$ transport-limited range to estimate $g_m$

Unlike the approaches of Ethier *et al.* (2006) and Sharkey *et al.* (2007), no Rubisco part of the FvCB model was involved in the curve fitting here to avoid more uncertainties because calculation of the Rubisco-limited  $A$  requires Michaelis-Menten constants for  $CO_2$  and  $O_2$  and Rubisco carboxylation capacity as inputs. An accurate *in vivo* estimation of the latter three parameters remains a challenge, as it may depend on  $g_m$  itself (Yin *et al.* 2009b). While the Rubisco part of the FvCB model can be theoretically used to estimate  $g_m$ , based only on data under non-photorespiratory conditions, the estimation of  $g_m$  would become dependent only on GE data if this part of the model is used alone because  $J$  is no longer a model input. Furthermore, for  $A-C_i$  curves, Rubisco limits  $A$  at low  $C_i$ , where the estimation of  $g_m$  based on an  $A-C_i$  curve would be theoretically more sensitive to any error in the estimated  $R_d$  or  $\Gamma_*$  (Harley *et al.* 1992). Any loss of activation of Rubisco at low  $C_i$  is another complication to consider. A further comparative disadvantage of using Rubisco-limited range of  $A-C_i$  data to estimate the average  $g_m$  is that variation of  $g_m$  with  $C_i$ , if any, is most striking at the low  $C_i$  range (Flexas *et al.* 2007; Yin *et al.* 2009b) so that the estimated average  $g_m$  is most afflicted by the variation of  $g_m$ .

As stated already, applying any Eqn 2-based approaches to the Rubisco-limited range is, in principle, not recommended either, because down-regulation of  $e^-$  transport rate may not be 100% (see data of Cheng *et al.* 2001). It is highly likely that, within this range, additional alternative  $e^-$  transport with a varying fraction of LET occurs in support of processes such as the Mehler ascorbate peroxidase reaction (Yin *et al.* 2009b). This additional alternative  $e^-$  transport, if significant, will cause an underestimation of  $g_m$ . This is an additional problem for the variable  $J$  method, if it is, as Long & Bernacchi (2003) proposed, applied to the Rubisco-limited range of an  $A-C_i$  curve. A dilemma with the standard variable  $J$  method is that it can be heavily afflicted by the restriction of  $10 < dC_i/dA < 50$  when applied to the  $e^-$  transport-limited range of an  $A-C_i$  curve, as shown in our

analysis. This is probably why the variable J method has usually been applied to an intermediate C<sub>i</sub> range of approximately 100–350 μbar (e.g. Niinemets *et al.* 2005). However, the variable J method remains the only ‘cheap’ method to calculate point-specific g<sub>m</sub> over a wide range of conditions, so it best serves as a first step to examine the pattern of any variation of g<sub>m</sub> with C<sub>i</sub> or with I<sub>inc</sub> before applying a model to describe this variation of g<sub>m</sub> (Yin *et al.* 2009b).

## CONCLUSIONS

The estimate of an average g<sub>m</sub> using GE- and CF-based approaches can be affected by both statistical artefacts in curve fitting and biological uncertainties in thylakoid stoichiometry. This means that (1) the estimated g<sub>m</sub> should not be considered to be absolute, especially if g<sub>m</sub> is high; and (2) comparison of g<sub>m</sub> estimates for different conditions is reliable only if they are based on the same approach and if they apply the same stoichiometry. The three variants using curve fitting, proposed here, are far less sensitive to random errors than the existing approaches are. The best way to estimate g<sub>m</sub> is probably to apply the NRH-A variant to the data within the e<sup>-</sup> transport-limited range. The value of g<sub>m</sub> obtained by using the data within the e<sup>-</sup> transport-limited range should be regarded as the average over the lower range of its estimates, if the reported variation of g<sub>m</sub> that increases with decreasing C<sub>i</sub> and with increasing I<sub>inc</sub> levels (Flexas *et al.* 2007; Yin *et al.* 2009b) is proven to be general.

