Mapping Intercellular CO₂ Mole Fraction (Cᵢ) in Rosa rubiginosa Leaves Fed with Abscisic Acid by Using Chlorophyll Fluorescence Imaging

Significance of Cᵢ Estimated from Leaf Gas Exchange

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Imaging of photochemical yield of photosystem II (PSII) computed from leaf chlorophyll fluorescence images and gas-exchange measurements were performed on Rosa rubiginosa leaflets during abscisic acid (ABA) addition. In air ABA induced a decrease of both the net CO₂ assimilation (Aᵣ) and the stomatal water vapor conductance (gₛ). After ABA treatment, imaging in transient nonphotorespiratory conditions (0.1% O₂) revealed a heterogeneous decrease of PSII photochemical yield. This decline was fully reversed by a transient high CO₂ concentration (7400 μmol mol⁻¹) in the leaf atmosphere. It was concluded that ABA primarily affected Aᵣ by decreasing the CO₂ supply at ribulose-1,5-bisphosphate carboxylase/oxygenase. Therefore, the Aᵣ versus intercellular mole fraction (Cᵢ) relationship was assumed not to be affected by ABA, and images of Cᵢ and gₛ were constructed from images of PSII photochemical yield under nonphotorespiratory conditions. The distribution of gₛ remained unimodal following ABA treatment. A comparison of calculations of Cᵢ from images and gas exchange in ABA-treated leaves showed that the overestimation of Cᵢ estimated from gas exchange was only partly due to heterogeneity. This overestimation was also attributed to the cuticular transpiration, which largely affects the calculation of the leaf conductance to CO₂, when leaf conductance to water is low.

Our understanding of the response of leaf photosynthesis to environmental factors is highly dependent on the interpretation of photosynthetic gas exchange and particularly on the relationship between net CO₂ assimilation and Cᵢ (Farquhar and Sharkey, 1982). Cᵢ estimations from gas exchange, which are formally estimations of the CO₂ mole fraction at the evaporating sites (Parkhurst, 1994), are based on the assumption that photosynthesis and transpiration are relatively uniform over the leaf area. This assumption appears to be adequate for most leaves when stomatal conductance is maximal, as most stomata are open. When stomatal conductance decreases, however, this assumption may not be valid. Upon application of ABA, short-term water stress, or low humidity, the occurrence of a nonuniform distribution or patchy distribution of the stomatal aperture and photosynthesis has been reported (Downton et al., 1988a, 1988b; Terashima et al., 1988; Daley et al., 1989; Raschke et al., 1990; Mott et al., 1993; Mott, 1995).

Upon ABA treatment, photosynthetic capacity measured under nonlimiting CO₂ availability remained unaffected, and since the patterns of distribution of stomatal aperture and photosynthesis were identical, the patchy response has been primarily attributed to a nonuniform distribution of stomatal closure (Terashima et al., 1988). In this context, the commonly reported unresponsiveness of Cᵢ relative to net CO₂ assimilation decline under these conditions, classically described as a nonstomatal inhibition of photosynthesis (Seemann and Sharkey, 1987), has been interpreted as an artifact due to patchy response (Downton et al., 1988a, 1988b; Terashima et al., 1988; Raschke et al., 1990; Mott, 1995).

Potential overestimation of Cᵢ when the distribution of stomatal aperture over the leaf is heterogeneous has been pointed out previously (Laisk et al., 1980). By modeling a bimodal distribution of leaf stomatal conductance, several authors (Downton et al., 1988a, 1988b; Terashima et al., 1988; Van Kraalingen, 1990) have concluded that the occurrence of heterogeneous stomatal distribution fully explains the behavior of Cᵢ under ABA or short-term water stress. Using a modeling approach, Cheeseman (1991) and very recently Buckley et al. (1997) have shown that such large overestimation of Cᵢ could not be fully accounted for by

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**Abbreviations**: (1 – Φ/Φₛᵢ), (1 – Φ/Φₛᵢ), local and mean Φₛᵢ; Aᵣ, Aᵣ, Aᵣ, Aᵣ, Aᵣ, Aᵣ, global (conventional gas-exchange data), local (pixel value), and mean (spatial average) gross and net rate of CO₂ assimilation, respectively; Cᵢ, ambient CO₂ mole fraction inside the cuvette; Cᵢ, Cᵢ, Cᵢ, Cᵢ, Cᵢ, Cᵢ, Cᵢ, global, local, and mean intercellular CO₂ mole fraction; E, E, E, global, local, and mean rate of transpiration; Sᵢ, Sᵢ, Sᵢ, Sᵢ, Sᵢ, Sᵢ, boundary layer conductance to H₂O; Sᵢ, Sᵢ, Sᵢ, Sᵢ, Sᵢ, Sᵢ, local, and mean stomatal conductance to H₂O; Sᵢ, Sᵢ, Sᵢ, Sᵢ, Sᵢ, Sᵢ, Sᵢ, global, local, and mean total conductance to H₂O; Φ, Φ, steady-state and maximal fluorescence yields; Φₛᵢ, photosynthetic yield of PSII; qN, non-photochemical quenching of chlorophyll fluorescence; Tᵥ, Tᵥ, Tᵥ, Tᵥ, Tᵥ, Tᵥ, Tᵥ, Tᵥ, local, and mean leaf temperature; wₛ, wₛ, wₛ, wₛ, wₛ, global, local, and mean intercellular mole fraction of water vapor.

