Rapid variations of mesophyll conductance in response to changes in CO₂ concentration around leaves

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ABSTRACT

The effects of short-term (minutes) variations of CO₂ concentration on mesophyll conductance to CO₂ (gₘ) were evaluated in six different C₃ species by simultaneous measurements of gas exchange, chlorophyll fluorescence, online carbon isotope discrimination and a novel curve-fitting method. Depending on the species, gₘ varied from five- to ninefold, along the range of sub-stomatal CO₂ concentrations typically used in photosynthesis CO₂-response curves (Aₙ–Cᵢ curves; where Aₙ is the net photosynthetic flux and Cᵢ is the CO₂ concentrations in the sub-stomatal cavity), that is, 50 to 1500 μmol CO₂ mol⁻¹ air. Although the pattern was species-dependent, gₘ strongly declined at high Cᵢ, where photosynthesis was not limited by CO₂, but by regeneration of ribulose-1,5-bisphosphate or triose phosphate utilization. Moreover, these changes on gₘ were found to be totally independent of the velocity and direction of the Cᵢ changes. The response of gₘ to Cᵢ resembled that of stomatal conductance (gₛ), but kinetic experiments suggested that the response of gₘ was actually faster than that of gₛ. Transgenic tobacco plants differing in the amounts of aquaporin NtAQP1 showed different slopes of the gₘ–Cᵢ response, suggesting a possible role for aquaporins in mediating CO₂ responsiveness of gₘ. The importance of these findings is discussed in terms of their effects on parameterization of Aₙ–Cᵢ curves.

Key-words: Aₙ–Cᵢ curves; aquaporins; photosynthesis; leaf internal conductance.

INTRODUCTION

Photosynthesis requires diffusion of CO₂ from the atmosphere into the leaf and finally to the site of carboxylation in the chloroplast stroma. From Fick’s first law of diffusion, the net photosynthetic flux (Aₛ) can be expressed as Aₛ = gₛ(Cᵢ – Cₛ) = gₘ(Cᵢ – Cₛ); where Cₛ, Cᵢ and Cₛ are the CO₂ concentrations (μmol mol⁻¹ air) in the atmosphere, the sub-stomatal cavity and the chloroplast stroma, respectively, with gₛ and gₘ being the stomata and mesophyll conductances, respectively (Long & Bernacchi 2003).

Gas-exchange studies usually assumed that gₘ was large and constant, that is, that Cᵢ = Cₛ (Farquhar, von Caemmerer & Berry 1980). However, there is now evidence that gₘ may be sufficiently small as to significantly decrease Cᵢ relative to Cₛ, therefore limiting photosynthesis (Evans et al. 1986; Di Marco et al. 1990; Harley et al. 1992; Loreto et al. 1992; Warren 2006). Moreover, gₛ is not constant, and it has been shown to acclimate during leaf development (Miyazawa & Terashima 2001) and senescence (Loreto et al. 1994; Grassi & Magnani 2005), as well as to the prevailing light (Hanba, Kogami & Terashima 2002; Niinemets et al. 2006), nutrient (Warren 2004) and watering conditions (Galmés, Medrano & Flexas 2006) during growth. There is also evidence of rapid variation of gₛ in response to leaf temperature (Bernacchi et al. 2002; Warren & Dreyer 2006), water stress (Flexas et al. 2002, 2004; Grassi & Magnani 2005), salinity (Bongi & Loreto 1989; Loreto, Centritto & Chartzoulakis 2003) and virus infections (Sampol et al. 2003). Recent studies have been compiled suggesting a role of aquaporins in the regulation of gₛ (Hanba et al. 2004; Flexas et al. 2006). Aquaporin activity can be regulated by different mechanisms, including direct phosphorylation of aquaporins (Kjellbom et al. 1999), an osmotically driven cohesion/tension mechanism (Ye, Wiera & Steudle 2004), pH-dependent gating of aquaporins (Tournaire-Roux et al. 2003), and transcriptional regulation and protein stability (Eckert et al. 1999), most of them operating in short times (seconds to hours). Therefore, as already shown for temperature (Bernacchi et al. 2002; Warren & Dreyer 2006), it is likely that rapid variations in gₛ can be induced by transient changes in the most common environmental conditions, including light intensity, relative humidity, wind speed or CO₂ concentration.

Regarding CO₂ concentration, in their early formulation of the two most common fluorescence methods used to estimate gₛ, Harley et al. (1992) explicitly stated that ‘The constant J method worked well over a large range of CO₂, but to resolve the effect of CO₂ on gₛ required the variable J method’. However, despite its evident interest to understand plant responses to climate change as well as for the
correct interpretation of $A_{N}$–$C_i$ curves, the response of $g_m$ to varying $CO_2$ has received only little attention over the past 15 years. The original data by Harley et al. (1992) showed that $g_m$ was almost halved when $C_i$ was increased from 100 to 300 bar in Quercus rubra, but not in Eucalyptus globulus. Moreover, using the isotopic method, Loreto et al. (1992) demonstrated that $g_m$ was reduced at 750 mbar $C_i$ as compared to ambient $CO_2$ in $Q. rubra$ and, specially, in Xanthium strumarium. Surprisingly, these authors did not discuss the implications of these apparent variations of $g_m$ at different $CO_2$ concentrations (Harley et al. 1992; Loreto et al. 1992). More than 10 years later, Düring (2003) was the first to show in grapevines that the $g_m$ estimated using the variable fluorescence method varied as much as sixfold in a range of $CO_2$ from 50 to 2000 $\mu$mol mol$^{-1}$ air during the performance of a typical $A_{N}$–$C_i$ curve (i.e. within minutes). On the other hand, Centritto, Loreto & Chartzoulakis (2003) showed in salt-stressed olives that, after maintaining the leaves for an hour at very low $CO_2$, they recovered an $A_{N}$–$C_i$ curve similar to control leaves. The effect could not be explained by the increased stomatal conductance only, but also required a concomitant increase in mesophyll conductance. It was therefore concluded that the response of $g_m$ might be as rapid and reversible as that of $g_s$ (Centritto et al. 2003). Flexas et al. (2004) showed a similar effect in drought-stressed sunflower. Regarding long-term acclimation to high $CO_2$, $g_m$ has been observed to decrease only in one species, but not in others (Singsaas, Ort & De Lucia 2004; Bernacchi et al. 2005) Finally, using a novel $A_{N}$–$C_i$ curve-fitting method to estimate $g_m$, the values obtained were reported to depend somewhat on the number and range of the specific points considered along the curve (Ethier et al. 2006), although these authors attributed it to a mathematical artefact rather than to changes in $g_m$ along the curve.