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## REFERENCES

- Allen J.F. (2003) Cyclic, pseudocyclic and noncyclic photophosphorylation: new links in the chain. *Trends in Plant Science* **8**, 15–19.
- Avenson T.J., Kanazawa A., Cruz J.A., Takizawa K., Ettinger W.E. & Kramer D.M. (2005) Integrating the proton circuit into photosynthesis: progress and challenges. *Plant, Cell & Environment* **28**, 97–109.
- Baker N.R. (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annual Review of Plant Biology* **59**, 89–113.
- Bernacchi C.J., Portis A.R., Nakano H., von Caemmerer S. & Long S.P. (2002) Temperature response of mesophyll conductance. Implication for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiology* **130**, 1992–1998.
- Bongi G. & Loreto F. (1989) Gas-exchange properties of salt-stressed olive (*Olea europaea* L.) leaves. *Plant Physiology* **90**, 1408–1416.
- Bunce J.A. (2008) Acclimation of photosynthesis to temperature in *Arabidopsis thaliana* and *Brassica oleracea*. *Photosynthetica* **46**, 517–524.
- von Caemmerer S. (2000) *Biochemical Models of Leaf Photosynthesis. Techniques in Plant Sciences*, No. 2. CSIRO Publishing, Collingwood, Victoria, Australia.
- von Caemmerer S. & Evans J.R. (1991) Determination of the average partial pressure of CO<sub>2</sub> in chloroplasts from leaves of several C<sub>3</sub> plants. *Australian Journal of Plant Physiology* **18**, 287–305.
- Cheng L., Fuchigami L.H. & Breen P.J. (2001) The relationship between photosystem II efficiency and quantum yield for CO<sub>2</sub> assimilation is not affected by nitrogen content in apple leaves. *Journal of Experimental Botany* **52**, 1865–1872.
- Di Marco G., Manes F., Tricoli D. & Vitale E. (1990) Fluorescence parameters measured concurrently with net photosynthesis to investigate chloroplastic CO<sub>2</sub> concentration in leaves of *Quercus ilex* L. *Journal of Plant Physiology* **136**, 538–543.
- Dubois J.-J.B., Fiscus E.L., Booker F.L., Flowers M.D. & Reid C.D. (2007) Optimizing the statistical estimation of the parameters of the Farquhar-von Caemmerer-Berry model of photosynthesis. *New Phytologist* **176**, 402–414.
- Epron D., Godard D., Cornic G. & Genty B. (1995) Limitation of net CO<sub>2</sub> assimilation rate by internal resistances to CO<sub>2</sub> transfer in the leaves of two tree species (*Fagus sylvatica* L. and *Castanea sativa* Mill.). *Plant, Cell & Environment* **18**, 43–51.
- Ethier G.J. & Livingston N.J. (2004) On the need to incorporate sensitivity to CO<sub>2</sub> transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. *Plant, Cell & Environment* **27**, 137–153.
- Ethier G.J., Livingston N.J., Harrison D.L., Black T.A. & Moran J.A. (2006) Low stomatal and internal conductance to CO<sub>2</sub> versus Rubisco deactivation as determinants of the photosynthetic decline of ageing evergreen leaves. *Plant, Cell & Environment* **29**, 2168–2184.
- Evans J.R. & von Caemmerer S. (1996) Carbon dioxide diffusion inside leaves. *Plant Physiology* **110**, 339–346.
- Farquhar G.D. & von Caemmerer S. (1982) Modelling of photosynthetic response to environmental conditions. In *Physiological Plant Ecology II, Water Relations and Carbon Assimilation. Encyclopaedia of Plant Physiology* (eds O.L. Lange, P.S. Nobel, C.B. Osmond & H. Ziegler) New Series, Vol. 12B, pp. 549–588. Springer Verlag, Berlin, Germany.
- Farquhar G.D., von Caemmerer S. & Berry J.A. (1980) A biochemical model of photosynthetic CO<sub>2</sub> assimilation in leaves of C<sub>3</sub> species. *Planta* **149**, 78–90.
- Flexas J., Diaz-Espejo A., Galmés J., Kaldenhoff R., Medrano H. & Ribas-Carbó M. (2007) Rapid variation of mesophyll conductance in response to changes in CO<sub>2</sub> concentration around leaves. *Plant, Cell & Environment* **30**, 1284–1298.
- Flexas J., Ribas-Carbó M., Díaz -Espejo A., Galmés J. & Medrano H. (2008) Mesophyll conductance to CO<sub>2</sub>: current knowledge and future prospects. *Plant, Cell & Environment* **31**, 602–621.
- Genty B., Briantais J. & Baker N. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990**, 87–92.
- Harley P.C., Loreto F., Di Marco G. & Sharkey T.D. (1992) Theoretical considerations when estimating the mesophyll conductance to CO<sub>2</sub> flux by analysis of the response of photosynthesis to CO<sub>2</sub>. *Plant Physiology* **98**, 1429–1436.
- Laisk A., Eichelmann H., Oja V., Rasulov B. & Rämme H. (2006) Photosystem II cycle and alternative electron flow in leaves. *Plant and Cell Physiology* **47**, 972–983.
- Laisk A., Eichelmann H., Oja V., Talts E. & Scheibe R. (2007) Rates and roles of cyclic and alternative electron flow in potato leaves. *Plant and Cell Physiology* **48**, 1575–1588.
- Long S.P. & Bernacchi C.J. (2003) Gas exchange measurements, what can they tell us about the underlying limitations to

