

## Update on Photosynthesis

## Carbon Dioxide Diffusion inside Leaves

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Leaves are beautifully specialized organs that enable plants to intercept light necessary for photosynthesis. The light is dispersed among a large array of chloroplasts that are in close proximity to air and yet not too far from vascular tissue, which supplies water and exports sugars and other metabolites. To control water loss from the leaf, gas exchange occurs through pores in the leaf surface, stomata, which are able to rapidly change their aperture. Once inside the leaf, CO<sub>2</sub> has to diffuse from the intercellular air spaces to the sites of carboxylation in the chloroplast (for C<sub>3</sub> species) (Fig. 1) or the cytosol (for C<sub>4</sub> species). These internal diffusion paths are the topic of this article.

There are several reasons why internal diffusion is of interest. First, Rubisco has a poor affinity for CO<sub>2</sub> and operates at only a fraction of its catalytic capacity in C<sub>3</sub> leaves. The CO<sub>2</sub> gradient within the leaf thus affects the efficiency of Rubisco and the overall nitrogen use efficiency of the leaf. Second, prediction of photosynthetic rates of leaves from their biochemical properties requires a good estimate of the partial pressure of CO<sub>2</sub> at the sites of carboxylation,  $p_c$ . Third, internal resistance to CO<sub>2</sub> diffusion results in a lower  $p_c$  and reduces carbon gain relative to water loss during photosynthesis (water-use efficiency). Considerable effort is being invested selecting and identifying plants with improved water-use efficiency using the surrogate measure of carbon isotope discrimination,  $\Delta$ , of plant dry matter (Ehleringer et al., 1993). The ratio of intercellular to ambient CO<sub>2</sub> partial pressure,  $p_i/p_a$ , and  $\Delta$  are both linearly related to water-use efficiency if  $p_c/p_i$  is constant. To date, we have little knowledge of genetic variation in  $p_c/p_i$ .

Until recently, it was not possible to directly measure the gradient in CO<sub>2</sub> partial pressure to the sites of carboxylation. The gradient could be inferred from a theoretical analysis of the diffusion pathway, but because several steps have unknown permeability constants, the values are uncertain. Opinion has oscillated from the existence of large to small gradients over the last 30 years. There are now two techniques that enable the gradient to be measured in C<sub>3</sub> leaves. After describing these techniques, we will consider diffusion through intercellular air spaces and diffusion across cell walls and liquid phase to sites of carboxylation. Finally, we will examine CO<sub>2</sub> diffusion into mesophyll cells and across the bundle sheath in C<sub>4</sub> leaves.

TECHNIQUES FOR MEASURING CO<sub>2</sub> TRANSFER CONDUCTANCE

Conventional gas-exchange techniques measure fluxes of water and CO<sub>2</sub> into and out of a leaf. The gradient in partial pressure of CO<sub>2</sub> from ambient air to the substomatal cavities (usually referred to as  $p_i$ ) is derived using Fick's law of diffusion, which states that the gradient in partial pressure is equal to the flux divided by the conductance, i.e.  $p_a - p_i = A/g$ , where  $A$  is the rate of CO<sub>2</sub> assimilation and  $g$  is the stomatal conductance to CO<sub>2</sub>. Stomatal conductance can vary rapidly as leaves adjust to changes in irradiance, CO<sub>2</sub>, or humidity. By contrast, CO<sub>2</sub> transfer conductance from the substomatal cavities to the sites of carboxylation ( $g_w$ ;  $p_i - p_c = A/g_w$ ) is approximately constant over 1 d.