Despite all this evidence, the possible effects of $CO_2$ on the regulation of $g_m$ are not usually taken into account. Actually, at least three commonly used methods for estimating $g_m$, that is, the constant J method (Harley et al. 1992), the slope-based variable fluorescence method (Terashima & Ono 2002) and the $A_{N}$–$C_i$ curve-fitting method (Ethier & Livingston 2004; Ethier et al. 2006), rely on the assumption that $g_m$ is not affected by $CO_2$ concentration. On the other hand, it is now recognized that a proper analysis using the Farquhar et al. (1980) model of photosynthesis must be done on a $C_i$ basis rather than on a $C_t$ basis (Long & Bernacchi 2003). However, most of the studies transform $A_{N}$–$C_i$ curves to $A_{N}$–$C_t$ curves using a single value of $g_m$ determined at ambient $CO_2$ concentration, that is, also neglecting the possible effects of $CO_2$ on $g_m$ (Flexas et al. 2002; Manter & Kerrigan 2004; Grassi & Magnani 2005).

Therefore, the objectives of the present work were (1) to characterize variations of $g_m$ in response to rapid (minutes) changes in $CO_2$ concentration in different species, (2) to validate the results using independent methods for the estimation of $g_m$ and (3) to discuss how these variations would affect the use of different methods to estimate $g_m$ and the correct analysis and interpretation of the most commonly used photosynthesis model (Farquhar et al. 1980), which is based on the response of net photosynthesis to $CO_2$ concentration at the site of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

**MATERIAL AND METHODS**

**Plant material and growing conditions**

Three-year-old olive trees (Olea europaea var. europaea) and 2-year-old plants of Richter-110 (a hybrid of Vitis berlandieri × Vitis rupestris) were growing outdoors under typical Mediterranean conditions in the experimental field of the University of the Balearic Islands. Olive trees were rooted in a clay-calcareous soil, while *Vitis* plants were growing in 40 L pots containing a mixture of perlite, horticultural substrate and clay. The measurements in olives were made during winter (early March), while in *Vitis* were made during summer (early August). The other species, including cucumber (Cucumis sativus L.), arabispidosis [Arabidopsis thaliana (L.) Heynh. genotype Col-0], tobacco (Nicotiana tabacum L. var. Samsun), flowering tobacco (Nicotiana sylvestris L.) and Limonium gibraltar (Sennem) Sennem were grown in a growth chamber in 10 L pots containing a mixture of perlite, horticultural substrate and clay. Transformed tobacco (N. tabacum L.) plants with different levels of aquaporin NtAQP1, differing in mesophyll conductance to $CO_2$ (Flexas et al. 2006), were also studied. Antisense and over-expressing plants were obtained from different lines: var. Samsun for the antisense (AS) lines (Siefritz et al. 2002) and line Hø 20.20 for the overexpressing (O) lines (Uehlein et al. 2003). Plants of each line with normal NtAQP1 expression were used as controls (CAS and CO). The environmental conditions were set to a 12 h photoperiod (25 °C day/20 °C night), 40–60% relative humidity and a photon flux density at plant height of ca. 900 $\mu$mol m$^{-2}$ s$^{-1}$ (halogen lamps), except in Arabidopsis (300 $\mu$mol m$^{-2}$ s$^{-1}$). All plants were daily irrigated at field capacity.

**Gas-exchange and chlorophyll fluorescence measurements**

All measurements were made on young, fully expanded leaves. Respiration in the light or ‘day’ respiration ($R_d$) and the apparent $CO_2$ photocompensation point ($C_i^*$) were determined according to the method of Laisk (1977) as described in von Caemmerer (2000). $A_{N}$–$C_i$ curves were measured, using an open gas-exchange system Li-6400 (Li-Cor Inc., Lincoln, NE, USA), at three different photosynthetically active photon flux densities (PPFDs) (50, 200 and 500 $\mu$mol m$^{-2}$ s$^{-1}$) at six different $CO_2$ levels ranging from 300 to 50 $\mu$mol $CO_2$ mol$^{-1}$ air. The intersection point of the three $A_{N}$–$C_i$ curves was used to determine $C_i^*$ (x-axis) and $R_d$ (y-axis). $C_i^*$ was used as a proxy for the chloroplastic $CO_2$ photocompensation point ($G^*$), according to Warren & Dreyer (2006). These values ranged from 33 $\mu$mol $CO_2$ mol$^{-1}$ air in *Olea* to 44 $\mu$mol $CO_2$ mol$^{-1}$ air in...
Figure 1. Example of the relationship between photochemical efficiency of photosystem II ($\phi_{PSII}$) and $\phi_{CO2}$ ([(An + $R_g$)/PPFD]) in tobacco leaves, obtained by varying either light intensity (empty symbols) or CO$_2$ concentration (filled symbols) under non-photorespiratory conditions in an atmosphere containing less than 1% O$_2$ (Valentini et al. 1995).

Arabidopsis, corresponding to Rubisco specificity factors between 85 and 112, that is, in good agreement with the reported values for C$_3$ plants (Galmés et al. 2005).

All other leaf gas-exchange parameters were determined simultaneously with measurements of chlorophyll fluorescence using the open gas-exchange system Li-6400 (Li-Cor Inc.) with an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc.). The actual photochemical efficiency of photosystem II ($\phi_{PSII}$) was determined by measuring steady-state fluorescence (Fs) and maximum fluorescence during a light-saturating pulse of ca. 8000 μmol m$^{-2}$ s$^{-1}$ (Fm$'$) following the procedures of Genty, Briantais & Baker (1989):

$$\phi_{PSII} = (Fm' - Fs)/Fm'$$

The electron transport rate ($J_{bs}$) was then calculated as

$$J_{bs} = \phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta$$

where PPFD is the photosynthetically active photon flux density, $\alpha$ is leaf absorbance and $\beta$ reflects the partitioning of absorbed quanta between photosystems II and I (PSI and PSII). $\alpha$ was measured using a spectroradiometer (HR2000CG-UV-NIR; Ocean Optics Inc., Dunedin, FL, USA) as described by Schultz (1996), using the light source from the Li-6400 and making the measurements inside a dark chamber. In addition, the product $\alpha \cdot \beta$ was determined from the relationship between $\phi_{PSII}$ and $\phi_{CO2}$ obtained by varying either light intensity or CO$_2$ concentration under non-photorespiratory conditions in an atmosphere containing less than 1% O$_2$ (Valentini et al. 1995). An example of such relationships is shown in Fig. 1 for tobacco. No differences were observed when light intensity or CO$_2$ concentration was changed. Therefore, only changes in light intensity were used for the other species. The resulting $\alpha$, as determined with the spectroradiometer, ranged between 0.88 (Arabidopsis) and 0.95 (Limonium), in agreement with other reports using the Li-6400 light source (Niinemets et al. 2005). The product $\alpha \cdot \beta$, as determined according to Valentini et al. (1995), ranged between 0.35 (Vitis) and 0.45 (Arabidopsis), depending on the species. The resulting value of light partitioning towards PSII ($\beta$) ranged between 0.39 and 0.51, that is, well within the range of typically reported values (Laisk & Loreto 1996).

