Dynamic changes of canopy-scale mesophyll conductance to CO₂ diffusion of sunflower as affected by CO₂ concentration and abscisic acid

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ABSTRACT

Leaf-level measurements have shown that mesophyll conductance (gₘ) can vary rapidly in response to CO₂ and other environmental factors, but similar studies at the canopy-scale are missing. Here, we report the effect of short-term variation of CO₂ concentration on canopy-scale gₘ and other CO₂ exchange parameters of sunflower (Helianthus annuus L.) stands in the presence and absence of abscisic acid (ABA) in their nutrient solution. gₘ was estimated from gas exchange and on-line carbon isotope discrimination (Δ₁₃C) in a ¹³CO₂/¹₂CO₂ gas exchange mesocosm. The isotopic contribution of (photo)respiration to stand-scale Δ₁₃C was determined with the experimental approach of Tcherkez et al. Without ABA, short-term exposures to different CO₂ concentrations (Ca 100 to 900 µmol mol⁻¹) had little effect on canopy-scale gₘ. But, addition of ABA strongly altered the CO₂-response: gₘ was high (approx. 0.5 mol CO₂ m⁻² s⁻¹) at Ca < 200 µmol mol⁻¹ and decreased to <0.1 mol CO₂ m⁻² s⁻¹ at Ca >400 µmol mol⁻¹. In the absence of ABA, the contribution of (photo)respiration to stand-scale Δ₁₃C was high at low Ca (7.2‰) and decreased to <2‰ at Ca >400 µmol mol⁻¹. Treatment with ABA halved this effect at all Ca.

Key-words: Helianthus annuus; canopy CO₂ exchange; mesocosm; mesophyll and stomatal conductance; photosynthesis; photosynthetic and (photo)respiratory ¹³C discrimination; respiration.

INTRODUCTION

Mesophyll conductance (gₘ) limits the diffusive flux of CO₂ from the substomatal air space to the sites of carboxylation (Lloyd et al. 1992; Epron et al. 1995; Evans & von Caemmerer 1996; Flexas et al. 2008). This limitation is complex, as it involves diffusion through intercellular airspaces and in the liquid phase through cell wall, plasma membrane and cytosol, and the envelope and stroma of the chloroplast (Evans et al. 2009). Recent research has revealed strong short-term, reversible and acclimation responses of gₘ to several environmental factors, such as light, temperature, drought, salinity and CO₂ (see Flexas et al. 2008 for a compilation of gₘ responses). This reversible or adaptive component appears to be associated with the conductance of membranes, which may be modified by the expression of cooporins (Hanba et al. 2004; Flexas et al. 2007), which are aquaporins capable of transporting CO₂ across plasma membranes (Terashima et al. 2006).

Interestingly, adaptive responses of gₘ to single environmental drivers do not always agree in direction and magnitude in different experiments with different species. In most cases, gₘ increases with decreasing atmospheric CO₂ (e.g. Flexas et al. 2007). However, gₘ did not respond to CO₂ in Triticum aestivum (Tazoe et al. 2009) and Raphanus sativus (von Caemmerer & Evans 1991). Finally, also a biphasic response of gₘ has been found in Helianthus annuus (Vrabl et al. 2009) with gₘ increasing with increasing CO₂ below 200 µmol mol⁻¹ and decreasing when CO₂ was increased beyond.

The negative gₘ response to water stress and salinity is fairly consistent among species (see compilation of Flexas et al. 2008). Also, the majority of studies agree that gₘ decreases with leaf age, decreasing nitrogen content and shading. However, these factors often co-vary, making it difficult to unravel the underlying mechanism. Piel et al. (2002) showed a marked difference in gₘ between sun and shade leaves of Juglans sp., but these leaves also differed in nitrogen content. A similar co-variation was observed by Niinemets et al. (2005) in Mediterranean evergreen species, where the decreasing nitrogen content with leaf age was accompanied by decreasing gₘ. In herbaceous plants older leaves become successively more shaded during canopy development when nitrogen is re-allocated to younger leaves formed near the top of the canopy (Connor, Sadras & Hall 1995). Both factors may affect gₘ.

The gₘ response to a particular environmental driver – for example, CO₂ – may well depend on interactions with other factors. However, such studies are rare. No interaction between irradiance and CO₂ was observed as either both parameters did not affect gₘ (Triticum aestivum; Tazoe et al. 2009) or lowering irradiance decreased gₘ but had no effect...