We have used  $g_w$  to emphasize the cell wall and liquid phase (Evans, 1983; von Caemmerer and Evans, 1991). However, it actually comprises resistance to diffusion through intercellular air spaces from substomatal cavities to the mesophyll wall,  $r_{ias}$ , as well as resistance through the wall and liquid phase,  $r_{liq}$ . When considering fluxes, it is convenient to use conductances because these vary in proportion to the flux. However, for a pathway with a series of limitations, the reciprocal of conductance, resistance, is more convenient because resistances can be summed to arrive at the total resistance for a pathway (although for distributed sinks and mixed pathways, this is not strictly true; see Parkhurst, 1994). CO<sub>2</sub> transfer resistance and conductance can be approximated by the equations (Evans et al., 1994)

$$r_w = r_{ias} + r_{liq} \quad \text{or} \quad (1a)$$

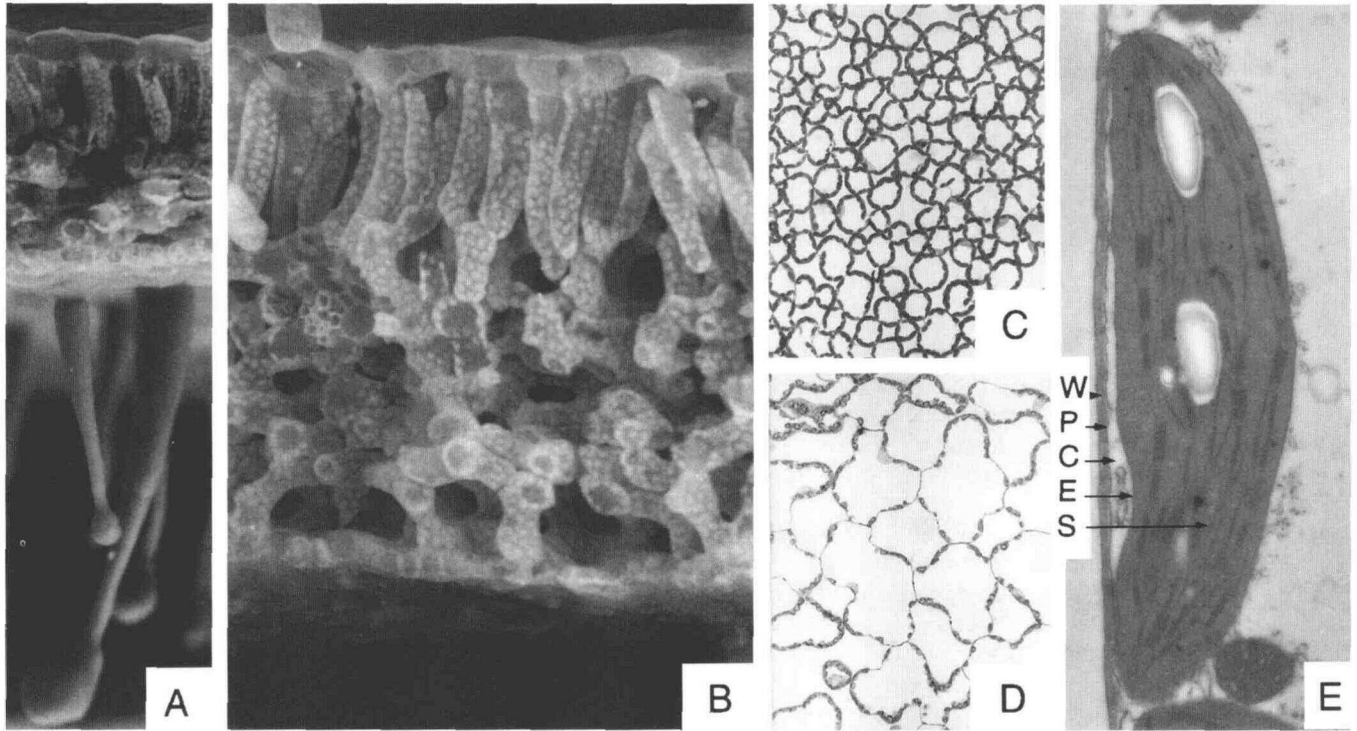
$$g_w = \frac{g_{liq}}{1 + g_{liq}/g_{ias}}, \quad (1b)$$

where  $g_{liq}$  and  $g_{ias}$  are the conductances through the wall and liquid phase and through intercellular air spaces, respectively.