CO$_2$-response curves were performed in light-adapted leaves of six different plants for each species. Except when specifically indicated, photosynthesis was induced with a PPFD of 1500 μmol m$^{-2}$ s$^{-1}$ (previously performed light-response curves had shown this to be sufficient light to saturate photosynthesis in all the species analysed) and 400 μmol mol$^{-1}$ CO$_2$ surrounding the leaf ($C_a$). The amount of blue light was set to 10–15% PPFD to maximize stomatal aperture. Leaf temperature was close to 25 °C, and leaf-to-air vapour pressure deficit was kept between 1.2 and 1.8 kPa during all measurements. Once steady state was reached (usually 30 min after clamping the leaf), a CO$_2$-response curve was performed. Gas exchange and chlorophyll fluorescence were first measured at 400 μmol mol$^{-1}$, then $C_a$ was either increased stepwise until 1800 μmol mol$^{-1}$ or decreased stepwise until 50 μmol mol$^{-1}$. Upon completion of measurements at high or low $C_a$, this was returned to 400 μmol mol$^{-1}$ to restore the original $A_N$. Then, $C_a$ was either decreased or increased (depending on the previous treatment) stepwise to complete the curve. The number of different $C_a$ values used for the curves ranged between 10 and 12, depending on the species, and the time lag between two consecutive measurements at different $C_a$ was restricted to 2–4 min, so that each curve was completed in 30–40 min.

Leakage of CO$_2$ into and out the leaf cuvette was determined for the range of CO$_2$ concentrations used in this study with photosynthetically inactive leaves of each species enclosed in the leaf chamber (obtained by heating the leaves until no variable chlorophyll fluorescence was observed), and used to correct measured leaf fluxes (Flexas et al. 2007).

Estimation of $g_m$ by gas exchange and chlorophyll fluorescence

The method by Harley et al. (1992) was used to make estimations of $g_m$ as

$$g_m = A_N/(C_i - (g^* \cdot (J_{bs} + 8 \cdot (A_N + R_d)))/(J_{bs} - 4 \cdot (A_N + R_d)))$$

were $A_N$ and $C_i$ are taken from gas-exchange measurements at saturating light and $g^*$ and $R_d$ were estimated using the Laisk (1977) method (see previous section).

The calculated values of $g_m$ were used to convert $A_N$–$C_i$ curves into $A_N$–$C_i$ curves using the following equation:

$$C_i = C_i - (A_N/g_m)$$
From $A_{c-C}$ curves, the maximum carboxylation capacity ($V_{c,max}$) and the maximum capacity for electron transport rate ($J_{max}$) were calculated using the temperature dependence of kinetic parameters of Rubisco described on a $C_{c}$ basis by Bernacchi et al. (2002), whereby net assimilation rate is given as

$$A = \min\{A_c, A_q\} - R_d \tag{5}$$

with

$$A_c = V_{c,max} \frac{C_c - \Gamma^*}{C_c + K_c[1 + (o_i/K_o)]} \tag{6}$$

$$A_q = \frac{J(C_c - \Gamma^*)}{4(C_c + 2\Gamma^*)} \tag{7}$$

where $A_c$ and $A_q$ represent photosynthesis limited by carboxylation and RuBP regeneration, respectively, $K_c$ and $K_o$ are the Rubisco Michaelis–Menten constants for carboxylation and oxygenation, respectively, and $o_i$ is the leaf internal oxygen concentration (assumed equal to the external).

**Estimation of $g_m$ by a curve-fitting method**

A new curve-fitting method (Ether & Livingston 2004; Ethier et al. 2006) was used to estimate $g_m$ in some experiments. In short, the method by Ether & Livingston (2004) fits $A-C$ curves with a non-rectangular hyperbola version of the Farquhar’s biochemical model of leaf photosynthesis. This is based on the hypothesis that $g_m$ reduces the curvature of the $A-C$ response curve. The combination of Eqn 4 with Eqns 6 and 7 yields the following quadratic equations, whose solutions are the positive roots:

$$A_c = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \tag{8}$$

where

$$a = -1/g_m$$

$$b = (V_{c,max} - R_d)/g_m + C_i + K_o(1 + O/K_c)$$

$$c = R_d(C_o + K_o) - V_{c,max}(C_i - \Gamma^*)$$

$$A_q = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \tag{9}$$

$$a = -1/g_m$$

$$b = (J/4 - R_d)/g_m + C_i + 2\Gamma^*$$

$$c = R_d(C_i + 2\Gamma^*) - J/4(C_i - \Gamma^*)$$

Values of $K_o, K_o, \Gamma^*$ and their temperature responses were the $C_{c}$-based in vivo values from Bernacchi et al. (2002). The $C_i$ cut-off point was determined based on the method proposed by Ethier et al. (2006). This method has been successfully used in several studies, showing good agreement with other independent estimates of $g_m$ (Niinemets et al. 2006; Warren & Dreyer 2006). Although some of the curves showed a third region, with photosynthesis limited by triose-phosphate use (e.g. Limonium in Fig. 1), the curve-fitting method was not applied to that region, because its parameterization is not straightforward (von Caemmerer 2000) and, depending on the equations chosen, it leads to arbitrary estimations of $g_m$ (data not shown).

**Estimation of $g_m$ by carbon isotope discrimination at near to ambient and high CO2**

Instantaneous carbon isotope discrimination was measured as previously described (Flexas et al. 2006). Leaves were placed in the chamber of the Li-6400 at 400 μmol mol⁻¹, a PPFD of 1500 μmol m⁻² s⁻¹ and at 25 °C. Gas-exchange parameters were measured as described with the Li-6400 system under steady-state conditions for a minimum of 45 min.

Once gas-exchange measurements were performed, the air exiting the cuvette was collected as follows: maintaining the leaf in the cuvette under steady state by maintaining the same conditions of light, CO2 concentration and temperature, the exhaust tube was disconnected of the match valve and connected through a series of Swagelock tube connectors to a magnesium perchlorate (water trap) tube and to a homemade 100 mL glass flask with Teflon stopcocks (RibasCarbo, Still & Berry 2002). Under steady-state conditions, the air passed through the desiccant and the open collecting bottle for 15 min at a flow above 150 mL min⁻¹, ensuring 20 full turnovers of air inside the collecting bottle before the stopcocks were closed and the bottle removed. In order to collect a reference air, the same procedure was followed with the cuvette empty.

Carbon isotope composition was determined in an isotope ratio mass spectrometer (IRMS) (Thermo Delta XPlus, Bremen, Germany) under dual-inlet mode. CO2 from the bottles (sample and reference) was first concentrated in a Precon loop under liquid nitrogen and then introduced in its corresponding fully expanded bellow. The bellows were then compressed to increase the signal for the $m/z$ 44 peak to a minimum of 1000 mV to maximize the signal/noise ratio. The dual-inlet IRMS compared the isotope ratio of the sample and reference CO2 introduced in its bellows. Firstly, the system performs a peak centre on ($m/z$ 45), then it equilibrates the sample and reference signal for the $m/z$ 44 peak and then both isotope ratios are compared 25 times. The SD of the $\delta^{13}C$ of the sample CO2 with respect to the reference CO2 was always below 0.03‰.