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heterogeneous stomatal conductance if a unimodal distribution of the stomatal aperture was assumed. Recently, a corresponding unimodal distribution of photosynthesis under ABA treatment has been reported (Meyer and Genty, 1995; Mott, 1995).

The aim of this study was to assess the significance of $C_i$ under patchy photosynthesis induced by ABA treatment in *Rosa rubiginosa* leaves. By using an experimental approach that allows the mapping of the photochemical yield of PSII from leaf chlorophyll fluorescence images (Genty and Meyer, 1995), we have demonstrated that ABA primarily affects photosynthesis by decreasing stomatal conductance.

Assuming the CO$_2$ assimilation versus $C_i$ relationship was not affected by ABA, high-resolution images of $C_i$ were constructed from images of the photochemical yield of PSII. Calculations of $C_i$ from gas exchange and images were compared and it was confirmed that $C_i$ estimated from gas exchange is largely overestimated. We have shown that this overestimation is only partly due to stomatal closure heterogeneity, in contrast to previous understanding. This overestimation also results from an overestimation of leaf conductance to CO$_2$ calculated from conductance to H$_2$O, which we attribute to cuticular transpiration. Important implications for the interpretation of photosynthetic gas-exchange data are discussed. A preliminary account of some of this work was presented earlier (Meyer and Genty, 1995).

**MATERIALS AND METHODS**

Plants of *Rosa rubiginosa* L. were cultivated in pots in a greenhouse under natural sunlight and photoperiod during the summer (1994 and 1995). Daily maximum and minimum air temperatures were 35 and 15°C, respectively. Detached, fully expanded leaves were used, with the petiole kept in distilled water throughout the experiment. Leaves were dark adapted for 1 h before the experiment and the apical leaflet was used for measurement.

Transverse leaflet sections and replicas of epidermis have shown that *R. rubiginosa* leaves were heteroboric and hypostomatous (Bro et al., 1996). In this early study, the mean leaf area was 6.3 ± 1.5 ($n=7$ leaves) cm$^2$, the mean surface of an areole (Terashima, 1992) was 0.221 ± 0.055 mm$^2$ ($n=9$), and the stomatal density was 112 ± 69 ($n=12$) stomata mm$^{-2}$.

**Gas-Exchange Measurements**

An open-flow gas-exchange system and a clamp on the leaf cuvette described in Genty and Meyer (1995) were used for the measurements of CO$_2$ and water vapor net exchanges. Cuvette foam gaskets were coated with paraffin for minimal water adsorption. Cuvette temperature was kept constant at 25°C. The vapor pressure deficit between the leaflet and air was maintained between 0.8 and 1.2 kPa. Gas-exchange parameters and leaflet temperature were calculated according to the method of von Caemmerer and Farquhar (1981) and Parkinson (1985), respectively, for the leaflet projected area and one transpiring surface (since *R. rubiginosa* leaflets are hypostomatous). By using a leaf energy-balance method, an average temperature for the area of the leaflet under consideration was provided. $g_w$ was 1.94 mol m$^{-2}$ s$^{-1}$.

**Mapping of PSII Photochemical Yield using Chlorophyll Fluorescence Imaging**

The method is described in detail in Genty and Meyer (1995). A video charge-coupled-device camera imaged the chlorophyll fluorescence of the upper side of the whole-leaf surface from which gas-exchange measurements were performed. Routine cycles of two images, one of steady-state relative fluorescence yield, $\Phi_i$, and the other of maximal relative fluorescence yield, $\Phi_{m,i}$, were digitized and used to construct in near-real time an image of the photochemical yield of PSII, $\Phi_{psii}$ by computing pixel by pixel $(1 - \Phi/\Phi_{m})$ (Genty et al., 1989).