Other terms synonymous with  $g_w$  are, first, internal conductance,  $g_i$ , or estimated internal conductance,  $g_{est}$  (Lloyd et al., 1992), and, second, mesophyll conductance,  $g_m$  (Harley et al., 1992; Loreto et al., 1992; Parkhurst, 1994). The latter is confusing because  $g_m$  has previously been used to mean the slope of the response of  $A$  to  $p_i$  near the compen-

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Abbreviation: CA, carbonic anhydrase.



**Figure 1.** The pathway for  $\text{CO}_2$  diffusion from the atmosphere into a tobacco leaf and ultimately the chloroplast. A, Scanning electron microscope image of an uncoated leaf cross-section showing the epidermal hairs ( $\times 195$ ). B, Scanning electron microscope cross-sectional view showing palisade tissue beneath the upper (adaxial) leaf surface and spongy tissue adjacent to the lower (abaxial) surface ( $\times 220$ ). Chloroplasts are clearly evident covering the majority of exposed mesophyll cell surfaces. C, Light micrograph of a paradermal section through palisade tissue showing air spaces between packed palisade cells ( $\times 200$ ). D, Light micrograph of a paradermal section through spongy tissue showing the lobed cells and absence of chloroplasts on walls adjacent to another cell ( $\times 200$ ). E, Transmission electron micrograph of a chloroplast showing the resistances encountered by  $\text{CO}_2$  diffusing between intercellular air space, across the cell wall (W), plasmalemma (P), cytosol (C), chloroplast envelope (E), and stroma (S) ( $\times 11,750$ ). Note at the bottom the mitochondrion on the side of the chloroplast away from the cell wall, a commonly observed location along with peroxisomes.

sation point and therefore includes both  $\text{CO}_2$  diffusion limitations and Rubisco activity.

Determining the  $p_c$ , and hence  $g_w$ , requires the combination of conventional gas-exchange with other measurements. The first method measures the change in carbon isotopic composition of  $\text{CO}_2$  passing over the leaf,  $\Delta$  (Evans et al., 1986). The second method independently assesses photosynthetic electron transport rate from Chl fluorescence and uses a biochemical model of  $\text{C}_3$  photosynthesis (Harley et al., 1992). An initial comparison between the two methods showed that they yield similar estimates of  $g_w$  (Loreto et al., 1992).

#### $\Delta$ Method

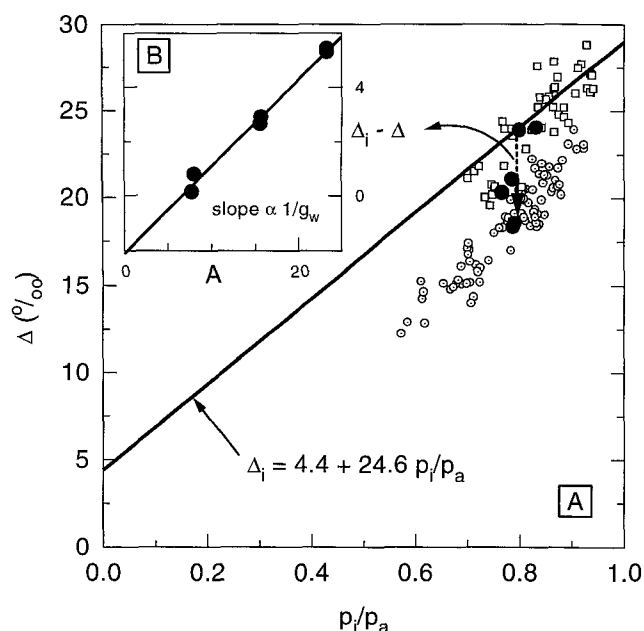
About 1.1% of atmospheric  $\text{CO}_2$  contains the heavy, stable isotope  $^{13}\text{C}$ .  $^{13}\text{CO}_2$  diffuses more slowly than  $^{12}\text{CO}_2$  and Rubisco discriminates against it during carboxylation reactions, both of which result in the carbon fixed during photosynthesis being depleted in  $^{13}\text{CO}_2$ . The preferential fixing of  $^{12}\text{CO}_2$  during photosynthesis enriches the surrounding air in  $^{13}\text{CO}_2$ . To estimate  $g_w$ , measurements are made at several irradiances (Fig. 2).  $p_i$  is calculated from  $A$  and stomatal conductance. Carbon isotope discrimination,