Carbon isotope discrimination was calculated as described by Evans et al. (1986), as

$$\Delta^{13}C_{obs} = \frac{[\xi(\delta^{13}C_o - \delta^{13}C_e)]/(1000 + \delta^{13}C_o - \xi(\delta^{13}C_o - \delta^{13}C_e))}$$

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where \( \xi = C_e/(C_e - C_o) \), and \( C_e \) and \( C_o \) are the CO\(_2\) concentrations of the air entering and leaving the chamber, respectively. Because of the dual-inlet comparison method used, the value of \( \delta^{13}C_e \) was equal to 0 and \( \delta^{13}C_o \) was the value obtained from the isotope analysis.

Mesophyll conductance values were determined by comparing predicted discrimination with observed discrimination. Predicted discrimination (\( \Delta_i \)) was calculated from the following equation by Evans *et al.* (1986):

\[
\Delta_i = a + (b - a) p_i / p_a
\]

where \( a \) is the fractionation occurring due to diffusion in air (4.4‰), \( b \) is the net fractionation by Rubisco and phosphoenolpyruvate carboxylase (PEPC) (29‰), and \( p_i \) and \( p_a \) are the intercellular and ambient partial pressures of CO\(_2\), respectively.

Finally, \( g_m \) was calculated from the equation (Evans & von Caemmerer 1996):

\[
\Delta_i - \Delta_{i, \text{obs}} = (29 - 1.8)(A_N/g_m) / p_a
\]

where 1.8‰ is the discrimination due to dissolution and diffusion of CO\(_2\) in water.

After collecting air from the cuvette at 400 \( \mu \text{mol CO}_2\text{ mol}^{-1}\text{ air} \), \( C_a \) was increased to 1500 \( \mu \text{mol CO}_2\text{ mol}^{-1}\text{ air} \), and the leaf allowed acclimating for 15 min. Then, air collecting and \( g_m \) determinations were repeated as described.

**RESULTS**

Fast \( A_N-C_i \) curves (30 to 45 min) were analysed in up to six different C\(_3\) species (Fig. 2). The initial portion of the curve showed almost linear dependence of \( A_N \) versus \( C_i \), indicating limitation by carboxylation. A second portion was clearly curvilinear, indicating limitation by regeneration of ribulose-1,5-bisphosphate. In three of the species (*Arabidopsis*, *Limonium* and *Vitis*), the curves displayed a third

![Figure 2](image-url)  
Figure 2. Response of net photosynthesis (\( A_N \), large symbols) and electron transport rate (\( J_{flu} \), crosshairs) to sub-stomatal CO\(_2\) concentrations (\( C_i \)) in (a) *Arabidopsis thaliana* (filled circles), (b) *Limonium giberrii* (empty circles), (c) *Nicotiana tabacum* (filled upwards triangles), (d) *Vitis berlandieri* × *Vitis rupestris* (empty upwards triangles), (e) *Cucumis sativus* (empty downwards triangles) and (f) *Olea europaea* var. *europaea* (open downwards triangles). Values are averages ± SE of three to six replicates, depending on the species.
portion consisting of constant or slightly declining $A_N$ with increasing $C_i$, indicating limitation by triose phosphate utilization. $J_{R_s}$ responded to $C_i$ in a biphasic mode in all species except Olea, with maximum $J_{R_s}$ being at $C_i$ between 200 and 600 μmol CO$_2$ mol$^{-1}$ air, depending on the species (Fig. 2). Such a biphasic response was already described by Sharkey, Berry & Sage (1988) under high light intensities, and it is thought to be due to feedback limitation by triose phosphate utilization. Because gas exchange was measured concomitantly with chlorophyll fluorescence, it was possible to estimate $g_m$ for most $C_i$ concentrations used, except at very low $C_i$, where $A_N$ was close to zero or negative, resulting in strongly variable and often not reliable $g_m$ estimates. At ambient CO$_2$, $g_m$ ranged from 0.127 mol CO$_2$ m$^{-2}$ s$^{-1}$ in Olea to 0.315 mol CO$_2$ m$^{-2}$ s$^{-1}$ in Limonium. If the bulk of the mesophyll resistance is in the liquid phase, it is theoretically more correct to include a pressure term in the units but for the purposes of this paper, we have used units without a pressure term for easier comparison between stomatal and mesophyll conductance values. Atmospheric pressure during these measurements was close to 101 kPa.

Contrary to what was usually assumed, $g_m$ was not constant along the range of $C_i$ used for $A_N$–$C_i$ curves (Fig. 3). It has been suggested that the reliability of $g_m$ data is questionable when $dC_i/dA_N$ is lower than 10 or higher than 50 (Harley et al. 1992). These values are shown by shaded areas in Fig. 3 (left, $dC_i/dA_N < 10$; right, $>50$). Taking into consideration the $g_m$ values within the reliable range, it is observed that $g_m$ decreases as $C_i$ increases in all the species studied (Fig. 3). At high $C_i$, $g_m$ values are as low as 5 to 30% those at lower $C_i$.

Transgenic tobacco plants differing in the expression of aquaporin NtAQP1 and $g_m$ (Flexas et al. 2006) also showed a similar dependence of $g_m$ on $C_i$ (Fig. 4). In these plants, the higher the $g_m$ at ambient CO$_2$, the stronger its dependency on $C_i$ was. Consequently, the largest differences between genotypes were observed at low $C_i$ and vice versa (Fig. 4).

Several tests were performed in Nicotiana to demonstrate the independence of this pattern on the experimental...
procedure. Firstly, the direction of changes in $C_a$ was analysed. Therefore, curves performed by initially decreasing $C_a$ from 400 $\mu$mol CO$_2$ mol$^{-1}$ air to 50 $\mu$mol CO$_2$ mol$^{-1}$ air, and then returned to 400 $\mu$mol CO$_2$ mol$^{-1}$ air and increased to 1800 $\mu$mol CO$_2$ mol$^{-1}$ air were compared to curves that were performed the opposite way. Curves of $A_S$, $J_{an}$ and $g_m$ versus $C_i$, were almost identical (data not shown).

The interference of light intensity to $g_m$ was also tested with several $A_S$–$C_i$, curves performed at lower light intensities (1000, 750 and 250 $\mu$mol m$^{-2}$ s$^{-1}$). Light intensity seemed to have some effect, because the dependency of $g_m$ on $C_i$ was larger at higher light intensity (Fig. 5). Nevertheless, $g_m$ declined with increasing $C_i$ in all cases. Finally, any possible effect that the short time employed between two consecutive measurements at different $C_i$ could produce on $g_m$ was tested. Different leaves were acclimated to different $C_i$ until steady state of both $A_S$ and $g_i$ was reached (typically 45 to 60 min after clamping the leaf). Data from different leaves at different $C_i$ were plotted together to produce an ‘artificial’ $A_S$–$C_i$ curve. The resulting plot was very similar to that of the ‘real’ $A_S$–$C_i$ curves shown in Fig. 3 (data not shown).

