

Lateral CO₂ Diffusion inside Dicotyledonous Leaves Can Be Substantial: Quantification in Different Light Intensities¹[W][OA]

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Substantial lateral CO₂ diffusion rates into leaf areas where stomata were blocked by grease patches were quantified by gas exchange and chlorophyll *a* fluorescence imaging in different species across the full range of photosynthetic photon flux densities (PPFD). The lateral CO₂ flux rate over short distances was substantial and very similar in five dicotyledonous species with different vascular anatomies (two species with bundle sheath extensions, sunflower [*Helianthus annuus*] and dwarf bean [*Phaseolus vulgaris*]; and three species without bundle sheath extensions, faba bean [*Vicia faba*], petunia [*Petunia hybrida*], and tobacco [*Nicotiana tabacum*]). Only in the monocot maize (*Zea mays*) was there little or no evident lateral CO₂ flux. Lateral diffusion rates were low when PPFD < 300 μmol m⁻² s⁻¹ but approached saturation in moderate PPFD (300 μmol m⁻² s⁻¹) when lateral CO₂ diffusion represented 15% to 24% of the normal CO₂ assimilation rate. Smaller patches and higher ambient CO₂ concentration increased lateral CO₂ diffusion rates. Calculations with a two-dimensional diffusion model supported these observations that lateral CO₂ diffusion over short distances inside dicotyledonous leaves can be important to photosynthesis. The results emphasize that supply of CO₂ from nearby stomata usually dominates assimilation, but that lateral supply over distances up to approximately 1 mm can be important if stomata are blocked, particularly when assimilation rate is low.

Photosynthesis in leaves depends on the diffusion of CO₂ from the ambient air through stomata and leaf air spaces into the chloroplasts in the mesophyll cells. This CO₂ diffusion poses an important limitation on photosynthesis, and much research is focused on understanding the various components, such as the factors that control stomatal aperture (Nilson and Assmann, 2007), the value of the so-called internal or mesophyll conductance (Yamori et al., 2006), and the role of aquaporins and carbonic anhydrase in CO₂ assimilation (Flexas et al., 2006). Stomata are discrete points of CO₂ entry that can be widely spaced (e.g. interpore distances 24–340 μm are cited in Willmer and Fricker, 1996). Therefore, CO₂ diffusion inside leaves is partly in a lateral (paradermal) direction (Parkhurst, 1994), although if stomata are open, diffusion in the vertical, transverse direction probably dominates due to the arrangement of cells and the connectivity of air spaces (Morison and Lawson, 2007). Stomatal apertures across leaves are often not

uniform (e.g. Weyers and Lawson, 1997; Mott and Peak, 2007) and differences in aperture might lead to substantial lateral diffusion with stomata in one region potentially supplying CO₂ to an adjacent region of the leaf, depending on the permeability of leaf tissue (Parkhurst, 1994). The permeability depends on leaf anatomy, particularly mesophyll cell size and shape, packing density, and the vascular anatomy (Pieruschka et al., 2005; Morison and Lawson, 2007). Many species have bundle sheath extensions (BSE) consisting of achlorophyllous parenchyma cells (Wylie, 1952; McClendon, 1992) that appear to provide a better supply of water to the epidermis (Kenzo et al., 2007) and may also allow local penetration of light into leaves (Vogelmann, 1989; Karabourniotis et al., 2000). In some species, these BSE can divide the mesophyll into discrete compartments or areoles leading to heterogeneous photosynthesis (Terashima, 1992; Morison and Lawson, 2007). It is usually concluded that no lateral diffusion will occur in heterobaric species with BSE but it will in homobaric leaf anatomies without BSE (Terashima, 1992; Evans and von Caemmerer, 1996; Jahnke and Krewitt, 2002). However, the importance of lateral CO₂ diffusion to photosynthesis in leaves has been debated recently (Morison et al., 2005; Pieruschka et al., 2005, 2006).

Previously, using combined chlorophyll *a* fluorescence imaging and gas exchange measurements, we showed that in moderate light there was strong CO₂ depletion in leaf areas where stomata were blocked with grease in one homobaric species (*Commelina communis*) and one heterobaric species (dwarf bean [*Phaseolus vulgaris*];

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Morison et al., 2005). While changing the external CO₂ concentration showed that there was some lateral diffusion in both species, we concluded that in the light, lateral diffusion could not support appreciable photosynthesis over distances of more than approximately 0.3 mm because of the marked decline in intercellular CO₂ mole fraction (C_i) when stomata were blocked. In contrast, Pieruschka et al. (2006) used chlorophyll *a* fluorescence images to examine light-shade boundaries in leaves of two homobaric species, faba bean (*Vicia faba*) and tobacco (*Nicotiana tabacum*), and two heterobaric species, dwarf bean and soybean (*Glycine max*). While they found that there was no detectable lateral diffusion in the heterobaric species, there could be sufficient lateral CO₂ diffusion in the homobaric species when stomatal conductance was low to increase photosynthesis over distances of up to 3 mm. Furthermore, they suggested that lateral CO₂ transfer from darkened to illuminated sections of leaves in patchy sunlight might be important in permitting some CO₂ assimilation when stomata are closed (Pieruschka et al., 2006). This would result in improved transpiration efficiency and could have some selective advantage.

Part of the disagreement in these recent articles may reside in the different techniques used and the different species examined and part in the importance of lateral CO₂ transfer varying with net CO₂ assimilation rate (A ; Lawson and Morison, 2006). In addition, we have argued that there is not a simple dichotomy between species with homobaric or heterobaric leaves but rather a gradation that depends on the extent and size of BSE and several other anatomical differences (Morison and Lawson, 2007). In this article, we reexamined the rate and spatial extent of lateral CO₂ diffusion during photosynthesis by using the artificial grease patch technique to simulate stomatal heterogeneity on a scale of a few millimeters. First, we examined lateral diffusion during different A by comparing the effect of grease patches on chlorophyll *a* fluorescence in wild-type and transgenic antisense Rubisco tobacco plants with normal and low A . Then we quantified the extent of lateral CO₂ diffusion using gas exchange across a range of light intensities in several species with different leaf anatomies. Using a two-dimensional (2-D) diffusion model, we explored the consequences of different A versus C_i relations and different simulated patch sizes. We conclude that lateral CO₂ diffusion over short distances up to approximately 1 mm can contribute substantially to leaf CO₂ assimilation rate in a number of dicotyledonous species, including some classified as heterobaric.