- photosynthesis? Procedures and sources of error. *Journal of Experimental Botany* **54**, 2393–2401.
- Loreto F., Harley P.C., Di Marco G. & Sharkey T.D. (1992) Estimation of mesophyll conductance to CO<sub>2</sub> flux by three different methods. *Plant Physiology* **98**, 1437–1443.
- Loreto F., Di Marco G., Tricoli D. & Sharkey T.D. (1994) Measurement of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. *Photosynthesis Research* **41**, 397–403.
- Miao Z., Xu M., Lathrop R.G. Jr & Wang Y. (2009) Comparison of the A-C<sub>i</sub> curve fitting methods in determining maximum ribulose 1,5-bisphosphate carboxylase-oxygenase carboxylation rate, potential light saturated electron transport rate and leaf dark respiration. *Plant, Cell & Environment* **32**, 109–122.
- Niinemets Ü., Cescatti A., Rodeghiero M. & Tosens T. (2005) Leaf internal diffusion conductance limits photosynthesis more strongly in old leaves of Mediterranean evergreen broad-leaves species. *Plant, Cell & Environment* **28**, 1552–1566.
- Niinemets Ü., Díaz-Espejo A., Flexas J., Galmés J. & Warren C.R. (2009a) Role of mesophyll diffusion conductance in constraining potential photosynthetic productivity in the field. *Journal of Experimental Botany* **60**, 2249–2270.
- Niinemets Ü., Díaz-Espejo A., Flexas J., Galmés J. & Warren C.R. (2009b) Importance of mesophyll diffusion conductance in estimation of plant photosynthesis in the field. *Journal of Experimental Botany* **60**, 2271–2282.
- Pons T.L., Flexas J., von Caemmerer S., Evans J.R., Genty B., Ribas-Carbó M. & Bruognoli E. (2009) Estimating mesophyll conductance to CO<sub>2</sub>: methodology, potential errors, and recommendations. *Journal of Experimental Botany* **60**, 2217–2234.
- Sacksteder C.A., Kanazawa A., Jacoby M.E. & Kramer D.M. (2000) The proton to electron stoichiometry of steady-state photosynthesis in living plants: a proton-pumping Q cycle is continuously engaged. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 14283–14288.
- Sharkey T.D., Bernacchi C.J., Farquhar G.D. & Singsaas E.L. (2007) Fitting photosynthetic carbon dioxide response curves for C<sub>3</sub> leaves. *Plant, Cell & Environment* **30**, 1035–1040.
- Singsaas E.L., Ort D.R. & Delucia E.H. (2003) Elevated CO<sub>2</sub> effects on mesophyll conductance and its consequence for interpreting photosynthetic physiology. *Plant, Cell & Environment* **27**, 41–50.
- Steigmiller S., Turina P. & Gräber P. (2008) The thermodynamic H<sup>+</sup>/ATP ratios of the H<sup>+</sup>-ATPsynthases from the chloroplasts and *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 3745–3750.
- Warren C.R. (2006) Estimating the internal conductance to CO<sub>2</sub> movement. *Functional Plant Biology* **33**, 431–442.
- Warren C.R. (2008) Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO<sub>2</sub> transfer. *Journal of Experimental Botany* **59**, 1475–1487.
- Yin X., van Oijen M. & Schapendonk A.H.C.M. (2004) Extension of a biochemical model for the generalized stoichiometry of electron transport limited C<sub>3</sub> photosynthesis. *Plant, Cell & Environment* **27**, 1211–1222.
- Yin X., Harbinson J. & Struik P.C. (2006) Mathematical review of literature to assess alternative electron transports and interphotosystem excitation partitioning of steady-state C<sub>3</sub> photosynthesis under limiting light. *Plant, Cell & Environment* **29**, 1771–1782. (Corrigendum on page 2252 of the same issue).
- Yin X., Harbinson J. & Struik P.C. (2009a) A model of the generalized stoichiometry of electron transport-limited C<sub>3</sub> photosynthesis: development and applications. In *Photosynthesis in Silico: Understanding Complexity from Molecules to Ecosystems* (eds A. Laisk & L. Nedbal & Govindjee) Volume 29, Book series 'Advances in Photosynthesis and Respiration', pp. 247–273. Springer, Dordrecht, Netherlands.
- Yin X., Struik P.C., Romero P., Harbinson J., Evers J.B., van der Putten P.E.L. & Vos J. (2009b) Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical C<sub>3</sub> photosynthesis model: a critical appraisal and a new integrated approach applied to leaves in a wheat (*Triticum aestivum*) canopy. *Plant, Cell & Environment* **32**, 448–464.

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## SUPPORTING INFORMATION

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**Figure S1.** An example of the generated ideal A-C<sub>i</sub> curve (line) and the response where A was imposed with a ±10% random 'noise' (points) (a), the plot of C<sub>i</sub> imposed with a ±10% random 'noise' against the ideal values of C<sub>i</sub> (b), and the plot of J imposed with ±10% random 'noise' against the ideal values of C<sub>i</sub> (c). The data in this figure are shown for the case where the preset g<sub>m</sub> is 0.20 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>. Ideal data set was created for the condition of 21% O<sub>2</sub>, assuming that J = 180 μmol electrons m<sup>-2</sup> s<sup>-1</sup>, R<sub>d</sub> = 1.5 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> and S<sub>c/o</sub> = 3.12 mbar μbar<sup>-1</sup>.

**Table S1.** Value of g<sub>m</sub> (mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>, standard error of the estimate in brackets if applicable) estimated by different approaches for the data set generated using different preset g<sub>m</sub> values (g<sub>m,set</sub>), where ±10% random 'noise' was imposed on J or on C<sub>i</sub> or on A, or on all the three (J + C<sub>i</sub> + A).

**Appendix S1.** SAS codes for estimating mesophyll conductance g<sub>m</sub> using three proposed variants.

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