Images of $\Phi_{psii}$ were stored as 512 × 512 pixels frames with an 8-bit resolution. Frequency distribution histograms were computed from native images of $\Phi_{psii}$ in floating-point mode with 32-bit precision using Labview software (National Instruments, Austin, TX) and dedicated processing routines (Concept VI, Graphitek, Mirmande, France). All images used for quantitative analysis of distribution of gas-analysis parameters correspond to the open area (6.25 cm$^2$) of the gas-exchange chamber, which comprised more than 75 to 90% of the total leaflet area. The single areoles of *R. rubiginosa* leaflets correspond to about 150 pixels, and 3 to 4 pixels refer to about 1 stoma and 10 epidermal cells.

**Mapping of Intercellular CO$_2$ Mole Fraction, CO$_2$ Assimilation, Water Vapor Conductance, Transpiration, and Leaf Temperature from Images of the Photochemical Yield of PSII**

\[
A_i, A_{nw}, C_i, g_i, w_i, T_i, E_i \text{, and } R \text{ correspond to global measurements (conventional gas-exchange data): } A, \text{ gross CO}_2 \text{ assimilation;} A_{nw}, \text{ net CO}_2 \text{ assimilation;} C_i, \text{ intercellular } \text{CO}_2 \text{ mole fraction;} g_i, \text{ total water vapor conductance;} w_i, \text{ intercellular mole fraction of water vapor;} T_i, \text{ leaf temperature;} E, \text{ rate of transpiration;} \text{ and } R, \text{ respiration in the dark.}
\]

\[
A_i, A_{nw}, C_i, g_i, w_i, T_i, E_i (1 - \Phi/\Phi_{m}), \text{ and } R_i \text{ correspond to local measurements (pixel i) and } A_i, A_{nw}, C_i, g_i, w_i, T_i, E_i, (1 - \Phi/\Phi_{m}), \text{ and } R \text{ correspond to the means of local measurements.}
\]

In nonphotorespiratory conditions, $(1 - \Phi/\Phi_{m})$, which is a relative estimate of the quantum yield of gross CO$_2$ assimilation (Genty et al., 1989), can be used to estimate $A_i$ as:

\[
A_i = \left( \frac{(1 - \Phi_{im}) - d}{k} \right) I
\]

where $I$ is the incident photon flux density, $k$ and $d$ are the slope, and the $Y$ is the intercept obtained from the linear regression of the relationship between the mean of the photochemical yield of PSII, $(1 - \Phi/\Phi_{m})$, and the quantum...
yield of gross CO₂ assimilation of the considered experiment (typical regression slope \( k \) was 11.4 with \( d = 0.06 \).

According to Farquhar et al. (1980), in ribulose-bisphosphate saturated and nonphotorespiratory conditions, \( A_i \) can be described by a hyperbolic function of \( C_i \) as:

\[
A_i = \frac{a C_i}{b + C_i}
\]

where \( a \) and \( b \) were obtained from the best fit of Equation 2 for the gas-exchange data obtained in uniform photosynthesis (before ABA feeding \( C_i \) and \( A_i \) equal \( C_i \) and \( A_i \), respectively). We have substituted \( A_i \) of Equation 2 by its expression in Equation 1 to resolve for \( C_i \). These calculations assume no change of the relationship between \( A_i \) and \( C_i \) during the course of a considered experiment. The validity of this assumption will be examined in “Results” and in “Discussion.”

Making the same assumption, the total conductance to H₂O, \( g_{iw} \), the transpiration, \( E_i \), and the leaf temperature, \( T_{li} \), were calculated from \((1 - \Phi/\Phi_m)\), for each pixel of images of the photochemical yield of PSII by solving numerically for the unknowns \( g_{iw}, E_i, T_{li} \), and \( w_{iw} \), the system of Equations 3, 4, 5, and 6:

\[
g_{iw} = \frac{1.6(E_i C_a + C_i A_i)}{C_a - C_i} \quad \text{(3)}
\]

\[
w_{a} = \frac{E_i \left(1 - \frac{w_a + w_s}{2}\right)}{w_s - w_a} \quad \text{(4)}
\]

where \( A_{mi} = A_i - R_e \), with \( R_e \) taken as \( R \) and where \( A_i \) and \( C_i \) were substituted by their expression in function of \((1 - \Phi/\Phi_m)\), using Equations 1 and 2. \( C_a \) is the ambient CO₂ mole fraction in the chamber.

\[
T_{bi} = T_{bi} + \frac{\alpha l_{bi} - \lambda E_i}{2(0.93 \rho C_{S_{bi}} + 4\epsilon \sigma (T_{bi} + 273)^4)} \quad \text{(5)}
\]

where \( T_{bi} \) is the air temperature (°C) in the chamber, \( l_{bi} \) is the total short-wave irradiance on the leaf, \( \alpha \) represents the fraction absorbed, \( \lambda \) is the latent heat of vaporization of water, \( \rho \) is the air density, \( C_p \) is its specific heat capacity, \( \epsilon \) is the thermal emissivity of the leaf, and \( \sigma \) is the Stefan-Boltzmann constant. The factor 0.93 is for converting the boundary layer conductance of water vapor (\( g_{iw} \)) into heat-transfer conductance for a laminar boundary layer. The factor 2 in the denominator accounts for the two-sided heat conductance of the leaf, since \( g_{iw} \) is a one-sided conductance in the first term of the denominator, and thermal absorption and emission by both sides of the leaf in the second term.

\[
w_{a} = \frac{6.1078 e^{17.27(T_{bi} + 273)}}{10P_a} \quad \text{(6)}
\]

where \( P_a \) is the ambient total pressure (kPa).