RESULTS

Imaging Photosynthesis in Regions with Blocked Stomata

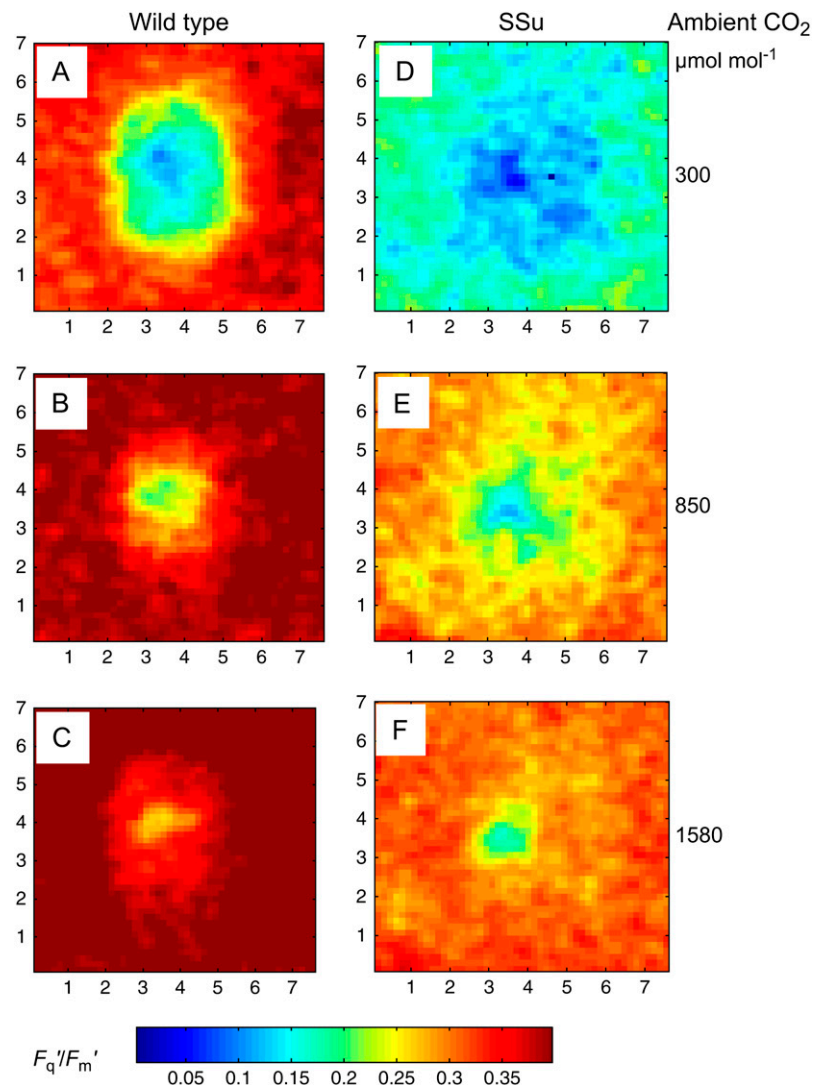
Applying a 4-mm diameter grease patch to mature leaves of tobacco in moderate photosynthetic photon flux density (PPFD; 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$) inhibited photo-

tosynthesis underneath the patch, as measured by the quantum efficiency of PSII (F_q'/F_m' ; Fig. 1). The chlorophyll *a* fluorescence images show a substantial reduction of F_q'/F_m' under the patch in the wild-type plants at an ambient CO₂ mole fraction (C_a) of 300 $\mu\text{mol mol}^{-1}$, and at the patch center, F_q'/F_m' dropped to <0.05 (Fig. 1A). The decrease of F_q'/F_m' was less severe and the area affected smaller when C_a was increased to 850 $\mu\text{mol mol}^{-1}$ (Fig. 1B). With $C_a = 1,580 \mu\text{mol mol}^{-1}$, the F_q'/F_m' decline was much smaller, although in the middle of the patch, F_q'/F_m' was only 0.25 compared to 0.4 outside the patch (Fig. 1C). When patches were applied to transgenic plants with reduced Rubisco (small subunit, SSu) and, consequently, reduced A , the F_q'/F_m' was substantially lower outside the patch but was still reduced to <0.05 in the patch center in normal C_a (Fig. 1D). In these transgenic plants, the grease patch effect became more noticeable in higher C_a (850 $\mu\text{mol mol}^{-1}$), because F_q'/F_m' outside the patch increased (Fig. 1E). Even in the highest C_a , there was still a noticeable small patch with a drop of F_q'/F_m' from approximately 0.3 to 0.17 (Fig. 1F).

In the 1% O₂ concentrations used, F_q'/F_m' was linearly related to A (Morison et al., 2005). Thus, each pixel's F_q'/F_m' value can be transformed to an A value using a calibration derived from A and F_q'/F_m' for the whole leaf area in the chamber prior to patch application. Transects of the derived A averaged from three leaves (Fig. 2) show that A in the wild-type plants dropped to only approximately 1 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the patch center in low C_a but was slightly higher in the SSu plants. The effect of the patch on A extended slightly beyond the area greased (approximately 0.3 mm), presumably because the additional CO₂ sink in the patch reduces C_i in the surrounding area. However, there is some uncertainty in the precise placement and the spread of grease at the patch margins on application, which makes it difficult to determine the exact zone of effect. Transects calculated when $C_a = 850 \mu\text{mol mol}^{-1}$ show an increase in A across the whole transect, and they are similar for wild-type and SSu plants because the increased C_a compensated for the lower Rubisco content in the transgenic plants. In even higher C_a , the reduction in F_q'/F_m' in the patch was slight, and the effect of the patch on photosynthesis in the two plant types was identical.

Assuming that the grease blocks stomata completely (see below), A should have declined to close to zero in these low O₂ conditions if there were no lateral CO₂ flux. Therefore, the A values in the patched area on these transects are lateral CO₂ flux rates (A_{lateral}) to a particular point. When $C_a = 300 \mu\text{mol mol}^{-1}$, the mean A_{lateral} was 5.6 and 4.7 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in wild-type and transgenic plants, respectively, compared to 14.1 and 8.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in ungreased areas (potential assimilation rate [A_p]). Therefore, the transgenic plants had higher values of A_{lateral}/A_p (0.58) compared to 0.40 in wild type. In the highest C_a , the patterns of the A transects were very similar in transgenic and wild-type plants and values of A_{lateral}/A_p were the same, approximately 0.82.

Figure 1. Images of F_q'/F_m' from chlorophyll *a* fluorescence in part of tobacco leaves with a 4-mm diameter grease patch applied to both sides at different C_a concentrations. Image scales are in millimeters. A to C are for a wild-type plant, and D to F are for an antisense plant with reduced Rubisco SSu. Leaf temperature approximately 25°C; 1% O_2 ; PPFD = $400 \mu\text{mol m}^{-2} \text{s}^{-1}$.



Gas Exchange Measurements of Lateral CO_2 Diffusion

The chlorophyll fluorescence imaging was performed with moderate PPFD to give high measurement sensitivity and under low O_2 to inhibit photorespiration and produce a linear relationship between A and F_q'/F_m' . To examine the magnitude of lateral CO_2 diffusion across a range of PPFD during normal CO_2 assimilation, we used a gas exchange approach. The response of CO_2 assimilation rate to PPFD was measured in areas of attached leaves before and after grease patches were applied to both leaf surfaces (Fig. 3A). The patches were evenly spaced and covered approximately one-half of the leaf, and five species were examined: petunia (*Petunia hybrida*), faba bean, sunflower (*Helianthus annuus*), dwarf bean, and maize (*Zea mays*). Normal ambient CO_2 and O_2 concentrations were used. In petunia, the transpiration rate (Tr) when greased (Tr^c) was reduced to 0.47 of that of the leaves prior to covering (Fig. 3B), exactly matching the proportion of leaf area not covered by grease, indicating that the grease blocked the stomata completely.