$\Delta_i$ , is then calculated assuming that  $p_i$  equals the partial pressure of  $\text{CO}_2$  at the sites of carboxylation,  $p_c$  (solid line in Fig. 2A). The difference between  $\Delta_i$  and measured  $\Delta$  is proportional to  $p_i - p_c$  and both are proportional to  $A$ . The slope of the relationship between  $\Delta_i - \Delta$  and  $A$  is inversely proportional to  $g_w$ . The measurements require  $\text{CO}_2$  to be cryogenically purified and subsequently analyzed with a ratio mass spectrometer.

#### Fluorescence Method

Measurement of steady-state Chl fluorescence during photosynthesis and during a saturating flash provides a noninvasive optical method for estimating photochemical efficiency of PSII,  $\phi\text{PSII}$ , i.e. the probability that an absorbed quantum is used in photosynthetic electron transport (Genty et al., 1989). The rate of electron transport,  $J_F$ , can be estimated from the product of  $\phi\text{PSII}$  and absorbed irradiance divided by 2 (because one quantum each is needed for both PSII and PSI per electron transferred from  $\text{H}_2\text{O}$  to NADPH). From Farquhar and von Caemmerer (1982), the rate of electron transport to NADPH,  $J$ , is given by

$$J = (A + R)(4p_c + 8\Gamma_*) / (p_c - \Gamma_*), \quad (2)$$



**Figure 2.** Estimation of  $g_w$  from  $\Delta$  measurements. A, Carbon isotope discrimination,  $\Delta$ , measured concurrently with  $\text{CO}_2$  exchange in tobacco at 1000  $\odot$  or 200  $\square$   $\mu\text{mol}$  quanta  $\text{m}^{-2} \text{s}^{-1}$  (Evans et al., 1994).  $\Delta = 4.4(p_a - p_i)/p_a + 1.8(p_i - p_c)/p_a + 29p_c/p_a$ , where 4.4‰ and 1.8‰ are the discrimination factors due to diffusion of  $\text{CO}_2$  in air and dissolution and diffusion in water, respectively, and 29‰ is discrimination by Rubisco (see Evans et al., 1986). The solid line,  $\Delta_i$ , is calculated assuming  $p_i = p_c$ , which almost occurs at low irradiance. The downward dashed arrow and filled circles show the change in  $\Delta$  as irradiance increases for a given leaf, and the data are replotted in the inset (B). B, When  $\Delta$  is measured at a number of irradiances, the difference between  $\Delta_i$  and measured  $\Delta$  is linearly related to A, and the slope is inversely proportional to  $g_w$ . From above,  $\Delta_i - \Delta = (29 - 1.8)(p_i - p_c)/p_a$ , and since  $(p_i - p_c) = A/g_w$ ,  $\Delta_i - \Delta = (29 - 1.8)(A/g_w)/p_a$ .