Equations 3 and 4 were derived from von Caemmerer and Farquhar (1981). In Equation 3, \( g_{iw} \) was directly calculated from the 1.6 ratio of diffusivities of water vapor and CO₂, since stomatal conductance was low in the presence of ABA; so the correction for the “laminar flow”-like transport of water vapor and CO₂ in the boundary layer can be neglected. Equation 5 was derived from the energy-balance method described by Parkinson (1985). In this expression, neither the possible heat-conduction exchange from a local site to another local site nor the small contribution of net energy storage were taken into account. Equation 6 was derived from Buck (1981).

From \( g_{iw} \), stomatal conductance to water vapor, \( g_{sw} \), was calculated by assuming that \( g_{sw} \) was constant over the leaflet. Images of \( C_{iw} \), \( A_{iw} \), \( g_{iw} \), \( g_{sw} \), \( T_{li} \), and \( E_i \) were constructed from a given image of \((1 - \Phi/\Phi_m)\), and the means \( C_{iw} \), \( A_{iw} \), \( g_{iw} \), \( g_{sw} \), \( T_{li} \), and \( E_i \) were computed from images. All calculations were computed from native images of PSII photochemical yield in floating-point mode with 64-bit precision using Labview software and dedicated processing routines (Concept VI, Graphtek).

**Protocol**

Experiments were performed at a photon flux density of 385 to 420 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) in air. After a steady state was reached, leaf chamber CO₂ mole fraction was varied in steps between ambient air composition and around 100 \( \mu \)mol mol\(^{-1}\) and back to air composition. \emph{cis-trans} ABA (99% purity, Sigma) was then added to the water supply of the leaf petiole to bring its concentration to \( 10^{-7} \) m. Sets of images of PSII photochemical yield were recorded just before and during a brief transition to 0.1% O₂ before ABA feeding, for the various external CO₂ mole fraction, and after ABA feeding, when a pseudo-steady state was reached.

A subsaturating photon flux density of 385 to 420 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) was chosen to remain in ribulose-bisphosphate-saturated condition for Rubisco activity at 340 \( \mu \)mol mol\(^{-1}\) CO₂ during transition in nonphotorespiratory conditions while keeping a large enough quantum yield of CO₂ assimilation for maximal accuracy of \((1 - \Phi/\Phi_m)\) imaging.

**RESULTS**

**Time Course of the Leaf Gas-Exchanges Response to ABA Treatment**

Figure 1 shows the time course of \( A_n \) and \( g_s \) measured by gas exchange during the ABA feeding of a \emph{R. rubiginosa} leaflet. The decline of \( g_s \) began 10 min after the addition of ABA and preceded the decrease of \( A_n \). A steady state was reached 1 h later. In response to ABA, \( A_n \) and \( g_s \) decreased by 80 and 90%, respectively, as commonly reported (Cummins et al., 1971; Farquhar and Sharkey, 1982; Raschke and Hedrich, 1985; Mott, 1995). When brief transitions to nonphotorespiratory conditions were performed (open symbols in Fig. 1), \( A_n \) increased by about 50 and 25% before and after ABA treatment, respectively, whereas \( g_s \) remained unchanged. Thus, stomatal conductance was not signifi-
siently affected by transition to an oxygen-depleted atmosphere. After each transition, 5 to 15 min were required for the recovery of steady \( \Delta \text{m} \) in air.

**Distribution of Photosynthesis in ABA-Fed Leaves**

\( \text{CO}_2 \) and \( \text{O}_2 \) Dependency

Figure 2 shows images of the photochemical yield of PSII obtained in air (A and B), and during transition to 0.1% \( \text{O}_2 \) (C and D), before (A and C) and after (B and D) addition of ABA at near-air external \( \text{CO}_2 \) concentration. Relative pixel intensity was scaled to an 8-bit gray scale, where black and white corresponded to a photochemical yield of 0 and 0.75 (A–C) or 0 and 0.4, respectively. Before adding ABA, the distribution of PSII photochemical yield was uniform and the mean photochemical yield was high both in air and during transition in 0.1% \( \text{O}_2 \) (Figs. 2, A and C, and 3, A and C). In response to the treatment in air, no heterogeneity was observed and photochemical yield was high (Figs. 2B and 3B), whereas a heterogeneous distribution of photochemical yield was revealed in nonphotorespiratory conditions (Fig. 2D). In this area (2% of all the mapped leaflet), which was not seen in air, \( \text{CO}_2 \) availability was presumably lower than in surrounding mesophyll because of a diffusive limitation (Bro et al., 1996).