However, the assimilation rate when greased (A^c) was only reduced to 0.60 of that prior to covering at PPFD $\geq 300 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 3A). In lower PPFD, A^c/A increased sharply, rising to 0.93 in the lowest PPFD. As a control experiment, the patch applicator was used on petunia leaves in exactly the same way but without any grease (Fig. 3, C and D). The ratios $A^c:A$ and $Tr^c:Tr$ were 0.94 and 0.86, respectively, when PPFD $\geq 300 \mu\text{mol m}^{-2} \text{s}^{-1}$, indicating that stomatal conductance was slightly reduced either by the procedure itself or by the time taken to carry out the two consecutive PPFD response curves. However, the reduction was clearly much smaller than when grease was applied, when Tr^c to Tr closely matched the ratio of covered to uncovered areas (Fig. 3B). Therefore, the simplest explanation for the results in the greasing experiment is that there was lateral CO_2 diffusion within the leaf into the mesophyll areas with greased epidermis. The relative contribution this made to the CO_2 assimilation in the whole leaf area measured was higher in low PPFD when there was low A .

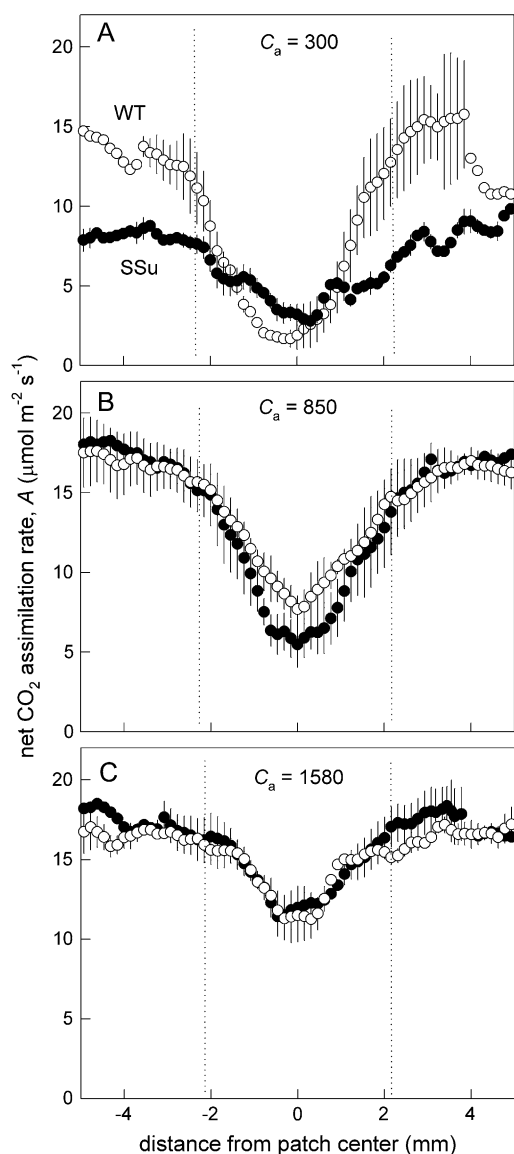


Figure 2. Transects of images of A in wild-type (white symbols) and antisense Rubisco SSu tobacco plants (black symbols) with a single 4-mm diameter grease patch at different C_a concentrations. Images were calculated from the linear relationship between F_q'/F_m' and A and transects are the mean of three plants with SEM shown; each transect was the average of three adjacent lines of pixels, each approximately 0.15×0.15 mm. Dotted vertical lines show the edge of the grease patch. Other conditions as given in Figure 1.

In faba bean (a homobaric species) and in sunflower and dwarf bean (heterobaric species) there were similar results (Supplemental Figs. S1–S3) to those with petunia. The values of A^c/A were significantly larger than Tr^c/Tr in all PPFD and increased to >0.8 at the lowest PPFD. Thus, the ratio of the change in CO₂ assimilation to the change in transpiration ($[A^c/A]/[Tr^c/Tr]$, equivalent to $A^c \times Tr/A \times Tr^c$; Fig. 4) was significantly larger than unity in all these four dicotyledonous species. This is strong evidence that lateral

CO₂ diffusion into the greased areas with blocked stomata was substantial. In contrast, in leaves of the heterobaric, monocot species maize, A^c/A was very similar to Tr^c/Tr (both approximately 0.55; Supplemental Fig. S4). Therefore, $A^c \times Tr/A \times Tr^c$ was close to 1.0 (Fig. 4), indicating very little lateral CO₂ flux.

The lateral diffusion rate was calculated as the CO₂ assimilation in excess of that expected from the remaining uncovered portion of the leaf (which was assumed to photosynthesize at the rate prior to patch application, i.e. A) as:

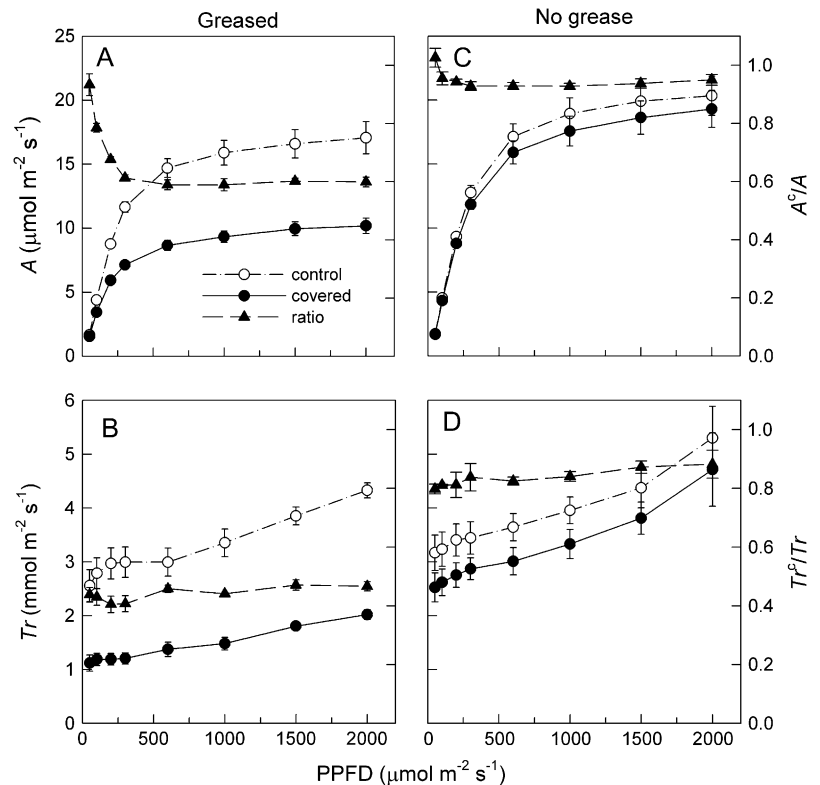
$$A_{\text{lateral}} = A^c - A \times (1 - f^c),$$

where f^c is the proportion of leaf area covered by grease. Figure 5A shows that A_{lateral} was significantly above 0 in all four dicot species. In moderate to high PPFD, A_{lateral} was approximately constant at 1.7 to 2.1 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. When PPFD was $<300 \mu\text{mol m}^{-2} \text{ s}^{-1}$, A_{lateral} declined, reaching a minimum of 0.8 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ in all four species in the lowest PPFD (Fig. 5A). In contrast, in maize, there was no measurable A_{lateral} in PPFD $>500 \mu\text{mol m}^{-2} \text{ s}^{-1}$, but in moderate to low PPFD it was detectable but peaking at only 0.7 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$.