Therefore, it seems that, regardless of the procedure and conditions to modify $C_i$, $g_m$ showed a dependency of $C_i$ similar to that of $g_i$. The velocity of response of both conductances was tested in *Nicotiana* (Fig. 6). Firstly, $A_S$, $J_{an}$, $C_i$, $g_i$ and $g_m$ were measured in steady state at almost ambient CO$_2$, then $C_i$ was suddenly increased to 1500 $\mu$mol CO$_2$ mol$^{-1}$ air, and the leaves allowed to acclimate to the new conditions for about 1 h. $A_S$, $J_{an}$, $C_i$, $g_i$ and $g_m$ were then determined. Finally, $C_i$ was returned to 400 $\mu$mol CO$_2$ mol$^{-1}$ air to follow $g_i$ and $g_m$ recovery (Fig. 6). Both $g_i$ and $g_m$ decreased at high CO$_2$.

Then, when $C_a$ was returned to lower values, both conductances recovered progressively to their initial values. $g_m$ almost fully recovered in about 15 min, while $g_i$ needed about 45 min to fully recover.

The concomitant decrease of both $g_i$ and $g_m$ in response to increasing CO$_2$ strongly reduced the chloroplast CO$_2$ concentration ($C_C$) with respect to ambient ($C_a$), particularly at high CO$_2$ concentrations. Overall, the $C_i/C_a$ ratio ranged between 0.50 and 0.76 at 400 $\mu$mol CO$_2$ mol$^{-1}$ air and, in most species, increased at 1800 $\mu$mol CO$_2$ mol$^{-1}$ air (data not shown). By contrast, the $C_i/C_a$ ratio ranged only between 0.25 and 0.57 at 400 $\mu$mol CO$_2$ mol$^{-1}$ air and usually decreased at 1800 $\mu$mol CO$_2$ mol$^{-1}$ air (data not shown).

To discard any possible artefact inherent to the fluorescence method, two totally independent methods were used to prove the effect of $C_i$ on $g_m$. One experimental comparison was performed in leaves of *N. tabacum* to determine $g_m$ by online carbon isotope discrimination (Evans et al. 1986) at two different CO$_2$ concentrations, 400 and 1500 $\mu$mol CO$_2$ mol$^{-1}$ air (Table 1). During this experiment, a second Li-6400 with chlorophyll fluorescence chamber was attached to the same leaves to simultaneously determine $g_m$ by the Harley et al. (1992) method. Carbon isotope discrimination and fluorescence measurements resulted in similar estimations of $g_m$ and confirmed that, at high $C_i$, $g_m$ was substantially reduced. However, these results could be affected by differences in respiration and photorespiration at the two CO$_2$ concentrations, because total carbon isotope discrimination is affected by discrimination during both processes (Ghashghaie et al. 2003). While the contribution of respiration may be relatively small and not very different between CO$_2$ concentrations, the rate of photorespiration is strongly affected by CO$_2$, and hence its associated discrimination. Therefore, an additional experiment was performed.
in leaves of *N. sylvestris* to determine *g*<sub>m</sub> by online carbon isotope discrimination at two different CO<sub>2</sub> concentrations, 400 and 1000 μmol CO<sub>2</sub> mol<sup>-1</sup> air, in atmospheres containing either 21 or 1% O<sub>2</sub> (Table 2). The values obtained for δ<sup>13</sup>C<sub>o</sub> – δ<sup>13</sup>C<sub>e</sub> and Δ<sup>13</sup>C<sub>obs</sub> were similar to those in the previous experiment, and did not differ significantly in the absence of O<sub>2</sub> (Table 2). The results confirmed a decline of *g*<sub>m</sub> at high CO<sub>2</sub> and no significant differences at *P* < 0.05 were observed when measurements were taken at 21 or 1% O<sub>2</sub> (Table 2), thus suggesting that discrimination during photorespiration can be neglected for *g*<sub>m</sub> estimations.

An additional independent method, consisting in a novel curve-fitting of *A*-<sup>N</sup>-*C<sub>i</sub> curves was applied. This method allows solving *g*<sub>m</sub> at the two different regions of the curves (Ethier & Livingston 2004; Ethier et al. 2006), that is, together with *V*<sub>max</sub> when photosynthesis is limited by carboxylation (*g*<sub>m</sub> *V*<sub>max</sub>) and with *J*<sub>max</sub> when it is limited by RuBP regeneration ( *g*<sub>m</sub> *J*<sub>max</sub>). In three of the species (*Nicotiana*, *Limonium* and *Olea*), a few additional *A*-<sup>N</sup>-*C<sub>i</sub> curves were performed to include a larger number of points (i.e. different *C<sub>i</sub>*), allowing the application of this method. In *Arabidopsis*, the method could be applied using the same curves as for fluorescence. Clearly, this method also supported that *g*<sub>m</sub> varies along the *C<sub>i</sub>* gradient during *A*-<sup>N</sup>-*C<sub>i</sub> curves. In fact, averaging fluorescence-derived *g*<sub>m</sub> values from Fig. 3 for each *C<sub>i</sub>* region in each species resulted in a highly significant correlation with values estimated using the curve-fitting method (Fig. 7).

**DISCUSSION**

Although rapid changes of *g*<sub>m</sub> in response to CO<sub>2</sub> have previously been suggested (Centritto et al. 2003; Düring 2003), no detailed analysis of this response has yet been performed. The present data show, in six different species, that *g*<sub>m</sub> varies in the short term (minutes) by as much as five- to ninefold in response to changes in *C<sub>i</sub>* between 50 and 1200 μmol CO<sub>2</sub> mol<sup>-1</sup> air, that is, well within the timing and values generally used during the performance of *A*-<sup>N</sup>-*C<sub>i</sub> curves. *g*<sub>m</sub> strongly declines at high *C<sub>i</sub>* that is, when photosynthesis is no longer limited by CO<sub>2</sub> availability. This response has been confirmed modifying the timing and conditions during *A*-<sup>N</sup>-*C<sub>i</sub> curves, as well as by two totally independent methods for *g*<sub>m</sub> estimation, that is, online carbon isotope discrimination method (Evans et al. 1986) and the curve-fitting method (Ethier & Livingston 2004; Ethier et al. 2006). Recently, we have been informed that similar results have been obtained by two other research groups (Ethier and Pepin, personal communication; Warren, personal communication).