where  $\Gamma_*$  is the  $\text{CO}_2$  photocompensation point in the absence of nonphotorespiratory  $\text{CO}_2$  evolution,  $R$ , and  $p_c = p_i - A/g_w$ . By assuming that photosynthesis is the sole sink for electrons and that the surface chloroplasts subsampled by fluorescence are representative of the leaf as a whole, this equation allows calculation of  $p_c$  and  $g_w$  from  $J_F$ ,  $A$ ,  $R$ ,  $p_i$ , and  $\Gamma_*$  (Fig. 3).  $\Gamma_*$  is related to the specificity factor of Rubisco and varies little among  $\text{C}_3$  species (Kane et al., 1994) but is dependent on temperature and the partial pressure of oxygen. Alternatively, the relationship between  $J_F$  and  $J$  calculated from gas exchange can be empirically established under nonphotorespiratory conditions (Epron et al., 1995). Another approach is to simply use Chl fluorescence to establish the range in  $p_i$  over which  $J_F$  is constant and seek the value of  $g_w$  that minimizes the variance in  $J$  calculated from Equation 2 (Harley et al., 1992).

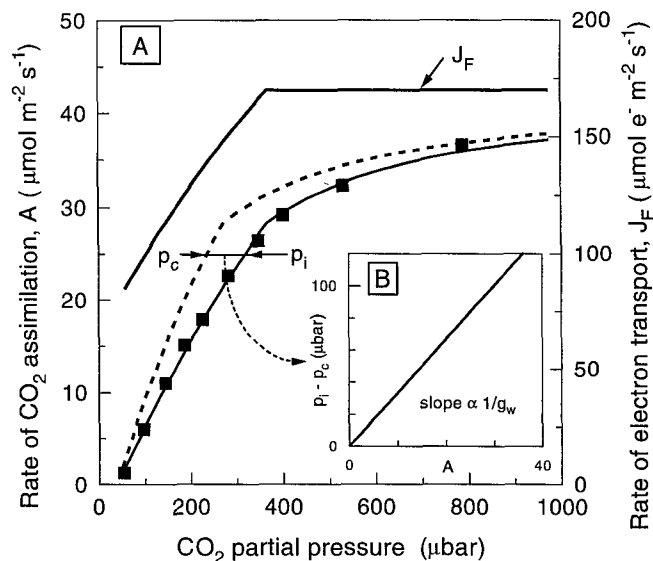
### $g_w$ Correlates with Photosynthetic Capacity

In our initial work with several species, a strong correlation between  $g_w$  and photosynthetic capacity was evident ( $g_w = 0.012 A$ ; Fig. 4). The correlation has been confirmed in several subsequent papers (Loreto et al., 1992, 1994;

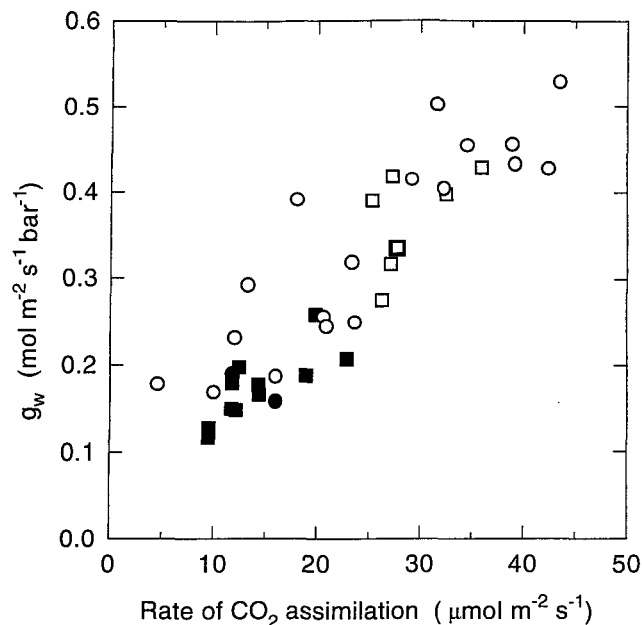
Epron et al., 1995). This has firmly established that  $p_c$  is about 30% lower than  $p_i$  for many species when leaves are actively photosynthesizing in high irradiance. The correlation holds not only for young leaves, but for older ones as well; as wheat leaves aged, both photosynthetic capacity and  $g_w$  declined in parallel (Loreto et al., 1994). For amphistomatous leaves, where intercellular air space resistance is probably the minor component (see below),  $g_w$  should be proportional to the surface area of chloroplasts exposed to intercellular air space per unit of leaf area,  $S_c$  (Laisk et al., 1970). There are only limited data available where both  $g_w$  and  $S_c$  have been measured (Evans et al., 1994; Syvertsen et al., 1995). The ratio of  $g_w/S_c$  is similar in tobacco, peach, and citrus but lower for *Macadamia*. It is significant that  $g_w/S_c$  for citrus is similar to that for peach and tobacco even though it has a much thicker leaf with lower porosity, features that should increase intercellular air space resistance. The similarity between sclerophyllous and mesophytic leaves in the relationship between  $g_w$  and photosynthetic capacity has also been noted (von Caemmerer and Evans, 1991; Loreto et al., 1992) (Fig. 4), suggesting that if intercellular air space resistance is more dominant in sclerophyllous leaves, it is offset by a smaller liquid phase resistance.