As the light-saturated CO₂ assimilation rates differed between species (Fig. 3; Supplemental Figs. S1–S4), we estimated the contribution that lateral flux made to A in the covered areas by expressing A_{lateral} as a proportion of A_p , i.e. the assumed rate of assimilation of the covered area if it had not been covered ($A_p = A \times f^c$; Fig. 5B). In sunflower, petunia, dwarf bean, and faba bean, the lateral CO₂ flux represented approximately 20%, 24%, 20%, and 15%, respectively, of the assimilation rates possible in moderate to high light intensities (PPFD $\geq 600 \mu\text{mol m}^{-2} \text{ s}^{-1}$). When PPFD was $\leq 200 \mu\text{mol m}^{-2} \text{ s}^{-1}$, the lateral CO₂ flux was a much larger proportion (up to 64%, 83%, 95%, and 87%, respectively). In maize, the lateral flux represented a significant proportion of A_p (maximum of 18%) only in low PPFD. However, it should be noted that with the low fluxes in these PPFD, the potential measurement errors increased and the errors in calculating the greased area and repositioning the leaf chamber exactly became relatively more important.

The calculated lateral CO₂ fluxes shown in Figure 5 are substantial and are the integrated fluxes into all the individual patches, each of which presumably shows spatial gradients similar to those measured in tobacco (Fig. 1). Figure 1 also shows that in normal C_a , the central area of the 4-mm patches used on the tobacco was contributing little to leaf photosynthesis. Given the 12- \times 5.8-mm diameter patches used for the light response experiments, it can be calculated that the A_{lateral} values represent an outer area of approximately 0.5 to 0.9 mm of each patch photosynthesizing at the potential rates. Therefore, smaller patches should produce higher lateral CO₂ fluxes, which we confirmed by comparing 4.5- and 5.8-mm diameter patches in faba bean leaves (Fig. 6A; Supplemental Fig. S5). With the smaller patches, the lateral CO₂ flux doubled to 3.4 $\mu\text{mol m}^{-2} \text{ s}^{-1}$, but

Figure 3. A and B, Response of A and Tr to PPFD in petunia with (covered) and without (control) 12×5.8 -mm diameter grease patches applied to both leaf surfaces covering approximately one-half the leaf area; $n = 4$. Bars = SEM. Ratios of $A^c:A$ and $Tr^c:Tr$ when covered and uncovered shown on right axes. C and D, Results for the same protocol of patch application but without grease; $n = 3$. Leaf temperature = 25°C , vapor-pressure difference = 0.8 kPa , chamber CO_2 concentration = $360 \mu\text{mol mol}^{-1}$ and $21\% \text{ O}_2$.



the shape of the A_{lateral} response to PPFD was very similar, showing saturation in approximately $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$. At higher PPFD, A_{lateral} represented some 24% of A_p in these leaves compared to 15% with larger patches (Fig. 6B). As the perimeter length of the 24 smaller patches was 56% longer than the perimeter of the 12 larger patches, the lateral flux per unit perimeter length was similar and in both cases is equivalent to a zone of approximately 0.5 mm in each patch photosynthesizing at the potential rate (or 1 mm at one-half the potential rate, etc.). Figure 6A also demonstrates the sensitivity of the calculated A_{lateral} to the exact determination of patch size. Although 5.8-mm diameter is the best estimate of the real grease patch size on the leaf, if the patch was just 0.2 mm larger or smaller, the calculated A_{lateral} would be up to 45% higher or lower in moderate to high PPFD.

The tobacco chlorophyll a fluorescence images and assimilation rate transects (Fig. 1) also showed that the lateral flux of CO_2 increased as the ambient CO_2 concentration increased. We confirmed this effect using the patches with gas exchange measurements, as A_{lateral} increased in sunflower almost in proportion to a 4-fold increase in C_a from 370 to $1,450 \mu\text{mol mol}^{-1}$ (Fig. 7A). Importantly, A_{lateral} increased substantially across the range of PPFD in the higher C_a , indicating that the saturation response of A_{lateral} to PPFD in lower C_a is due to the limited supply of CO_2 . However, A_{lateral}/A_p showed saturation at the same PPFD as at lower C_a (Fig. 7B). In these experiments in high C_a , the reduction in transpiration rate when covered was more than

f^c (Supplemental Fig. S6). While there is no obvious explanation, it suggested an alternative estimate of A_{lateral} using the Tr^c/Tr measured at each PPFD rather than the fixed f^c (see Eq. 1). However, this modification produced estimates of A_{lateral} similar to those from Equation 1 (shown as “recalculated” in Fig. 7A).

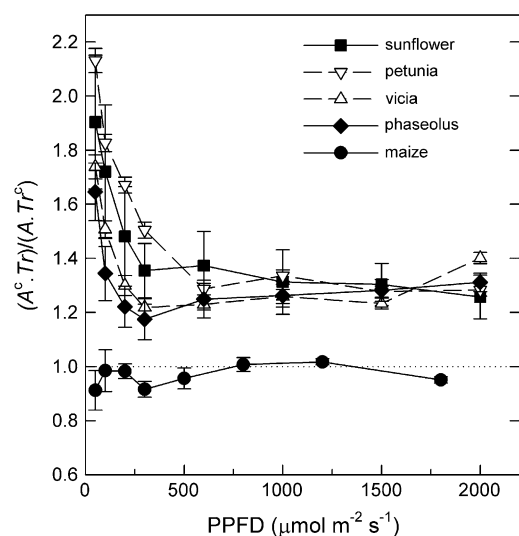


Figure 4. Change with PPFD of the relative effect of grease patches on A compared to the effect on Tr in five species. Relative effect ratio calculated as $[A^c/A]/[Tr^c/Tr]$, equivalent to $[A^c \times Tr]/[Tr^c \times A]$. Data calculated from Figure 3 and Supplemental Figures S1 to S4. Bars = SEM.

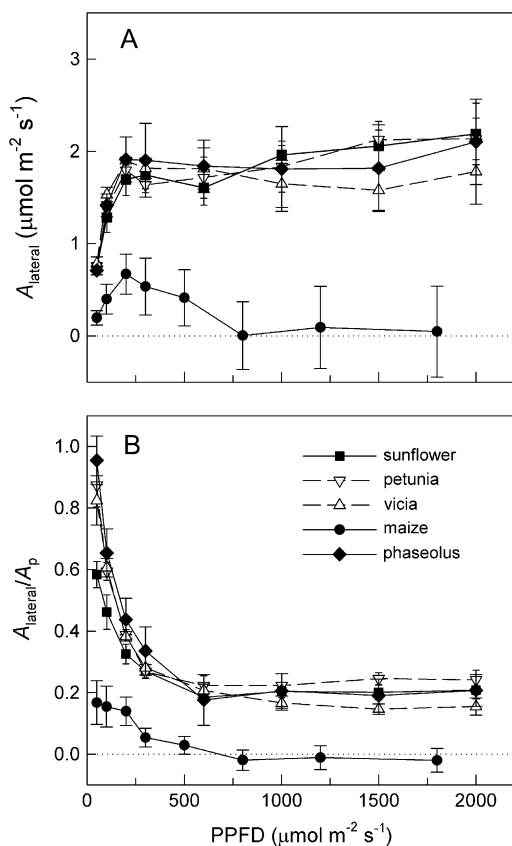


Figure 5. A, Response of A_{lateral} into circular greased areas of leaf to PPFD in five species. B, A_{lateral} expressed as a proportion of the A_p in the covered leaf with a particular PPFD.