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on the general response to CO₂ (Banksia species; Hassiotou et al. 2009). When studying the interaction of CO₂ and drought on gₑ, care must be taken for co-variating parameters as explained earlier. Drought induced changes in relative water content might affect gₑ via anatomical or morphological changes without altering the physiology. In this respect, exogenous application of abscisic acid (ABA) is a useful tool to mimic drought effects on stomatal conductance without affecting the morphology of leaves.

Thus, in this work, we addressed the following main questions: (1) does canopy-scale gₑ respond dynamically to short-term variation of CO₂ similar to the response of leaves? and (2) does ABA influence the gₑ response to CO₂?

To this end, we used a mesocosm-scale ¹³CO₂/¹²CO₂ gas exchange method (Schnyder et al. 2003) and we estimated gₑ using the carbon isotope-based approach of Evans et al. (1986), Evans & von Caemmerer (1996) and Flexas et al. (2007), as modified for canopy-scale measurements according to Tcherkez et al. (2010). Finally, we minimized isotopic non-steady states (which can cause artefacts in the determination of gₑ; Tazoe et al. 2009) by growing plants and performing all ¹³CO₂/¹²CO₂ exchange measurements in the same isotopic environment.

MATERIALS AND METHODS

Plant material and growth conditions

Plants of sunflower (Helianthus annuus L., cv. Optisol, CARG, BSA-Nr. 241) were sown individually in plastic pots (35 cm deep, 5 cm diameter), filled with 0.8 kg of washed quartz sand (0.4–0.8 mm particle size), transferred to four growth cabinets (Conviron E15, Conviron, Winnipeg, Canada) and arranged at a density of 78 plants m⁻². Throughout the experiment an automatic drip irrigation system supplied nutrient solution [modified half-strength Hoagland solution with 7.5 mM N L⁻¹; composition: 2.5 mM KNO₃, 2.5 mM Ca(NO₃)₂, 1 mM MgSO₄, 0.5 mM KH₂PO₄, 0.1 mM Fe–ethylene-diaminetetraacetic acid (EDTA), 23 mM H₂BO₃, 4.6 μM MnCl₂, 0.4 μM ZnSO₄, 0.16 μM CuSO₄, 0.26 μM H₂MoO₄] to every pot every 4 h. The system allowed a high frequency of sampling (approx. one sample per 2 min), so that each inlet and outlet of the four growth cabinets was sampled every 25 min. Each sample was compared with a VPDB-gauged working standard (IRMS, Delta plus, Finnigan MAT, Bremen, Germany). Air for mass spectrometric analysis was pumped continuously via a steel capillary from the gas sampling system to a 300 μL sample loop which was attached to a six-port Valco valve (Valco Instruments Co. Inc., Houston, TX, USA) mounted in a GC interface (GP-GC Interface, Finnigan MAT, Bremen, Germany). Air samples were fed to the mass spectrometer via periodically flushing the sample loop with He carrier gas and flushing the content through a Nafion® water trap and a GC column (25 m x 0.32 mm Poraplot Q, Chrompack, Middelburg, Netherlands). Then, the CO₂ was introduced into the ion source of the IRMS via a glass capillary (0.1 mm i.d.) connected to the interface via an open split.

The system allowed a high frequency of sampling (approx. one sample per 2 min), so that each inlet and outlet of the four growth cabinets was sampled every 25 min. Each sample was compared with a VPDB-gauged working standard reference CO₂ gas. The external precision (standard deviation) at 300 μmol mol⁻¹ CO₂ was <0.2‰ for δ¹³C.

During the entire experiment, including the establishment phase of the stands, two of the growth cabinets were supplied with CO₂ from a fossil-organic source (δ¹³C = –47.1‰) and two with CO₂ from a mineral source (δ¹³C = –5.4‰; both CO₂: Messer Griesheim, Frankfurt a.M., Germany).