Using three totally independent methods, based on different assumptions, which show similar results decreases the possibility that the observed *g*<sub>m</sub> variations with CO<sub>2</sub> are due to artefacts during gas-exchange measurements or result from misleading estimations of the parameters involved in the calculations, particularly when using the fluorescence method. Several limitations of current portable gas-exchange systems have been considered and studied (Long & Bernacchi 2003): lateral leakage inside and outside the chamber which was determined and used to correct values (Flexas et al. 2007), lateral CO<sub>2</sub> diffusion outside and inside the leaf between the darkened area of the leaf under the chamber gasket and the leaf area of the light, although difficult to quantify, is considered to be minor especially when measurements are done at high light intensity (Jahnke & Krewitt 2002; Galmés et al. 2006). Regarding the parameters involved in *g*<sub>m</sub> calculations using the fluorescence method, the critical assumptions are: day respiration (*R*), CO<sub>2</sub> photocompensation point (*I*<sub>0</sub>), leaf absorptance (*α*), light partitioning between photosystems (*β*) and the absence of alternative electron-consuming reactions, such as the Mehler reaction. The fact that the relationship between φ<sub>PSII</sub> and φ<sub>CO2</sub> under low O<sub>2</sub> was similar when changing light intensity or CO<sub>2</sub> concentrations (see Fig. 1) suggests that *α* and *β* were not affected by CO<sub>2</sub> concentration. However, in addition to photosynthesis and photorespiration, other reactions such as the Mehler reaction or nitrite reduction have been shown to consume as much as 10% of the total *I*<sub>0</sub> (Miyake & Yokota 2000; Laisk...
and their incidence can be higher at ambient than at high CO₂ concentration (Miyake & Yokota 2000). Moreover, \( R_d \) may be underestimated when using the Laisk method (Pinelli & Loreto 2003), and high CO₂ has been sometimes reported to substantially reduce \( R_d \) (Gonzalez-Meler et al. 1996; Bruhn, Wiskich & Atkin 2007). Finally, using \( C_i^* \) instead of \( \Gamma^* \) may lead to further errors. These two parameters relate to each other following the equation: \( \Gamma^* = C_i^* + R_d/g_m \). As explained in M&M, \( g_m \) was not estimated close to the compensation point, and hence \( C_i^* \) was used as a proxy for \( \Gamma^* \). To assess the possibility that our conclusions were due to the interference of the possible bias in assumed parameters, their effects on \( g_m \) for several leaves of the different species were simulated.

An example of such simulations is shown in Table 3 for a leaf of Arabidopsis (simulations for other species resulted in qualitatively similar results, not shown). At ambient (400 \( \mu \text{mol} \text{CO}_2 \text{mol}^{-1} \text{air} \)) and high (1500 \( \mu \text{mol} \text{CO}_2 \text{mol}^{-1} \text{air} \)) CO₂, respectively, leaf temperature was 25.2 and 25.7 °C, \( A_N \) was 13.0 and 23.0 \( \mu \text{mol} \text{CO}_2 \text{m}^{-2} \text{s}^{-1} \), \( C_i \) was 283 and 1165 \( \mu \text{mol} \text{CO}_2 \text{mol}^{-1} \text{air} \), and \( J_{\text{an}} \) was 112 and 125 \( \mu \text{mol} \text{CO}_2 \text{m}^{-2} \text{s}^{-1} \). Using these data, together with similar simulations, their effects on \( g_m \) for several leaves of the different species were simulated.

\( g_m \) was estimated by two independent methods, a gas-exchange–chlorophyll fluorescence method \((g_m, \text{Harley})\) and online carbon isotope discrimination method \((g_m, \text{Evans})\). Values are averages ± SE of five replicates per CO₂ concentration. The \( C_i \) shown was calculated using the \( g_m \) values obtained using the Harley method.

\( g_m \) was estimated by online carbon isotope discrimination method. Values are averages ± SE of five replicates per CO₂ concentration.

### Table 1. Net photosynthesis (\( A_N \)), stomatal conductance (\( g_s \)), mesophyll conductance (\( g_m \)), sub-stomatal (\( C_i \)) and chloroplast (\( C_c \)) CO₂ concentrations, the difference in \( \delta^{13} \text{C} \) between the air leaving and entering the leaf cuvette (\( \delta^{13} \text{C}_{\text{air}} - \delta^{13} \text{C}_{\text{leaves}} \)) and the observed \( \delta^{13} \text{C} \) discrimination (\( \delta^{13} \text{C}_{\text{leaves}} \)) by leaves of Nicotiana sylvestris acclimated for 15 min to either 400 or 1500 \( \mu \text{mol} \text{CO}_2 \text{mol}^{-1} \text{air} \).

<table>
<thead>
<tr>
<th></th>
<th>( A_N ) (( \mu \text{mol} \text{CO}_2 \text{m}^{-2} \text{s}^{-1} ))</th>
<th>( g_s ) (( \mu \text{mol} \text{CO}_2 \text{m}^{-2} \text{s}^{-1} ))</th>
<th>( \delta^{13} \text{C}<em>{\text{air}} - \delta^{13} \text{C}</em>{\text{leaves}} ) (‰)</th>
<th>( \delta^{13} \text{C}_{\text{leaves}} ) (‰)</th>
</tr>
</thead>
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<tr>
<td>( A_N )</td>
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<td>( g_s )</td>
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<td>( \delta^{13} \text{C}<em>{\text{air}} - \delta^{13} \text{C}</em>{\text{leaves}} ) (‰)</td>
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<td>0.71 ± 0.08</td>
<td>12.4 ± 0.7</td>
</tr>
<tr>
<td>( \delta^{13} \text{C}_{\text{leaves}} ) (‰)</td>
<td>22.7 ± 0.7</td>
<td>0.075 ± 0.002</td>
<td>0.32 ± 0.02</td>
<td>11.2 ± 0.9</td>
</tr>
</tbody>
</table>

\( g_m \) was estimated by online carbon isotope discrimination method. Values are averages ± SE of five replicates per CO₂ concentration.

### Table 2. Net photosynthesis (\( A_N \)), stomatal conductance (\( g_s \)), mesophyll conductance (\( g_m \)), sub-stomatal (\( C_i \)) and chloroplast (\( C_c \)) CO₂ concentrations, the difference in \( \delta^{13} \text{C} \) between the air leaving and entering the leaf cuvette (\( \delta^{13} \text{C}_{\text{air}} - \delta^{13} \text{C}_{\text{leaves}} \)) and the observed \( \delta^{13} \text{C} \) discrimination (\( \Delta^{13} \text{C}_{\text{leaves}} \)) by leaves of Nicotiana sylvestris acclimated for 15 min to either 400 or 1000 \( \mu \text{mol} \text{CO}_2 \text{mol}^{-1} \text{air} \) and, in an atmosphere containing 21 or 1% O₂.