Modeling Lateral Diffusion into Patched Areas

The difference in lateral CO₂ diffusion observed between the four dicot species and the monocot maize (Fig. 5A) was probably due largely to anatomical differences. However, it may also be affected by the different response of A to C_i between plants with C3 and C4 photosynthesis, because mesophyll cells along the diffusion pathway can reduce C_i to much lower values in a C4 species. The effect of such different A/C_i relationships was explored using the 2-D model previously developed to understand lateral CO₂ diffusion into leaf patches with blocked stomata at this scale (Gallouët and Herbin, 2005; Morison et al., 2005). The model computes C_i values across a patch averaged across the depth of the leaf. Empirical A/C_i relationships were determined at low, medium, and high PPFD in petunia and maize leaves with no patches. These relationships were used to calculate the effect of selecting different values of the effective lateral CO₂ diffusion coefficient (D_c') on the CO₂ assimilation rate across a patch (Fig. 8). In both high and low PPFD, the decline in A across the patch was less steep in the C3 species petunia than in the C4 maize at any particular D_c' . Thus, even at low light, if D_c' was 25% of that in free air, the calculated A ($=A_{\text{lateral}}$) was reduced to 0 in the patch center in

maize, while in petunia it was 3 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In high light, even an unfeasibly high D_c' of 50% produced very low A values in the patch center in maize, while there were substantial CO₂ assimilation rates in the petunia patch. However, for all D_c' values used ($\geq 6\%$), the model suggests there should be appreciable lateral diffusion into the periphery of the patch in maize (Fig. 8), which does not agree with the very low measured A_{lateral} rates in Figure 5A for this species. This suggests that there are substantial physical barriers to lateral CO₂ diffusion in maize and other monocotyledonous species with similar vascular patterns.

The modeled transects were used to calculate the lateral fluxes into the greased mesophyll area (in the same proportion to the ungreased area as in the gas exchange experiments), assuming different D_c' (Fig. 9A). This demonstrates that for any feasible D_c' value ($\leq 40\%$), the modeled lateral CO₂ diffusion in petunia is largely unaffected by PPFD over the range used here (200–1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$), agreeing with the measurements in Figure 5. Furthermore, using the A_{lateral} rates in Figure 5 to interpolate in Figure 9A suggests that an appropriate D_c' value for petunia is approximately 20% of the free air value. The model was also used to examine the sensitivity of A_{lateral} to the patch size, assuming

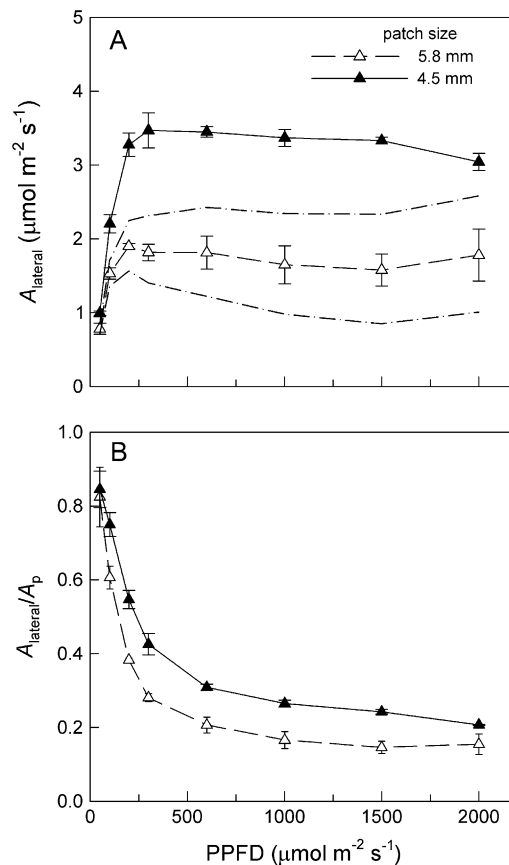


Figure 6. A, Response of A_{lateral} to PPFD in faba bean with either large (5.8 mm) or small (4.5 mm) patches. B, A_{lateral} expressed as a proportion of the A_p in the covered leaf with a particular PPFD.

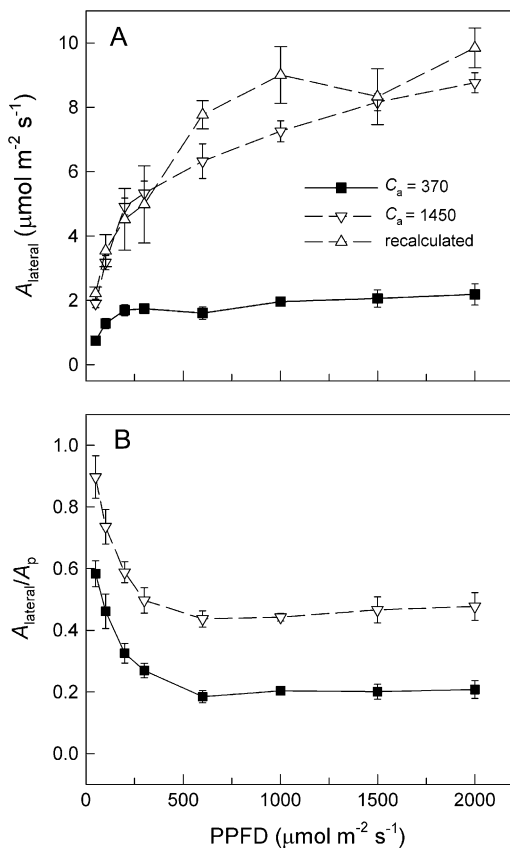


Figure 7. A, Response of A_{lateral} to PPFD in sunflower in either 370 or 1,450 $\mu\text{mol mol}^{-1}$ ambient CO_2 . B, A_{lateral} expressed as a proportion of the A_p in the covered leaf with a particular PPFD.

a D_c' of 20% (Fig. 9B). The modeled values of A_{lateral}/A_p match well those measured for petunia with 5.8-mm diameter patches at the three PPFD used (see Fig. 5). The values of A_{lateral}/A_p also matched the figures calculated for the wild-type tobacco with 4-mm patches (0.4) and the relative lateral CO_2 flux increased sharply with smaller patches, as the measurements with faba bean showed (compare Figs. 9B and 6B). The relative lateral diffusion was slightly reduced with larger patches when the preindustrial atmospheric CO_2 concentration was used (Fig. 9B), although the calculation did not take into account any alterations of the A/C_i response parameters with growth in a different CO_2 concentration.