Rates of net CO₂ exchange in light (A, μmol CO₂ m⁻² s⁻¹) were calculated as the difference between the CO₂ fluxes entering (Fₑ, μmol CO₂ s⁻¹) and leaving (Fₑat) the cabinets divided by the cabinets’ ground area (s, 1.4 m²):

$$A = (Fₑ - Fₑat)/s.$$  (1)

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Calculation of $g_m$

According to Fick’s first law, $g_m$ is expressed as,

$$g_m = \frac{A}{C_i - C_e},$$  

with $A$ the net photosynthesis rate of the stand and $C_i$ and $C_e$ the CO$_2$ concentrations in the intercellular airspace and in the chloroplast. Canopy-scale on-line carbon isotope discrimination ($\Delta_{obs}$) is (Tcherkez et al. 2010):

$$\Delta_{obs} = a_0 \frac{C_a - C_i}{C_a} + a \frac{C_i - C_e}{C_a} + a_m \frac{C_i - C_e}{C_a} + b \frac{C_e}{C_a} \frac{d^*}{C_a},$$  

(5a)

where

$$d^* = eR_e/k + e_hR_h/C_a + A + f\Gamma^*,$$  

(5b)

with $C_a$ and $C_e$ denoting the CO$_2$ concentration in the atmosphere and at the leaf surface, $a_0$ the fractionation during diffusion through the leaf boundary layer; $a$ that during diffusion in air, $a_m$ the combined fractionation during dissolution of CO$_2$ in water and fractionation during diffusion of dissolved CO$_2$ in water; $b$ the fractionation associated with carboxylations, $R_e$ the rate of leaf dark respiration in light; $R_h$ the dark respiration of heterotrophic plant parts (root and stem), $k$ the carboxylation efficiency, $\Gamma^*$ the CO$_2$ compensation point in the absence of dark respiration, $f$ the fractionation associated with photorespiration, $e$ the fractionation associated with leaf dark respiration in light and $e_h$ the fractionation associated with respiration of heterotrophic plant parts. $d^*/C_a$, the last term on the right-hand side of Eqn 5a, represents the effect of total mesocosm respiration in light on $\Delta_{obs}$. This includes photorespiration and daytime dark respiration of autotrophic (leaf) and heterotrophic plant parts and soil, and was termed (photo)respiration (in accordance with Tcherkez et al. 2010). This term is a potentially large component of $\Delta_{obs}$ as respiration is a much bigger fraction of net CO$_2$ exchange at mesocosm than at leaf scale, and carbon isotope fractionation in photorespiration and dark respiration of different plant parts is significant (Ghashghaie et al. 2003; Klumpp et al. 2005). The contribution of (photo)respiration to $\Delta$, $d^*/C_a$ was obtained as:

$$d^*/C_a = \Gamma(b - \Delta^*)/C_a$$  

(6)

(cf. Eqn 3 in Tcherkez et al. 2010), where $\Delta^*$ denotes $\Delta$ at the CO$_2$ compensation point, $\Gamma$ of the mesocosm. $\Delta^*$ was estimated as the intercept of a linear regression of $\Delta_{obs}$ versus $A$ (‘second method’ in Tcherkez et al. 2010) using the data obtained during the CO$_2$ response measurements (as presented in Fig. 2a,c). This method has the advantage, that it does not require assumptions on the rates of (photo)respiration of leaves, and respiration of roots and stems and their specific fractionation factors (see discussion in Tcherkez et al. 2010).

Mesophyll conductance was calculated using the difference between $\Delta_{obs}$ (Eqn 3) and an estimate of $\Delta$ assuming infinite $g_m$ ($\Delta$):

$$\Delta_i = a_0 \frac{C_a - C_i}{C_a} + a \frac{C_i - C_e}{C_a} + a_m \frac{C_i - C_e}{C_a} + b \frac{C_e}{C_a} \frac{d^*}{C_a},$$  

(7)
within the top 20 cm of the canopy. Measured using six thermocouples evenly distributed between

The last term in Eqn 7 \[ \Delta \equiv \Delta_{\text{ba}} \] is the same as the last term in Eqn 5a. Thus, any difference between \( \Delta \) and \( \Delta_{\text{obs}} \) was caused by the difference between \( C_i \) and \( C_a \):

\[
\Delta_{\text{obs}} - \Delta_{\text{ba}} = (b - a_m) \frac{C_i}{C_a} - (b - a_m) \frac{C_i}{C_a} \tag{8}
\]

with \( a_m = 1.8 \% \) (Vogel, Grootes & Mook 1970; O’Leary 1984).

Equation 8 could be solved for \( C_a \) as the only unknown and used to calculate \( g_m \) using Eqn 1.