### INTERCELLULAR AIR SPACE RESISTANCE

$\text{CO}_2$  must diffuse from substomatal cavities through tortuous interstices between cells to reach all of the surfaces that have adjacent chloroplasts. Primitive leaves have stomata only on the lower surface (see Mott et al., 1982). Since light is absorbed mainly at the upper surface,  $\text{CO}_2$  must



**Figure 3.** Estimation of  $g_w$  from gas exchange and Chl fluorescence. A, The responses of  $A$  (from gas exchange) and  $J_F$  (from fluorescence) to intercellular  $\text{CO}_2$  partial pressure. By rearranging Equation 2, we can calculate  $p_c = \Gamma_* [J_F + 8(A + R)] / [J_F - 4(A + R)]$ , enabling the  $A:p_c$  curve to be replotted on the basis of  $p_c$  (dashed curve). B, The difference between  $p_i$  and  $p_c$  is proportional to  $A$ , and the slope is proportional to  $1/g_w$ , analogous to Figure 2.



**Figure 4.** Relationship between  $g_w$  and rate of  $\text{CO}_2$  assimilation under  $1 \text{ mmol quanta m}^{-2} \text{ s}^{-1}$ , and  $350 \mu\text{bar CO}_2$  at  $25^\circ\text{C}$ . Data are from von Caemmerer and Evans (1991) (○, ●) and Lloyd et al. (1992) (□, ■) and are for sclerophyllous leaves (●, ■) and mesophytic leaves (○, □). The units of conductance depend on the units used for  $\text{CO}_2$ . When  $\text{CO}_2$  is dissolving to reach the sites of carboxylation, the amount depends on the partial pressure of  $\text{CO}_2$  and conductance has the units  $\text{mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ . For air space conductance, the units could be  $\text{mol m}^{-2} \text{ s}^{-1}$  if  $\text{CO}_2$  is given as a mole fraction (see Harley et al., 1992).

diffuse across the bulk of the leaf. Generally, leaves with greater photosynthetic capacities have additional stomata on their upper epidermis, reducing the diffusion path length (Mott et al., 1982). The resistance to  $\text{CO}_2$  diffusion can be either in the plane of the leaf surface, with limited lateral movement, or vertically across the leaf.

#### Lateral Resistance

Certain leaves have extensions to their vascular bundles that span across the leaf, forming a physical barrier to gaseous diffusion. This creates discrete gaseous compartments in the leaf, and such leaves are called heterobaric (e.g. grapevine, *Vitis vinifera*; cocklebur, *Xanthium strumarium*; and sunflower, *Helianthus annuus*). Most leaves do not possess these complete barriers and are called homobaric. When stomatal closure is unevenly distributed across the leaf surface, compartmentation can result in patches of the leaf having different  $p_i$  values (Terashima, 1992).