DISCUSSION

The experiments here using both chlorophyll *a* fluorescence imaging and gas exchange measurements with grease patches on leaves allowed the quantification of a substantial lateral CO_2 diffusion rate across a range of light intensities in five dicotyledonous species, two of them previously classified as homobaric and two heterobaric (Fig. 5). Only in the monocot maize was there very little lateral CO_2 diffusion. In all the dicot

species, the lateral diffusion rate saturated at moderate PPFD in present day CO_2 concentrations. The results emphasize that local stomata usually supply CO_2 to the adjacent mesophyll, but when stomata are blocked, significant lateral CO_2 diffusion can occur over short distances up to approximately 1 mm. While in our recent fluorescence imaging work (Morison and Lawson, 2007) we showed qualitatively that there were differing amounts of lateral diffusion in different species, we could not quantify the rates, as the images were not calibrated with simultaneous measurements of A . Furthermore, in our previous work with fluorescence imaging on two species, dwarf bean and *C. communis* (Morison et al., 2005), we emphasized the decline in C_i to low concentrations within approximately 0.3 to 0.5 mm of the grease patch periphery and thus did not assess the overall contribution of lateral CO_2 diffusion to photosynthesis in the whole patch directly. The results here modify our earlier interpretation of the quantitative significance of lateral CO_2 diffusion to photosynthesis.

Lateral CO_2 Diffusion Depends upon the Rate of Photosynthesis of the Mesophyll Cells

In our work with tobacco, we used the linear relationship of A and F_q'/F_m' to calculate A directly from the fluorescence images. Although the decline in A was sharp in the 0.3- to 0.8-mm periphery of patches (Figs. 1 and 2), the overall assimilation supported by lateral CO_2 flux within the patch was substantial, and in wild-type plants in normal C_a it was 40% of the assimilation rate outside the patch. Furthermore, the comparison of the wild-type and transgenic tobacco (Figs. 1 and 2) emphasizes that lateral CO_2 diffusion depends on both the CO_2 gradient (and hence on C_a) and the rate of CO_2 uptake by the mesophyll cells along the pathway. Previous work with these transgenic Rubisco antisense plants has shown that the leaf anatomy was similar to that of the wild type but their A was only 25% of wild type in normal C_a (von Caemmerer et al., 1994). Thus in normal C_a , the CO_2 uptake by mesophyll cells was lower in the transgenic plants than in the wild type (Fig. 2), creating a higher C_i outside the patch and removing less CO_2 along the lateral pathway, resulting in relatively more lateral diffusion (58% of the uncovered assimilation rate compared to 40% in the wild type).

Lateral CO_2 Diffusion Rate Is Quantitatively Similar in the Dicotyledonous Species Studied

In the four dicotyledonous species measured with gas exchange, the A_{lateral} values were quite similar despite the very different leaf anatomies and the presence and absence of BSE. In high PPFD, A_{lateral} represented some 15% to 24% of the A_p (Fig. 5). These values are specific to the particular patch size and conditions used, as A_{lateral} and A_{lateral}/A_p both increased when smaller patches or higher C_a were used (Figs. 6 and 7). In higher C_a , the lateral C_i gradient was increased, enabling more

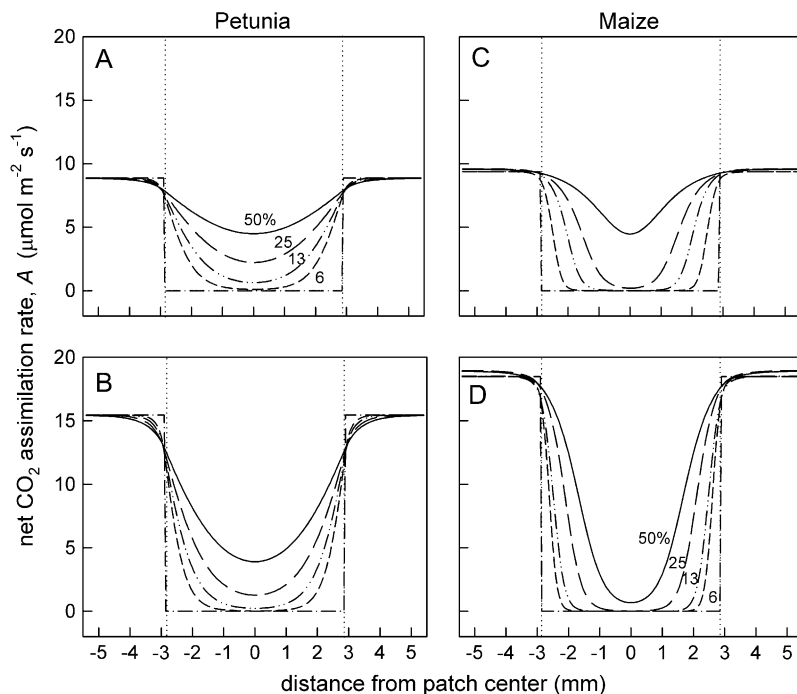


Figure 8. Modeled transects of A across circular patches 5.8-mm diameter for different assumed reductions in the effective D_c' inside the leaves compared to the free air value. Modeled using photosynthetic parameters for petunia in low light (A; $200 \mu\text{mol m}^{-2} \text{s}^{-1}$) or high light (B; $1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$) and for maize in low light (C; $200 \mu\text{mol m}^{-2} \text{s}^{-1}$) or high light (D; $1,200 \mu\text{mol m}^{-2} \text{s}^{-1}$). Ambient $\text{CO}_2 = 360 \mu\text{mol mol}^{-1}$.

lateral diffusion deeper into the patched area, and the slope of the A/C_i relationship for the cells along the pathway was reduced, with consequent higher A in the patch, as is also evident in the tobacco chlorophyll fluorescence images (Fig. 1).

The lateral CO₂ diffusion rates approached saturation in relatively low PPFD despite some differences in photosynthetic rates and in growing conditions (glasshouse or growth cabinet). The saturation was caused by the balance between the integrated assimilation rate of cells at different distances from the patch edge and the higher photosynthetic demand for CO₂ at higher PPFD, as is illustrated in the modeled A transects (Fig. 8). The diffusion model calculations (Fig. 9A) showed that with large patches there is a small increase in A_{lateral} at the highest PPFD (also evident in some of the data sets in Fig. 5). However, when expressed relative to A when not patched, A_{lateral} was constant across PPFD $>600 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 9B).

Interestingly, light response curves of leaves usually show a light saturation of calculated C_i (e.g. Morison and Jarvis, 1983), which is similar to the saturation shown here in A_{lateral} . This suggests that the calculated C_i is partly influenced by the relative photosynthetic rates of the different layers of cells as PPFD increases and the local supply of CO₂ within the leaf, which becomes limiting at higher PPFD.

The good agreement between the measured and modeled A_{lateral} values and their responses to PPFD and patch diameter gives confidence in the observations presented here (compare Figs. 5 and 9, and 2 and 8). However, the model is only a 2-D approximation and does not represent the vertical gradients in C_i and A that must exist in leaves because of the light and photosyn-

thetic characteristics profiles and the differences between cell types such as found in C4 species. The model calculations used only gas exchange measurements and A/C_i data obtained on unpatched leaves for parameterization and a range of assumed effective D_c' values. Previous evaluation of the model showed that when fitted to measured C_i patterns in patched leaves, it estimated feasible D_c' values (Gallouët and Herbin, 2005; Morison et al., 2005). When used with petunia data here, the model suggested a D_c' value of approximately 20%, which is plausible given the typical porosities of leaves (Morison and Lawson, 2007).