**RESULTS**

**Canopy gas exchange and \( ^{13}C \) discrimination under growth conditions**

\( A \) and \( \Delta_{\text{obs}} \) of sunflower canopies were measured in four different mesocosms under growth conditions. Measurements started after canopy closure when \( A \), and \( \Delta_{\text{obs}} \) were near-constant on a day-by-day basis (Fig. 1 and Table 1). \( A \) averaged 15.6 \( \mu \text{mol} \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1} \) in the four chambers and varied little between them (Table 1). Similarly, \( A \) was quite stable during the course of individual light periods. In general, it decreased slightly between 1 and 15 h in light (Fig. 1a). \( \Delta_{\text{obs}} \) was high in all growth chambers (average 24.1%). But it was virtually the same in growth chambers with different \( \delta^{13} \text{CO}_2 \) (Table 1).

**Effect of ABA on gas exchange and \( ^{13}C \) discrimination under growth conditions**

ABA had strong effects on \( A \) and \( \Delta_{\text{obs}} \) (Fig. 1). \( A \) and \( \Delta_{\text{obs}} \) started to decrease immediately after ABA addition to the nutrient solution (data not shown). The effect of ABA on \( A \) and \( \Delta_{\text{obs}} \) saturated after about 2 to 3 d when \( A \) and \( \Delta_{\text{obs}} \) stabilized at near-constant values. At that time \( A \) was 11.8 \( \mu \text{mol} \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1} \), or 25% less than the rate just prior to ABA addition. Again, there was a slight decrease in \( A \) throughout the light period. \( \Delta_{\text{obs}} \) was 17.2\%, i.e. 6.9\% less than before ABA addition (Table 1).

ABA also strongly affected the relationship between \( \delta^{13} \text{C} \) of net CO2 exchanged (\( \delta_{\text{am}} \)) and the CO2 respired in the night (\( \delta_{\text{an}} \)) or the (photo)respired CO2 (\( \delta_{\text{ap}} \)). In control canopies, \( \delta_{\text{am}} \) was approximately 5% enriched relative to \( \delta_{\text{an}} \) (Table 2). Conversely, \( \delta_{\text{ap}} \) was depleted by 2.6% relative to \( \delta_{\text{an}} \). After ABA application, \( \delta_{\text{am}} \) was still slightly enriched relative to \( \delta_{\text{an}} \) (approx. 1%\%; Table 2). ABA had a particularly strong effect on \( \delta_{\text{ap}} \), which was 3%\% enriched relative to \( \delta_{\text{an}} \) after ABA addition, but was significantly depleted before addition.

**Effect of ABA on the CO2 response of \( A, \Delta_{\text{obs}} \) and transpiration**

The CO2 response of \( A, \Delta_{\text{obs}} \) and canopy conductance was measured on the day before first ABA addition, and after 3 d of ABA addition, when \( A \) and \( \Delta_{\text{obs}} \) had decreased to a stable level. All measurements were performed between 2 h of ‘lights on’ and 6 h before the end of the light period, when \( A \) and \( \Delta_{\text{obs}} \) were near constant. In the absence of ABA, \( A \) followed the characteristic response of photosynthesis to changes in CO2 partial pressures (\( C_i \)) as described by leaf or cell-based models of Farquhar, von Caemmerer & Berry (1980) and von Caemmerer and Farquhar (1981) (Fig. 2a). Transpiration also showed a strong response to increasing \( C_i \) (Fig. 2b), with the rate decreasing by 30% between 100 and 900 \( \mu \text{mol} \text{ mol}^{-1} \text{ CO}_2 \). Furthermore, \( \Delta_{\text{obs}} \) increased greatly with increasing \( C_i \) (Fig. 2c). At 900 \( \mu \text{mol} \text{ mol}^{-1} \text{ CO}_2 \) \( \Delta_{\text{obs}} \) was 28.3\% [± 0.1 standard deviation (SD)], close to the \( ^{13} \text{C} \) discrimination value of Rubisco.
ABA strongly modified the short-term CO₂ responses of A, Δobs and transpiration. ABA had no effect on A at C₅ < 150 μmol mol⁻¹, but at higher C₅, A was significantly reduced relative to the control (Fig. 2a). At a C₅ of 900 μmol mol⁻¹ A was reduced by 25% relative to the control.

Transpiration (E) was affected more strongly by ABA addition (Fig. 2b) and the reduction, relative to the control, was similar (near 40%) over the whole range of C₅. Overall, ABA caused a substantial improvement of water use efficiency (A/E) at all C₅ (Supporting Information Fig. S1). ABA did not affect the sensitivity of E to C₅ when this sensitivity was expressed as the change in E produced by a small change in C₅ (dE/dC₅). However, ABA did affect the response of E to C₅; an increase from 120 to 860 μmol mol⁻¹ CO₂ caused a reduction in E by 20% in the absence of ABA, while the same change caused a reduction of 40% in the presence of ABA.