\( \text{O}_2 \) dependency of PSII photochemical yield was markedly enhanced after the ABA treatment. The suppression of photorespiration induced a decrease of the mean photochemical yield of 62% (Fig. 3, B and D), which indicates that the allocation of electron transport to the fixation of \( \text{CO}_2 \) chemical yield only decreased by 13.5% (Fig. 2, A and C), although total conductance to water vapor did not decline. This suggests that \( \text{CO}_2 \) fixation was an electron transport sink almost large enough to compensate for the lack of photorespiration, as predicted by models based on kinetic properties of Rubisco for subsaturating irradiances (Farquhar et al., 1980). In air, \( \text{O}_2 \)-dependent processes other than photorespiration may also have occurred, however, they are not considered in this discussion, since their contribution is likely to be very small in steady-state experiments where \( \text{Ci} \) never dropped below \( \text{CO}_2 \) compensation (see Genty and Harbinson, 1996). During the transition to nonphotorespiratory conditions, the PSII photochemical yield especially decreased in mesophyll cells bordering the midrib of the leaflet (Fig. 2C) corresponding to the “tail” of low values of the \( (1 - \Phi/\Phi_m) \) distribution (Fig. 3C). In this area (2% of all the mapped leaflet), which was not seen in air, \( \text{CO}_2 \) availability was presumably lower than in surrounding mesophyll because of a diffusive limitation (Bro et al., 1996).

Figure 2. Images of \( (1 - \Phi/\Phi_m) \) taken before (A and C) and during (B and D) treatment of a leaf of \text{R. rubiginosa} with \( 10^{-4} \text{ M ABA} \). Leaflet atmosphere was 340 \( \mu\text{mol} \text{ mol}^{-1} \text{ CO}_2 \), 21% (A and B) or 0.1% (C and D) \( \text{O}_2 \), and 70% RH. The photon flux density was 390 \( \mu\text{mol} \text{ m}^{-2} \text{ s}^{-1} \). The bar indicates 1 cm. Three to four pixels represent about 1 stoma and 10 epidermal cells. Pixel intensity was scaled using an 8-bit gray scale where black and white correspond to a photochemical yield of 0 and 0.75, respectively (A–C), or 0 and 0.4, respectively (D). \( \Delta \text{m} \) was 9.1, 2.9, 14.3, and 3.6 \( \mu\text{mol} \text{ m}^{-2} \text{ s}^{-1} \) for A through D, respectively, and \( g_s \) was 142, 23, 153, and 22 \( \mu\text{mol} \text{ m}^{-2} \text{ s}^{-1} \) for A through D, respectively.
had markedly decreased, as shown by the reduction of net CO₂ assimilation after ABA treatment in an O₂-depleted atmosphere. On the other hand, in air, O₂ reduction by the glycolate pathway and internal reassimilation of evolved CO₂ were able to uniformly drive a large photochemistry over the ABA-treated leaflet. Actually, in air, photorespiration almost masked the heterogeneous decrease in CO₂ fixation, which was revealed by imaging \( (1 - \Phi/\Phi_m) \) during the transition in nonphotorespiratory conditions (Figs. 2D and 3D). It is worth noting that a slight decrease of mean photochemical yield occurred in air, in response to ABA (Fig. 3, A and B). This was similar to the one seen after O₂ depletion in the control leaflet (Fig. 3, A and C). However, in contrast to what happens in nonphotorespiratory conditions (Fig. 2C), this slight decrease in photochemical yield occurred randomly distributed over the leaflet area (Fig. 2B) and the distribution of frequencies was narrower and symmetrical (Fig. 3B). This further supports the suggestion that O₂-dependent processes were the major sink for electron transport in ABA-treated leaves, since local internal O₂ concentration is likely to be uniform over the leaflet area.