The large A_{lateral} values determined here might be surprising, as two of the species (sunflower and dwarf bean) are normally described as heterobaric and have BSE on some veins. However, determining the distribution of such extensions on different vein orders and their continuity and, hence, their effectiveness as diffusion barriers is difficult (Weyers and Lawson, 1997). The spacings between the smallest veins visible in macro photographs of the sunflower and dwarf bean leaves used here were typically 0.5 to 0.9 mm, agreeing with the mean spacing between BSE of $635 \mu\text{m}$ reported by Wylie (1952) in a large survey of 52 temperate herbaceous species. The grease patches applied were large and circular, so even if there are discrete areoles formed by veinlets and BSE, there will be parts of areoles underlying the edge of the patches where lateral diffusion is possible. In homobaric species without such barriers, diffusion will more readily occur and may be possible over longer distances of several millimeters (Pieruschka et al., 2005, 2006). However, with normal photosynthetic rates, even in these homobaric species, effective diffusion will only be possible over short distances because

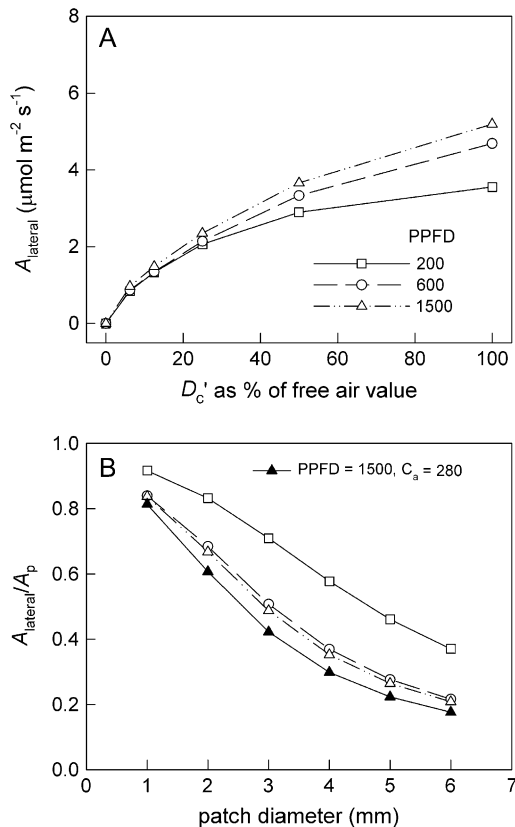


Figure 9. A, Change of modeled A_{lateral} with different reductions in the effective D_c' inside the leaves compared to the free air value. B, Change of modeled relative A_{lateral}/A_p for petunia with patch diameter assuming $D_c' = 20\%$. Modeled using parameters of A/C_i response curves measured in petunia at the three PPFD shown, in ambient CO_2 concentration of $360 \mu\text{mol mol}^{-1}$, except for the curve shown in B with black symbols, where a preindustrial CO_2 of $280 \mu\text{mol mol}^{-1}$, a 25% increase in conductance, and a 15% reduced leaf thickness was used.

of the CO_2 consumption along the pathway (as exemplified in Fig. 1 in tobacco, and see figure 1 in Morison and Lawson [2007] for other species).

These results both agree and contrast with those of Pieruschka et al. (2006), who used chlorophyll *a* imaging across light-shade boundaries at moderate light intensities (PPFD = $290 \mu\text{mol m}^{-2} \text{s}^{-1}$). They found differences in F_q'/F_m' that suggested substantial lateral CO_2 diffusion in the two homobaric species, tobacco and faba bean, as observed here, although the effect was evident over longer distances than here. However, they did not find evidence for lateral CO_2 diffusion in the heterobaric species dwarf bean or soybean. Part of these differences can be attributed to the different experimental system and conditions, as they mainly used drought-stressed plants with low photosynthetic rates and considerably higher vapor-pressure difference (1.9 kPa). Figure 5 shows that with low photosynthetic rates, A_{lateral} is small in absolute terms. In addition, it is possible that part of the effect they observed was due to localized changes in water status close to nontranspiring areas,

which might have affected photosynthesis in the homobaric species without BSE more than in the heterobaric species with BSE and the consequent better water supply.

Lateral CO_2 Diffusion Is Low in Maize

The monocot maize was markedly different from the four dicots and showed almost no lateral CO_2 diffusion. This difference agrees with the contrasting effect of C_a on F_q'/F_m' in patched areas of these species (Morison and Lawson, 2007, Fig. 1). Although high C_a restored F_q'/F_m' in dwarf bean, faba bean, tobacco, and sunflower, there was no such effect in maize. While on a large scale maize and other monocots are often described as having a parallel venation, the longitudinal veins are linked by numerous small transverse veins, so they have an essentially reticulate pattern (Nelson and Dengler, 1997). The measurements here suggest this vein network acts as an effective lateral diffusion barrier. In addition, measurements by Long et al. (1989) showed very limited transverse gas diffusion across maize leaves because of the limited connectivity between upper and lower surface airspaces, which would further restrict any lateral diffusion. However, we have previously found substantial lateral diffusion in another nongrass monocot species, *C. communis* (Morison et al., 2005), which suggests that a range of other monocots should be examined before a generalization is made.

The Role of Lateral Gas Diffusion in Leaves

It may be suggested that the lateral CO_2 fluxes found here are particular to the experimental system used with the artificial grease patches. Certainly, if patchy stomatal behavior occurs in particular areoles bounded by veins with continuous BSE, then lateral CO_2 transfer may not be appreciable. This is evident in many chlorophyll fluorescence images of patchy stomatal behavior (e.g. Eckstein et al., 1996; Beyschlag and Eckstein, 1998, 2001). However, there are several natural situations where heterogeneity of photosynthesis takes place without such organized stomatal patchiness, e.g. in light and shade flecks, when there are rain drops on leaf surfaces, during some types of herbivory (Aldea et al., 2005), or during pathogen infection. The results here suggest that in such situations, significant lateral CO_2 diffusion may occur over short distances of up to 1 mm. In particular, if CO_2 assimilation rates are low the relative size of these lateral fluxes may be high.

Pieruschka et al. (2006) have suggested that any lateral CO_2 diffusion when stomata are closed confers an increase in transpiration efficiency, which may have a selective advantage. Certainly, the artificial stomatal patchiness created here caused an increase in transpiration efficiency of approximately 25% in moderate to high PPFD (Fig. 4). However, there was a substantial reduction in the overall assimilation rate when leaves were patched, supporting a strong selection for an optimal stomatal spacing to ensure sufficient local CO_2 supply to the mesophyll. The presence or absence of

BSE in the herbaceous species we examined did not affect the degree of lateral diffusion. While we have not examined any evergreen, perennial species, it is notable that a large survey of 250 evergreen species in a tropical rainforest recently showed that leaves with BSE were much more prevalent in species from gap or upper canopy and emergent habitats (Kenzo et al., 2007). In contrast, species without BSE were predominantly understory or subcanopy species. This suggests that possessing BSE improves either water distribution or mechanical support for leaves in drier, more exposed environments and that lateral gas diffusivity is not a primary selection factor. In addition, Nikolopoulos et al. (2002) have suggested that BSE can increase light penetration into thicker leaves (typical of drier habitats) and improve photosynthesis despite the loss of photosynthetic leaf area that the achlorophyllous BSE represents. These observations emphasize the complexity of the task of understanding the ecological distribution of different leaf forms from photosynthetic characteristics (Wright et al., 2004) and leaf hydrology (Sack and Holbrook, 2006).