#### Vertical Resistance

It is possible to independently measure the substomatal  $\text{CO}_2$  partial pressure of the lower and upper leaf surfaces of amphistomatous leaves. Differences across the leaf depend on the coordination between photosynthesis and stomatal

conductance by each leaf surface and the extent to which  $\text{CO}_2$  diffusion across the leaf is restricted. With  $350 \mu\text{bar}$  of  $\text{CO}_2$  outside the leaf, differences between the upper and lower surface  $p_i$  values amount to 0 to  $20 \mu\text{bar}$ , or less than  $10 \mu\text{bar}$  on average (Mott and O'Leary, 1984; Wong et al., 1985; Parkhurst et al., 1988). A more direct way to assess the restriction to diffusion across the leaf is to follow an inert gas such as helium or  $\text{N}_2\text{O}$ . This has yielded values of intercellular resistance across the leaf from  $3 \text{ m}^2 \text{ s}^{-1} \text{ mol}^{-1}$  for *Xanthium* (Farquhar and Raschke, 1978; Mott and O'Leary, 1984) up to  $59 \text{ m}^2 \text{ s}^{-1} \text{ mol}^{-1}$  for *Zea mays* (Long et al., 1989). However, it is possible to determine this only with amphistomatous leaves, and since intercellular resistance to  $\text{CO}_2$  diffusion involves lateral as well as vertical movement, these resistances are probably overestimates.

Another approach is to compare gas exchange in air with that in Helox (air that has helium instead of nitrogen), because rates of diffusion in Helox are 2.3 times faster than in air. Resistance to diffusion in intercellular air spaces can be reduced in Helox, and this is reflected in an increase in photosynthetic rate at a given substomatal  $\text{CO}_2$  partial pressure. Helox increased photosynthetic rates by an average of 2 and 12% for amphistomatous and hypostomatous leaves, respectively (Parkhurst and Mott, 1990). If it is assumed that  $g_w = 0.012 \text{ A}$  (Fig. 4), it can be shown that intercellular air space resistance accounts for roughly 17 and 67% of the  $\text{CO}_2$  transfer resistance in amphistomatous and hypostomatous leaves, respectively. A large intercellular air space resistance was inferred for the thick *Metrosideros* leaves up an elevational gradient (Vitousek et al., 1990), but for amphistomatous leaves, it may be much less than 17% (e.g. sunflower, Mott and O'Leary, 1984; tobacco, Evans et al., 1994).

#### CELL WALL AND LIQUID PHASE RESISTANCE

Haberlandt (1914; fig. 107) observed "in the photosynthetic tissues of higher plants, the chloroplasts adhere exclusively, or in great part, to those walls which abut upon airspaces; by this means they evidently obtain the most favorable conditions for the absorption of carbon dioxide." The reason for this is that  $\text{CO}_2$  diffuses 10,000 times more slowly in water than air, so short liquid pathways are essential if rapid  $\text{CO}_2$  exchange is to occur. The other way to reduce resistance is to increase the surface area available for gas exchange. Both methods are employed by the leaf (Fig. 1). Photosynthetic capacity of a leaf declines with age and can be changed in many species by growth irradiance. Increases in photosynthetic capacity require extra Rubisco and thylakoid proteins so that the chloroplast volume per unit of leaf area increases. At the same time, the internal leaf surface increases so that changes in the ratio of photosynthetic capacity to chloroplast surface area exposed to intercellular air space are small (Nobel et al., 1975; Evans et al., 1994).