ABA-treated leaves were exposed to a short pulse of high-CO₂ concentration in nonphotorespiratory conditions to test whether a CO₂-supply limitation of photosynthesis could be ruled out. Within 1 min, transient high CO₂ (7400 μmol mol⁻¹ CO₂) fully reversed the heterogeneous decrease of \( (1 - \Phi/\Phi_m) \) in response to ABA (Figs. 4 and 5). The fast kinetics of the reversion of the photochemical quantum yield of PSII and therefore of CO₂ assimilation showed that the response of photosynthesis to ABA only involved diffusive phenomena and was unlikely to result from a down-regulation of carboxylation demand, hypothesized as a long-term effect of leaf CO₂ deficiency (Terashima et al., 1988; Ort et al., 1994). This confirmed the intactness and the uniformity of the photosynthetic capacity in ABA-treated leaves. These data provide unambiguous support for the hypothesis that ABA treatment only reduced the diffusion of CO₂ into the leaflet through the closure of stomata, as suggested by previous studies (Downton et al., 1988a; Terashima et al., 1988; Ward and Drake, 1988; Graan and Boyer, 1990; Lauer and Boyer, 1992; Raschke et al., 1990). This also supported the assumption we used in subsequent calculations and discussion of significance of \( C_i/A \) relationship during the course of the experiment.

**Pattern of Heterogeneity**

In response to ABA, the decrease in PSII photochemical yield was seen all over the leaflet in nonphotorespiratory conditions (Figs. 2D and 3D). However, the extent of the decrease varied spatially, resulting in a mosaic of patches of about 0.2 mm² (Figs. 2D and 6). In the experiment depicted in Figure 2, patches of similar yield were grouped in such a way that they formed larger areas that drew the net of the major veins that diverged from the midrib; \( (1 - \Phi/\Phi_m) \) was the highest in groups of patches close to major

![Diagram of frequency distributions of \( (1 - \Phi/\Phi_m) \) corresponding to the images A through D of Figure 2.](image)
veins (Fig. 2D). This pattern varied from experiment to experiment.

Figure 6 shows a detail of images of $\Phi$ and $\Phi_m$ and the corresponding image of $(1 - \Phi/\Phi_m)$ for an ABA-treated leaflet during a transition in O$_2$-depleted atmosphere. Contrast of the image of $\Phi$ has been enhanced to better illustrate the local variations of $\Phi$. Images of $\Phi_m$ and $(1 - \Phi/\Phi_m)$ show a similar pattern of heterogeneity. $\Phi$ also varied according to similar pattern but to a lesser extent. Thus, the patches in which $\Phi$ and $\Phi_m$ were low also showed a low $(1 - \Phi/\Phi_m)$. It is worth noting that in five out of nine experiments, in some patches (no more than 1.5% of all the leaflet area), in nonphotorespiratory conditions, the photochemical yield could be very low (values of about 30% of the mean $(1 - \Phi/\Phi_m)$). This was due to a large increase in $\Phi$ and $\Phi_m$, which became almost equal (not shown). It mimicked the action of an inhibitor of electron transport-like DCMU (Genty and Meyer, 1995), and was reversible following either a brief addition of high CO$_2$ or the restoration of normal O$_2$ concentration. This suggests that ABA could locally inhibit the photochemical efficiency of PSII in some areoles of the leaflet under nonphotorespiratory conditions. This may be the consequence of the severe depletion of sinks that drive electron transport in nonphotorespiratory conditions in these areoles (Genty and Harbinson, 1996). In most experiments after ABA treatment, $\Phi_m$ decreased by about 30% and $\Phi$ increased by about 10%. In all experiments the percentage of heterogeneity (estimated by the ratio of the sd versus the mean of pixel values over the leaflet area) of $\Phi$ and $\Phi_m$ distributions was greater than that of $(1 - \Phi/\Phi_m)$, before the ABA treatment (Fig. 6, $\Phi$, $\Phi_m$, $(1 - \Phi/\Phi_m)$; 7, 9, and 3%, respectively), and lower after the treatment (Fig. 6, $\Phi$, $\Phi_m$, $(1 - \Phi/\Phi_m)$; 6, 11, and 14%, respectively). Variations of $(1 - \Phi/\Phi_m)$ were not linearly related to variations of $\Phi_m$. This is a consequence of the fact that the quantum yield of
PSII is not linearly related to the qN of \( F_m \) (Genty et al., 1989; Siebke and Weis, 1995). Using Equations 1 to 6, images of \( A_n \), \( g_s \), \( C_i \), \( E_i \), and \( T_l \) were derived from the image of \((1 - \Phi/\Phi_m)\) obtained for an ABA-treated leaflet in nonphotorespiratory conditions. Figure 7 shows the image of \( g_s \) computed from image of \((1 - \Phi/\Phi_m)\) taken during ABA treatment (Fig. 2D). Image was calculated according to the method detailed in “Materials and Methods.” The bar indicates 1 cm. The 3-bit color scale corresponds to a \( g_s \) scale from 0 to 0.04 mol m\(^{-2}\) s\(^{-1}\). \( g_s \) with SD are indicated. Major veins are shown by the white pixels of the image.