ABA and C₅ had strong interactive effects on Δobs. Δobs was unaffected by ABA when C₅ was low. But, the responses of Δobs to C₅ diverged strongly when C₅ was increased above 150 μmol mol⁻¹; Δobs decreased in the presence of ABA, but increased in its absence. At a C₅ of 500 μmol mol⁻¹, Δobs was 15% less in the presence of ABA, 12% less than in its absence. In both treatments, changes of C₅ above 500 μmol mol⁻¹ had little effect on Δobs.

Effect of ABA on the components of Δobs

Using δ¹³C/C₅ (Eqn 6), the (photo)respiratory contribution to Δobs could be calculated for each measurement taken during the CO₂ response (Fig. 3). This term described the total effect of all respiratory activities of the mesocosm (including photorespiration) on Δobs measurements in light. In control canopies (photo)respiratory discrimination was a very significant component of Δobs, at low C₅, i.e. 7.2‰ at C₅ of 120 μmol mol⁻¹ decreasing strongly with increasing C₅ (1‰ at the highest C₅). After ABA application (photo)respiratory discrimination showed a similar shape of the response to a change in C₅. However, at each CO₂ concentration, the contribution to Δobs was only half of that in control canopies (3.8 and 0.6‰ at the lowest and highest C₅, respectively).

Effect of ABA on the CO₂ response of mesophyll conductance

Canopy-scale mesophyll conductance was calculated from estimates of C₃ and C₅, and the net rate of CO₂ fixation (A), using Fick’s first law (Eqn 1). Estimates of C₅ were obtained from E, leaf temperature and the vapour pressure deficit of chamber air. Canopy conductance was very high and was ignored in the estimation of C₅. Estimates of C₅ were obtained with Eqn 8, using parameters as given in the Materials and Methods section.

In control canopies, the difference ΔA – Δobs was highest at low C₅ and decreased nearly linearly with increasing CO₂ concentration (Fig. 4a). Again, there was virtually no difference in ΔA – Δobs between ABA-treated and control canopies at low C₅. But ΔA – Δobs increased with ABA application up to 400 μmol mol⁻¹ C₅, but did not change with higher CO₂ concentration.

Accordingly, gₚ in control canopies averaged 0.45 mol CO₂ m⁻² s⁻¹ and hardly responded to C₅ (Fig. 4b). In contrast, gₚ in ABA-treated canopies strongly responded to C₅: at low C₅, gₚ was in the range observed in control canopies, but it decreased exponentially with C₅ and attained 0.1 mol CO₂ m⁻² s⁻¹ at the highest CO₂ concentration.
Taken together, the CO2 responses of gs (not shown, but see Fig. 2b) and gm implied that the Cc to Ca ratio of ABA-treated canopies decreased with Ca, while it increased in controls (Fig. 5a). In ABA-treated canopies the ratio was ~0.8 at a Ca of <200 µmol mol⁻¹, decreased to 0.6 up to 400 µmol mol⁻¹ and remained at ~0.6 beyond 400 µmol mol⁻¹. Conversely, in the control treatment, the ratio increased from ~0.8 at a Ca of <200 µmol mol⁻¹ to ~0.95 at 850 µmol mol⁻¹. Nevertheless, Cc (and Ci) increased continuously with Ca in both treatments (although to different extents), meaning that the degree of CO2 limitation of Rubisco decreased with Ca (Fig. 5b,c). Decreasing CO2 to low Ca reversed this effect.

As discussed by Warren (2006), the estimation of gm with the isotopic method depends on the values of the parameters used for the estimation of ∆. These include the rate of (photo)respiration, the fractionation associated with photorespiration and dark respiration in light and the fractionation by carboxylases (cf. Eqn 7), which are all associated with uncertainties. Therefore we elected to use the approach of Tcherkez et al. (2010) to estimate discrimination associated with total mesocosm respiration in light (termed ‘(photo)respiration’ and including all photorespiration, and dark respiration by autotrophic and heterotrophic components of the system in the light). This approach avoided assumptions about individual respiratory fractionation factors and rates of photo- and dark respiration in light. The integral contribution of these respiratory activities to mesocosm-scale ∆obs was especially important.