<table>
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<th>( g_s ) (( \mu \text{mol} \text{CO}_2 \text{m}^{-2} \text{s}^{-1} ))</th>
<th>( \delta^{13} \text{C}<em>{\text{air}} - \delta^{13} \text{C}</em>{\text{leaves}} ) (‰)</th>
<th>( \Delta^{13} \text{C}_{\text{leaves}} ) (‰)</th>
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\( g_m \) was estimated by online carbon isotope discrimination method. Values are averages ± SE of five replicates per CO₂ concentration.
strongly reduces the chloroplast CO₂ concentration (Cᵳ) with respect to ambient (Cᵢ), particularly at high CO₂ concentrations. The function of this regulation is unknown, and can only be speculated. One possibility is that this response serves to match CO₂ availability and photosynthetic capacity. When photosynthesis is limited by CO₂ availability, gᵳ tends to be higher (with some exceptions at low Cᵢ), which may result in increased CO₂ availability in the chloroplast stroma. Consequently, gᵳ decreases when photosynthesis is no longer limited by CO₂ availability, that is, at high Cᵢ, which may explain also why it seems to be lower when light is limiting photosynthesis (Fig. 5). A sustained high gᵳ at high Cᵢ, when photosynthesis cannot increase the rate of CO₂ consumption, would almost double the CO₂ concentration in the chloroplast stroma (Cᵳ) according to C/Cᵢ and Cᵳ/Cᵢ ratios. Maintaining both gᵳ and gᵳ high would result in about fourfold increase in Cᵢ. According to the known relationship between CO₂ concentration in water and pH, this could result in a significant decrease of stromal pH (up to about 0.3 to 0.5 units if stromal content is assumed to be pure water), which could be detrimental to photosynthesis due to the extreme pH sensitivity of photosynthetic enzymes (Berkowitz, Chen & Gibbs 1983; Pfanz 1995). While this possibility cannot be discarded at present, it is unlikely because efficient pH regulation mechanisms have been shown to operate in chloroplasts (Hauser et al. 1995; Oja et al. 1999; Savchenko et al. 2000). Alternatively, perhaps the mechanism leading to a high gᵳ requires energy, although this is unknown at present (see further discussion). If so, at high CO₂ where photosynthesis is limited by energy availability, increasing gᵳ would compete with photosynthesis for energy, and would be inefficient as the added CO₂ reaching the chloroplast would result in little increase of CO₂ fixation. The opposite would be true at low CO₂, that is, energy is in excess to that required for photosynthesis, and increased CO₂ availability would be beneficial for photosynthesis.

Despite substantial knowledge about the regulation of stomatal opening, the mechanisms leading to its response to Cᵢ are still unclear. It has been hypothesized that these may be related to malate-induced regulation of anion channels (Hedrich et al. 1994), to CO₂-related pH and membrane potential changes in guard cells (Hedrich et al. 2001), or to CO₂-mediated changes in photosynthesis in guard cells (Messinger, Buckley & Mott 2006), in a mechanism mediated either by zeaxanthin (Zeiger et al. 2002) or ATP (Buckley, Mott & Farquhar 2003). Much less is known about the regulation of mesophyll conductance variations, and until recently it was assumed that leaf structural properties were causing most gᵳ variations (von Caemmerer & Evans 1991; Lloyd et al. 1992). One of the few physiological bases known is the recent discovery that some aquaporins can be involved in the regulation of gᵳ (Hanba et al. 2004; Flexas et al. 2006). Actually, transgenic tobacco plants differing in the amounts of aquaporin NtAQP1 differ in their slope of gᵳ response to Cᵢ (Fig. 2), suggesting that NtAQP1 may also be involved in this response. While the responses of aquaporin physiology to

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**Figure 7.** Relationship between estimates of mesophyll conductance (gᵳ) by combined gas exchange and chlorophyll fluorescence (gᵳ Harley) and the curve-fitting method (gᵳ Ethier) at different regions of net photosynthesis and sub-stomatal CO₂ concentration (Aᵦ-Cᵦ) curves [maximum carboxylation capacity (Vᵦ), maximum capacity for electron transport rate (Jᵦ)]. For the Harley method, all values of gᵳ shown in Fig. 2 within each interval were averaged to get an 'average' gᵳ for each region. Notice that estimates by the two methods were made in the same species and Cᵢ intervals, but not in the same leaves and measuring time.

between 0.118 and 0.145 µmol CO₂ m⁻² s⁻¹ (i.e. close to the original estimation of 0.110 µmol CO₂ m⁻² s⁻¹) except when a Γ* of 58.3 µmol CO₂ mol⁻¹ air was considered, which yielded a much higher gᵳ (0.317 µmol CO₂ m⁻² s⁻¹). However, the latter value is unlikely, because a Γ* of 58.3 µmol CO₂ mol⁻¹ would reflect a specificity factor of only 64, that is, the lowest by far ever described for a C₃ species (Galmés et al. 2005). In addition, at high CO₂, all the simulated values ranged between 0.031 and 0.050 µmol CO₂ m⁻² s⁻¹. Therefore, although misleading assumptions in any of the parameters involved in gᵳ calculations may lead to variations in the absolute values of estimated gᵳ, none of them would impair the conclusion that gᵳ declines at high CO₂. In the case of the isotope method, the major limitation would be the interference of carbon isotope discrimination during photorespiration (Ghashghaie et al. 2003), which may be substantially different at ambient and high CO₂. However, the fact that similar gᵳ estimations were obtained under 21% and 1% O₂ regardless of CO₂ concentration, suggests that the interference of photorespiration does not affect gᵳ estimations. Altogether, it provides convincing evidence that decreased gᵳ at high CO₂ has a physiological basis, and does not result from artefacts in the methods used for its estimation.

Except for the species-dependent differences at low Cᵢ, the response of gᵳ to increasing Cᵢ resembles that known for gᵳ (Raschke 1979). Moreover, these data suggest that gᵳ responds faster to varying Cᵢ than does gᵳ. The data shown in Fig. 5 suggest that, as observed for gᵳ, gᵳ responds to changes in light intensity, although further studies are needed. Together, decreasing gᵳ and gᵳ as Cᵢ increases

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Table 3. Simulation of the effects of possible errors in the model parameters assumptions on mesophyll conductance ($g_m$) estimates using the chlorophyll fluorescence method by Harley et al. (1992) in a leaf of *Arabidopsis thaliana* at 400 $\mu$mol CO$_2$ mol$^{-1}$ air (ambient CO$_2$) or 1500 $\mu$mol CO$_2$ mol$^{-1}$ air (high CO$_2$).

A. Effect of possible alternative electron flow and its dependence on CO$_2$. In the first row, alternative electron-consuming reactions are assumed to use 10% of total electron transport rate ($J_{ha}$), while in the second row alternative electron-consuming reactions are assumed to use 10% of total $J_{ha}$ only at ambient CO$_2$, being negligible at high CO$_2$.

<table>
<thead>
<tr>
<th>$J_{ha}$ Ambient CO$_2$ ($\mu$mol e$^{-}$ m$^{-2}$ s$^{-1}$)</th>
<th>$J_{ha}$ High CO$_2$ ($\mu$mol e$^{-}$ m$^{-2}$ s$^{-1}$)</th>
<th>$g_m$ Ambient CO$_2$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
<th>$g_m$ High CO$_2$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>113</td>
<td>0.145</td>
<td>0.050</td>
</tr>
<tr>
<td>101</td>
<td>125</td>
<td>0.145</td>
<td>0.032</td>
</tr>
</tbody>
</table>

B. Effect of possible misleading estimates of $R_d$ and its dependence on CO$_2$. In the first row, $R_d$ is assumed to be equal to respiration in the dark ($R_o$). In the second row, in addition, $R_d$ at high CO$_2$ is assumed to be only 30% that at low CO$_2$.