Figure 6. Detail of images of \( \Phi \) and \( \Phi_m \) and of the corresponding \((1 - \Phi/\Phi_m)\), taken during the treatment of leaflet of \( R. \) rubiginosa with ABA (10\(^{-4}\) m). Typical surface of an areole was about 0.2 mm\(^2\). Leaflet atmosphere was 340 μmol mol\(^{-1}\) CO\(_2\), 0.1% O\(_2\), and 70% RH. The photon flux density was 385 μmol m\(^{-2}\) s\(^{-1}\). The bar indicates 4 mm. Eight to 10 pixels correspond to about 1 stoma and 10 epidermal cells. \((1 - \Phi/\Phi_m)\) was 0.329 ± 0.046 for the leaf area depicted. For the \( \Phi \) and \( \Phi_m \) images, the scaling of pixel intensity was done using an 8-bit gray scale where black and white pixels correspond to a relative fluorescence yield of 0 and 0.72, respectively (\( \Phi \)), or 0 and 1, respectively (\( \Phi_m \)). For the image of \((1 - \Phi/\Phi_m)\), black and white pixels correspond to 0 and 0.5, respectively.
Computed from images of A from images of (1 2F the image of C 2F 0.022 mol m 6 0.019 0.006, respectively, after the treatment. The ABA treatment did not change the assumptions, taking into account heterogeneous distribution of stomatal conductance, another estimation of stomatal conductance, another estimation of global CO2 supply induced by stomatal closure only, as evidenced in the previous section.

Figure 9 also shows the corresponding C predicted for gas exchange can be derived from images of (1 - 1.6 + E/2)C - A/ [2/1.6 + E/2] (rearrangement of Eq. 3 with A, and E, computed from images of A and E, respectively). In Figure 9, the C predicted from these calculations are the C values that should be obtained using gas-exchange techniques if ABA solely induced heterogeneous stomatal closure. This predicted value corresponds to the C described by equation 30b in Farquhar (1989), where C was simply modeled as (C - 1.6A/2) (see also Meyer and Genty, 1995). The difference between C and the C predicted for gas exchange was rather small and the C predicted for gas exchange remained substantially smaller than the global C estimated from gas exchange (Fig. 9). Consequently, the ABA induced large departure from control A versus conventional C relationship could only be partly explained by heterogeneity of stomatal closure.

DISCUSSION

In this study we interpreted the decline of photosynthesis after ABA treatment as having been caused by stomatal closure only. The short time scale of the reversion (within less than 1 min) of low assimilation rate under transient high-CO2 availability was the strongest indication that stomatal closure was determining the ABA-induced inhibition of photosynthesis. In our experimental conditions the reversion was always almost complete, such that a significant inhibition of metabolic capacity for photosynthesis caused by low-CO2 supply was unlikely to occur. The weak dependency of photosynthetic electron transport activity on O2 shown in air, before and after ABA treatment, provides further evidence for the maintenance of a fully competent photosynthetic apparatus. Thus, O2-dependent processes were able to drive almost as large a photochemical activity as before ABA treatment when maximal A occurred. Maintenance of Rubisco activity via O2 reduction by the
glycolate pathway and reassimilation of evolved CO₂ appears as a likely interpretation for such a response. A similar interpretation has been proposed to explain the occurrence of a large photochemical activity in water-stressed leaves (Cornic and Massacci, 1996).

By imaging PSII photochemical yield during a short transition to O₂-depleted air, it was possible to accurately quantify the distribution of A, Cᵢ, and gᵢ. Thus, in our study, maximal deviation between Aᵢ, measured by gas exchange and Aᵥ, estimated by fluorescence imaging was 8%. Such a precision was a prerequisite for an accurate analysis of the significance of Cᵢ.

Continuous frequency distributions of gᵢ were always seen in contrast to reports of bimodal distributions observed using imaging of qN (Daley et al., 1989). Recently, Mott (1995) using a similar qN imaging also found no evidence for bimodal distribution after ABA treatment. However, in these two earlier studies no experimental basis was provided to characterize the diffuse or mutative origin of the inhibition of photosynthetic activity induced by ABA treatment. A pure diffuse limitation is a prerequisite for the validity of the assumption used in these imaging studies: the relationship between fluorescence parameters Aᵢ and Cᵢ, did not change during the experiment, which allowed the derivation of distributions of A, Cᵢ, and gᵢ from heterogeneous fluorescence images. In this context, it is important to note that an ABA treatment in O₂-depleted air (Daley et al., 1989) and in air (Mott, 1995: present study) may provide different results (e.g. stomatal closure in O₂-depleted air do not allow a maintenance of Rubisco and photochemical activities). This remark is also valid for many studies where long-term nonphoto respiratory conditions have been used to reveal a heterogeneous photosynthetic activity from fluorescence imaging (e.g. Raschke et al., 1990; Mott et al., 1993; Cardon et al., 1994; Genty and Meyer, 1995; Siebke and Weis, 1995). Equally important is the nonlinearity of the relationship between qN and A, gᵢ, or Cᵢ both in air and in nonphoto respiratory conditions, which warrants a correct quantification from straight interpretation of qN distribution. Thus, Mott (1995) noted that in air, qN was almost insensitive to Cᵢ at C_CUR near the CO₂ compensation point. Finally, leaf area probed by gas exchange and imaging may need to be identical for a valid quantification of heterogeneity, which was not the case in previous reports. A selective image of a small fraction of the leaf area may provide a different distribution pattern than an image of a whole leaf or leaflet.