DISCUSSION

This work revealed a significant co-limitation of canopy photosynthesis by gm in sunflower under ambient CO2. Short-term variation of CO2 had little effect on gm in control conditions, but addition of ABA led to dynamic CO2-responses of gm. In the presence of ABA, gm decreased very strongly and rapidly with increasing CO2, significantly decreasing Cc below Ca at ambient to elevated Ca. Decreasing CO2 to low Ca reversed this effect.

As discussed by Warren (2006), the estimation of gm with the isotopic method depends on the values of the parameters used for the estimation of ∆. These include the rate of (photo)respiration, the fractionation associated with photorespiration and dark respiration in light and the fractionation by carboxylases (cf. Eqn 7), which are all associated with uncertainties. Therefore we elected to use the approach of Tcherkez et al. (2010) to estimate discrimination associated with total mesocosm respiration in light (termed ‘(photo)respiration’ and including all photorespiration, and dark respiration by autotrophic and heterotrophic components of the system in the light). This approach avoided assumptions about individual respiratory fractionation factors and rates of photo- and dark respiration in light. The integral contribution of these respiratory activities to mesocosm-scale ∆obs was especially important.
at low $C_a$, where the (photo)respiratory signal accounted for up to 35% of $\Delta_{obs}$ in the control mesocosm (Fig. 3). This derived from the fact that the ratio of (photo)respiration to gross photosynthesis is highest at low $C_a$ and decreases with increasing $C_a$.

Importantly, the ranking of the treatments and the shape of the response functions of $g_m$ to $C_a$ were also not altered when we modified the fractionation factors affecting $d^p$ ($b$ and $\Delta^p$, see Eqn 6) in a wide range. Increasing (decreasing) $\Delta^p$ led to decreasing (increasing) $g_m$ in both treatments without affecting their ranking. In ABA-treated canopies, for example, an increase of $\Delta^p$ from 21.4‰ (i.e. the measured value) to 25‰ caused a decrease in $g_m$ of 13%. Increasing (decreasing) $b$, led to lower (higher) estimates of $g_m$ in both treatments, again with no effect on treatment ranking. The same was true for the shape of the $g_m$ response to $C_a$. Also, consideration of canopy-scale heterogeneity in stomatal conductance of leaves (which may vary as a function of leaf position/irradiance, age and ABA effects) would not alter our conclusions. Lloyd et al. (1992) have shown that even in situations of very high heterogeneity of stomatal conductance the error in the difference between $\Delta$ and $\Delta_{obs}$ would not exceed 1‰. This error is only a small fraction of the ABA effect on $\Delta - \Delta_{obs}$ observed in our study. Finally, we are very confident that artefacts in $\Delta_{obs}$ measurements were insignificant: we observed the same $\Delta_{obs}$ for canopies growing in the presence of CO$_2$ with different $\delta^{13}C$ (Table 1), meaning that there were no leaks in the chambers, which could have influenced mesocosm $^{13}$CO$_2$/$^{12}$CO$_2$ exchange and, hence, $\Delta_{obs}$ measurements (Schnyder 1992). Moreover, the external precision of $^{13}$CO$_2$/$^{12}$CO$_2$ measurements was very high (SD < 0.2‰). This measure of precision included all errors associated with CO$_2$-free air generation, source CO$_2$ composition, gas mixing, sampling and determination of $^{13}$CO$_2$/$^{12}$CO$_2$ ratios (see Schnyder et al. 2003). Lastly, all $^{13}$CO$_2$/$^{12}$CO$_2$ exchange measurements were performed in the same isotopic environment as stand growth.