<table>
<thead>
<tr>
<th>$R_d$ Ambient CO$_2$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
<th>$R_d$ High CO$_2$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
<th>$g_m$ Ambient CO$_2$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
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</tr>
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<tbody>
<tr>
<td>0.92</td>
<td>0.92</td>
<td>0.118</td>
<td>0.033</td>
</tr>
<tr>
<td>0.92</td>
<td>0.31</td>
<td>0.118</td>
<td>0.031</td>
</tr>
</tbody>
</table>

C. Effect of using apparent CO$_2$ photocompensation point ($C^*$) instead of chloroplastic CO$_2$ photocompensation point ($G^*$). Two estimations are made. In the first row, $g_m$ near the compensation point is assumed to be 0.1 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$, resulting in a $G^*$ of 58.3 $\mu$mol CO$_2$ mol$^{-1}$ air. In the second row, $g_m$ near the compensation point is assumed to be 0.4 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$, resulting in a $G^*$ of 46.4 $\mu$mol CO$_2$ mol$^{-1}$ air.

<table>
<thead>
<tr>
<th>$G^*$ Ambient CO$_2$ ($\mu$mol CO$_2$ mol$^{-1}$ air)</th>
<th>$G^*$ High CO$_2$ ($\mu$mol CO$_2$ mol$^{-1}$ air)</th>
<th>$g_m$ Ambient CO$_2$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
<th>$g_m$ High CO$_2$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
</tr>
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<tr>
<td>58.3</td>
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<td>0.317</td>
<td>0.047</td>
</tr>
<tr>
<td>46.4</td>
<td>46.4</td>
<td>0.144</td>
<td>0.037</td>
</tr>
</tbody>
</table>
varying CO₂ have not been studied, it has been shown that a change in cytosolic pH of 0.5 to 1.0 units can induce a strong gating of aquaporins through protonation of a histidine residue of the protein (Tournaire-Roux et al. 2003), therefore providing a potential mechanism for the observed CO₂-induced regulation. In addition, an energy-dependent gating based on phosphorylation has been described (Kjellbom et al. 1999), which would match the energy-dependent hypothesis for gₘ regulation raised earlier. An alternative mechanism, not based on aquaporin function, could be related to chloroplast swelling and shrinkage. Modifications in chloroplast shape, preventing close association between chloroplast and cell surface, have been shown to alter gₘ in phytochrome mutants of tobacco (Sharkey et al. 1991). Moreover, gₘ could be affected by chloroplast swelling or movements (Sharkey, personal communication; Tholen et al. 2007). Whether this occurs under high CO₂ remains unknown. Clearly, further studies would be needed to elucidate which mechanisms can mediate gₘ responses to CO₂.

Regardless of the physiological reasons for and the mechanisms involved in the regulation of the response of gₘ to CO₂, the observed variations during the performance of typical Aₚ–Cᵣ curves may induce errors on the photosynthesis parameterization. These Aₚ–Cᵣ curves are commonly used to develop prediction models of CO₂ assimilation for crops (Díaz-Espejo et al. 2006) and natural vegetation (Xu & Baldocchi 2003), to help predicting the effects of climate change on photosynthesis (Rogers et al. 2001), for scaling up from leaf to whole plant and/or ecosystem carbon assimilation models (Harley & Baldocchi 1995), and to assess the influence of stresses on the photosynthetic capacity of plants (Centritto et al. 2003; Loreto et al. 2003). Therefore, its correct parameterization is important, and this should take into account mesophyll conductance to CO₂, as already highlighted (Ethier & Livingston 2004). However, Aₚ–Cᵣ curves are often transformed to Aₚ–Cᵣ curves using a value for gₘ determined at ambient CO₂ and assuming a constant gₘ for the entire range of Cᵣ (Flexas et al. 2002; Manter & Kerrigan 2004; Grassi & Magnani 2005; Galmés et al. 2006), which may not be true according to the present results. The effect of neglecting changes of gₘ with varying Cᵣ on parameterization of Aₚ–Cᵣ curves is illustrated with a few examples in Fig. 8. In some cases, when gₘ is large, like in Arabidopsis (Fig. 8a) or Vitis (Fig. 8b), the Aₚ–Cᵣ curve determined by concomitant gas-exchange and chlorophyll fluorescence measurements along the entire range of Cᵣ differs from that estimated from a single gₘ value at ambient CO₂ at most on the maximum value of Cᵣ attained, while the differences in parameterization of Vₑ,max and Jₑ,max are negligible. In other cases, however, when gₘ is largely reduced, such as under severe water stress, the effect is expected to be substantial, because the difference between Cᵣ and Cₑ becomes greater. This is illustrated by the response of a Vitis leaf subjected to severe water stress (Fig. 8c). Assuming a constant gₘ may have led to an estimation of Vₑ,max of only 19 μmol m⁻² s⁻¹, accompanied by a very low Jₑ,max. However, taking into account the variations of

![Figure 8](image-url)  
Figure 8. Examples of the effects of neglecting mesophyll conductance (gₘ) variations with sub-stomatal CO₂ concentration (Cₑ) on the parameterization of Aₚ–Cᵣ curves. Aₚ–Cᵣ curves were determined by concomitant gas-exchange and chlorophyll fluorescence measurements along the entire range of CO₂ concentrations in the atmosphere (Cₑ, filled circles) or from a single gₘ value determined at ambient CO₂ (empty circles). The curves shown are for well-irrigated Arabidopsis (a), a well-irrigated Vitis (b) and a severely water-stressed Vitis (c). At 400 μmol CO₂ mol⁻² air, estimated gₘ values were 0.200, 0.291 and 0.005 μmol CO₂ m⁻² s⁻¹ for Arabidopsis, irrigated Vitis and stressed Vitis, respectively. Values of maximum carbon evolution capacity (Vₑ,max) and maximum capacity for electron transport rate (Jₑ,max) estimated for each curve are shown in the inset.
with $C_i$ results in an estimation of $V_{c,max}$ of up to 107 mol m$^{-2}$ s$^{-1}$, that is, only slightly depressed as compared to the irrigated plant, while $J_{max}$ cannot be determined. The latter result matches better biochemical evidence in water-stressed plants (Flexas et al. 2004).

CONCLUSION

In conclusion, the present study clearly demonstrates that mesophyll conductance to CO$_2$ changes in response to varying CO$_2$; even faster than does stomatal conductance, and this should be taken into account for a correct parameterization of $A_{NP}$--$C_i$ curves, particularly when $g_m$ is low as, for instance, under stress. These results are striking and urge the need for studies regarding both the function and the physiological and molecular basis of such regulation.

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