MATERIALS AND METHODS

Plant Material

Seeds of sunflower (*Helianthus annuus*) 'Rigasol' and maize (*Zea mays*) 'Adonis' were sown in a peat- and loam-based compost and grown in controlled environment cabinets in Orsay at 23°C/18°C day/night, 250 to 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 16 h/d. Seedlings of petunia (*Petunia hybrida*) mixed hybrids were planted into a bed of loam soil in a heated glasshouse in Orsay in late March. Tobacco (*Nicotiana tabacum*) 'W38' plants were either wild type or from selfed T₂ progeny of a tobacco transformant with an antisense gene directed against the Rubisco SSu driven by the cauliflower mosaic virus 35S promoter (Hudson et al., 1992). Seeds were sown in controlled environment cabinets in Colchester at a PPFD = 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 18-h photoperiod, with 25°C/18°C day/night temperatures and C_a = 800 $\mu\text{mol mol}^{-1}$ to produce wild-type and transgenic plants of similar appearance and growth characteristics (von Caemmerer et al., 2004). Faba bean (*Vicia faba*) 'Aquadulce' and dwarf bean (*Phaseolus vulgaris*) 'Vilbel' were sown in pots in a peat- and loam-based compost (F2, Levington Horticulture) and grown in a naturally illuminated, heated glasshouse in Colchester. Temperature was maintained above 20°C at night and rarely exceeded 30°C during the day. Supplementary lighting (350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the height of the plants) was provided from 7 AM to 7 PM by sodium vapor lamps.

All plants were well watered throughout, and young but fully expanded leaves were used for all measurements. Measurements were made on attached fourth or fifth leaves of 3- to 5-week-old faba bean plants and the fully expanded primary leaves of dwarf bean, although very similar results were obtained with the terminal leaflet of the first trifoliolate. Petunia shoots were excised from the 12- to 16-week-old plants in the glasshouse and recut under water before measurement. For maize, the third leaves of 6-week-old plants were used, still attached, and the chamber was placed on the first third of the leaf from the tip. For sunflower, excised second or third leaves of 4- to 6-week-old plants were used.

Chlorophyll *a* Fluorescence Imaging with Single Patches

Images of chlorophyll *a* fluorescence were obtained on attached tobacco leaves essentially as described previously by Morison et al. (2005) using a CF Imager (Technologica) with a spatial resolution of approximately 0.15 × 0.15 mm. The LEDs in the imaging system provided a PPFD of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Steady-state fluorescence (*F'*) was continuously monitored, while maximum fluorescence in the actinic light (*F_m'*) was measured during an 800-ms exposure to a saturating pulse of 4,800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Using the images captured at *F'* and *F_m'*, images of $F_q'/F_m' = (F_m' - F')/F_m'$ were constructed by the

imaging software. Leaf conditions during imaging were controlled using a 10-cm² area stirred leaf chamber [model PLC(B), PP Systems] with a low reflectance window attached to a portable infrared gas analysis system (CIRAS1, PP Systems). A single grease patch 4 mm in diameter was applied to both leaf surfaces using neoprene foam discs mounted onto forceps and leaves were illuminated and imaged from above. Leaves were placed in the chamber and allowed to stabilize for at least 30 min to the chamber conditions, which were CO₂ mole fraction of 360 $\mu\text{mol mol}^{-1}$, water vapor pressure of approximately 1.8 kPa, and leaf temperature of 24°C to 27°C, giving leaf-air vapor pressure differences in the range of 0.7 to 1.4 kPa. Oxygen concentration was maintained at 1% (v/v) by supplying air from a cylinder of 1% oxygen in nitrogen.

Using the CF imager software, parts of the F_q'/F_m' images with the patch and some surrounding leaf areas were isolated, smoothed using a loss-less, low pass spatial filter, and the data transferred to MATLAB (v. 7.0, The MathWorks). For comparison between conditions, average transects of F_q'/F_m' were calculated from three rows of pixels along the horizontal axis passing through the center of the patch image. Transects were averages of three wild-type and three transgenic plants.

A/Q Measurements with One-Half of the Leaf Covered in Patches

Responses of *A* to PPFD (*A/Q* curves) were measured using identical portable gas exchange systems at Colchester and Orsay. They were the Li-6400 (LiCor) with standard leaf chamber, using white chamber seals and red and blue LED light source. Air humidity was supplied and controlled using a dew-point generator (Li-610, LiCor). Initially, leaves were allowed to stabilize for approximately 30 to 45 min to the leaf chamber conditions, where CO₂ was maintained at 360 $\mu\text{mol mol}^{-1}$, 21% O₂, leaf temperature of 25°C, a PPFD of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a controlled water vapor pressure difference of approximately 0.8 kPa. After stabilization, an *A/Q* curve was constructed by first decreasing, then increasing the PPFD in steps lasting approximately 5 to 10 min. The final measurement was a repeat at 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD. Then, approximately one-half the leaf area was covered with grease in 12- × 5.8-mm diameter patches and applied to both leaf surfaces using neoprene foam discs as before. The grease used depended on the experiment, as the silicone grease (Rhodosil) damaged faba bean leaves, so nonsilicone grease was used (Glisseal, Borer Chemie). The leaf was placed back in the chamber in an identical position and allowed to stabilize for approximately 30 min. After stabilization, a second *A/Q* curve was constructed, identical to that described above. With faba bean, an additional set of experiments was done with 24- × 4.4-mm diameter patches. Grease patches had no noticeable effect on leaf appearance for several days after plants were returned to the glasshouse or cabinet. For petunia and maize, *A/C_i* response curves were also measured at three different PPFD: 200, 600, and 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Modeling CO₂ Diffusion into Circular Patches

The 2-D mathematical model implemented in MATLAB developed and described by Gallouët and Herbin (2005) and previously used by Morison et al. (2005) was used to calculate gradients in C_i and *A* across patches, assuming different reduction factors for D_{c'} from that in free air. The model used an empirical hyperbolic function relating *A* to C_v ($A = [(aC_i)/(b + C_i)] - c$) with these three parameters derived from measurements at three different PPFD. The spatial step size was 50 nm and the modeled area and patch size were chosen to represent the relative patched and unpatched areas in the leaf chamber.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Response of *A* and *Tr* to PPFD in sunflower with (covered) and without (control) 12- × 5.8-mm diameter grease patches applied to both leaf surfaces covering approximately one-half the leaf area; *n* = 3.

Supplemental Figure S2. Response of *A* and *Tr* to PPFD in faba bean with (covered) and without (control) grease patches covering approximately one-half the leaf area; *n* = 3.

Supplemental Figure S3. Response of *A* and *Tr* to PPFD in dwarf bean with (covered) and without (control) grease patches covering approximately half the leaf area; *n* = 4.

Supplemental Figure S4. Response of *A* and *Tr* to PPF in maize with (covered) and without (control) grease patches covering approximately one-half the leaf area; *n* = 5.

Supplemental Figure S5. Response of *A* and *Tr* to PPF in faba bean with (covered) and without (control) small grease patches (4.5-mm diameter) covering approximately one-half the leaf area; *n* = 3.

Supplemental Figure S6. Response of *A* and *Tr* to PPF in sunflower with (covered) and without (control) grease patches covering approximately one-half the leaf area; *n* = 5.

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