The data also demonstrate that daytime (photo)respired CO$_2$ was $^{13}$C-depleted by a few permil relative to CO$_2$ net fixed, whereas CO$_2$ respired in darkness was enriched by several permil (Table 2). This variation was also observed in previous work at mesocosm-scale with sunflower growing at 200 and 1000 µmol mol$^{-1}$ CO$_2$ (Tcherkez et al. 2010) and in leaf-scale studies (Ghashghaie et al. 2003; Tcherkez et al. 2004) and is probably related (at least in part) to diurnal variations in the isotopic composition of sucrose (Gessler et al. 2008), the main substrate for respiratory metabolism.
These variations result from an isotope effect of aldolases (Gleixner & Schmidt 1997) that catalyse the production of fructose-1,6-bisphosphate from triose phosphates. This causes a circadian variation in the $^{13}$C content of sucrose, depleting sucrose formed in light, and enriching sucrose formed from transitory starch at night (Tcherkez et al. 2010). Perhaps, the divergent effect ABA on $\delta_{13}$C is also related to an ABA effect on C metabolism. Water stress can change C partitioning between starch and soluble sugars (sucrose and hexoses) synthesis in favour of sugars (Vassey & Sharkey 1989; Kaneki et al. 1998), to assist osmotic adjustment (Villadsen, Rung & Nielsen 2005). External addition of ABA can also cause changes in the activity of enzymes associated with primary carbohydrate metabolism (Yang et al. 2004). In this context, it is interesting to note that after ABA application, the (photo)respiratory flux was slightly $^{13}$C-enriched relative to that net fixed (Table 2). This effect could be related to an inhibition of starch biosynthesis by ABA, which would lead to less negative $\delta^{13}$C of triose phosphates exported from chloroplasts during the light period. This effect would also cause a less negative $\delta^{13}$C of sucrose synthesized in the cytoplasm and exported to other plant parts.

The $g_m$ responses observed here were highly dynamic and fully reversible, and were unrelated to anatomical features. This corroborates the view that $g_m$ has a protein- or enzyme-facilitated component (Bernacchi et al. 2002; Warren & Dreyer 2006; Flexas et al. 2007) with aquaporins as likely candidates. In tobacco leaves, the highest concentration was found in cells adjoining to the substomatal cavities (Otto & Kaldenhoff 2000). Aquaporins can mediate CO$_2$ transport across membranes (Maurel et al. 2008), and in tobacco were shown to enhance membrane permeability for CO$_2$ (Uehlein et al. 2003). Overexpression in rice (Hanba et al. 2004) and tobacco (Flexas et al. 2006) significantly increased $g_m$. Flexas et al. (2007) hypothesized that maintaining high $g_m$ could be an energy consuming process. Thus, at low $C_{i}$, when CO$_2$ availability is limiting photosynthesis, the excess energy could be used to increase $g_m$. At high $C_{i}$, diverting energy to maintain a high $g_m$ would be less efficient, as the extra CO$_2$ at the site of carboxylation would result in only little additional CO$_2$ fixation. In our study, the modulation of $g_m$ seemed strictly dependent on the presence of ABA. The mechanism underlying this effect is presently unclear. However, if aquaporins played a role, then it should be interesting to investigate ABA effects on their CO$_2$ transport function. Interestingly, the CO$_2$ response of $g_m$ observed at the mesocosm scale, and the effect of ABA on this response, differed from that observed at leaf-scale by Vrabl et al. (2009). In the presence of ABA, they found a similar CO$_2$ response of $g_m$ in the reliable range of their data. However, in controls, they also observed a decrease of $g_m$ with increasing CO$_2$, while we found no effect. Absence of a CO$_2$ effect on $g_m$ has also been observed by others (see compilation in Flexas et al. 2008). It is well possible, that the difference between the leaf- (Vrabl et al. 2009) and the present mesocosm-scale CO$_2$ response of $g_m$ was related to interactions with other external factors, such as light and CO$_2$ exposure of plants. The leaf measurements of Vrabl et al. occurred in high light (irradiance was $>$2 times higher than during growth), whereas our measurements were performed at the same irradiance as during growth (600 $\mu$mol m$^{-2}$ s$^{-1}$ PPDF). Interestingly, Flexas et al. (2007) showed that in tobacco the response of $g_m$ to a change in CO$_2$ concentration was far less pronounced at low compared with high light intensity during measurements. Furthermore, Vrabl et al. (2009) measured the CO$_2$ response on a portion of a leaf, whereas the remaining part and all other leaves experienced a different CO$_2$ concentration. This contrasts with the mesocosm-scale response, which was obtained by exposing all leaves to the same CO$_2$. Lastly, Vrabl et al. (2009) measured only young leaves, whereas all leaf-age categories of plants contributed to the mesocosm-scale measurement. It is known that physiological responses to ABA addition can depend on interactions with particular environmental scenarios (Tardieu, Parent & Simonneau 2010), and availability of light could be an important factor.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Water-use efficiency (WUE) in ABA-treated (open symbols) and control (full symbols) sunflower canopies as a function of $C_a$. WUE was calculated as the ratio of $A$ to $E$. Lines are fitted for ease of comparison.

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