From our comparative analysis of Cᵢ predicted from imaging and global Cᵢ estimated conventionally from gas exchange, it is clear that the large overestimation of Cᵢ observed after ABA treatment can only be partly explained by heterogeneous stomatal closure, in contrast to conclusions of previous studies but in support of the modeling work of Cheseman (1991) and Buckley et al. (1997). The remaining large difference between global and predicted Cᵢ for gas exchange corresponded to a difference in E and gᵢ of 58 ± 9 μmol m⁻² s⁻¹ and 3.3 ± 0.8 mmol m⁻² s⁻¹ for the data of Figure 9, respectively. A difference in gᵢ of 5.6 ± 2.1 mmol m⁻² s⁻¹ was obtained for four experiments (gᵢ of the control and ABA treated leaflet were 209 and 26 mmol m⁻² s⁻¹, respectively) (Table I). This difference agrees well with the value of cuticular water permeance given for leaves of trees (Kerstiens, 1995). Considering that cuticular conductance to CO₂ is likely to be insignificant (Holmgren et al., 1965), this indicates that the main overestimation of Cᵢ may result from cuticular transpiration, which is not taken into account in the usual calculation of leaf conductance to CO₂ from total conductance to H₂O. As cuticular transpiration occurred on both sides of the hypostomatous R. rubiginosa leaf, we estimated cuticular conductance as 2.8 ± 1.0 mmol m⁻² s⁻¹. Recent data have shown that in Vitis vinifera cuticular conductance to CO₂ was measurable and was approximately 6% of cuticular conductance to H₂O (Boy er et al., 1997). This indicates that the difference between global and predicted gᵢ for gas exchange may slightly under estimate cuticular conductance to H₂O. Water vapor adsorption by the leaf chamber and the sealing gaskets may also contribute to a residual water vapor net flux we interpreted as cuticular transpiration. However, this problem was minimized by coating the chamber and gaskets with Teflon and paraffin, respectively (maximal error due to water vapor adsorption and release from foam gaskets and walls of the leaf chamber was lower than 9% of the mean Δgᵢ).

Reduction of stomatal pore size induced by ABA treatment may change the relationship between molecular diffusivity of water vapor and CO₂ and offer an alternative explanation for a break of the relationship between stomatal conductance to water and CO₂. However, this possibility is unlikely, since the ratio of conductance to water over the one to CO₂ has been shown to remain close to the ratio of the free diffusion coefficient for a wide range of width of the stomatal pore (0.01–5 μm) and insensitive to the aperture-dependent change of the diffusion coefficients (Milthorpe and Penman, 1967).

Figure 10 depicts the control relationship between Aᵢ and Cᵢ in nonphotorespiratory (A) and photorespiratory (B) conditions and shows the consequence of not taking into account the cuticular transpiration for the estimation of Cᵢ during ABA treatment for the experiment of Figure 9. The overestimation of Cᵢ resulted mainly from the occurrence of cuticular transpiration when most of the stomata were closed. However, during the early phase of ABA

<table>
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<tr>
<th>Δgᵢ</th>
<th>gᵢ Control</th>
<th>gᵢ + ABA</th>
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<tr>
<td>5.6 ± 2.1</td>
<td>209 ± 55</td>
<td>26.2 ± 9.5</td>
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treatment in air when stomatal conductance remained high, heterogeneous stomatal closure was likely to be the only cause of this overestimation (Fig. 10B).

In conclusion, we show that both nonuniform stomatal closure and cuticular transpiration have to be taken into account to accurately predict \( C_i \) from gas-exchange measurements when stomatal conductance is low. Our data clearly show that after ABA treatment, heterogeneous decline of \( \Delta n \) results only from stomatal closure. In mildly water-stressed leaves where a maintenance of the full integrity of photosynthetic capacity remains questionable (see e.g. contradictory reports of Cornic and Massacci [1996] and Lauer and Boyer [1992]), our approach appears very promising for characterizing the significance of \( C_i \) and for separating stomatal from nonstomatal inhibition of photosynthesis.

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