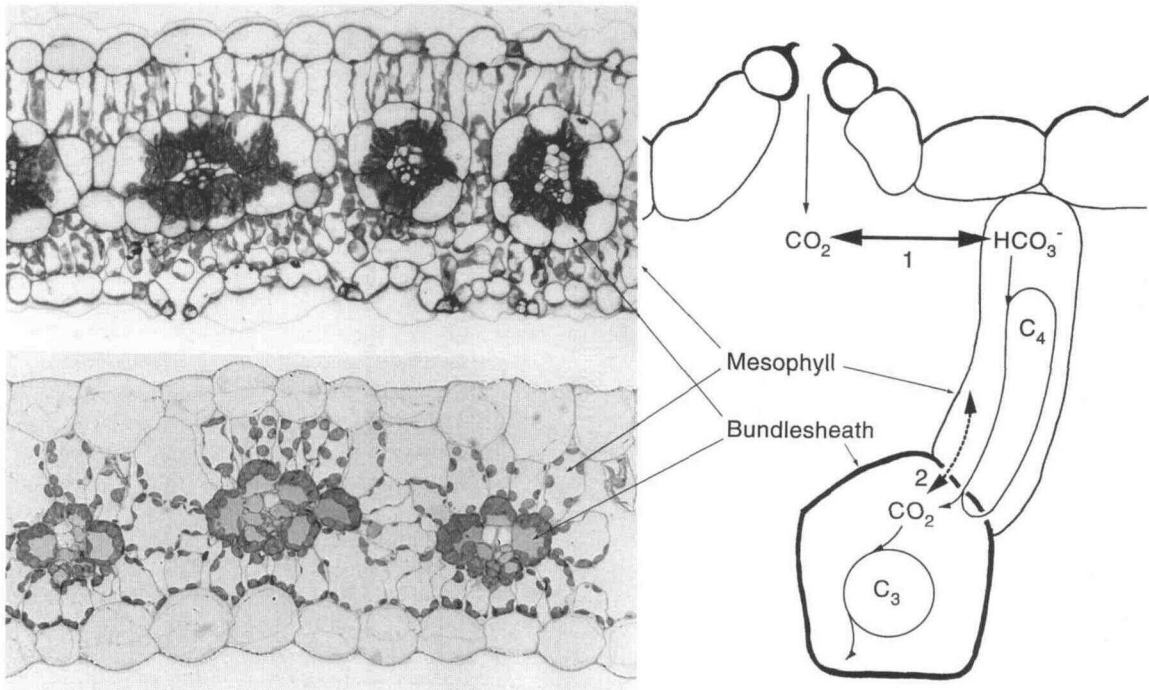
There is a striking analogy between  $\text{CO}_2$  exchange across mesophyll cell walls in leaves and that in mammalian lungs. The resting exchange rate of  $\text{CO}_2$  in mammals is  $3 \mu\text{mol m}^{-2} \text{ alveolar surface s}^{-1}$ , regardless of animal size over 4 orders of magnitude (Tenney and

Remmers, 1963). Wheat photosynthesizing in sunlight has a similar exchange rate per unit of chloroplast surface area adjacent to intercellular air space. Rapid exchange requires large surface areas to reduce gradients across the interfaces in both lungs and leaves. However, the similarity in  $\text{CO}_2$  exchange rate per unit of surface area is not due to similar conductances. In the lung, the  $\text{CO}_2$  partial pressure is, on average, 20 mbar, whereas the partial pressure of  $\text{CO}_2$  in the blood declines from 60 to 53 mbar as it passes through the lungs. The average gradient is thus 36 mbar. In wheat leaves, the gradient from substomatal cavities to the sites of carboxylation is only 90  $\mu\text{bar}$ . This suggests that the conductance to  $\text{CO}_2$  from the air to the liquid phase is 400 times greater in leaves than in lungs. Allowing for the fact that the  $\text{CO}_2$  exchange rate increases 10-fold in an active mammal over the resting rate, the conductance in leaves is still about 40 times greater than in mammalian lungs. The reason for this difference is unclear given that the path lengths appear similar. Plants require a greater conductance to support the rapid exchange of  $\text{CO}_2$  during photosynthesis, but at present we do not understand exactly how they achieve this.

The resistances imposed by the cell wall and segments of the liquid phase can be roughly calculated (see Fig. 1E). This requires assumptions about the effective diffusivities of  $\text{CO}_2$  through the cell wall and cytosol and the

permeabilities of the plasmalemma and chloroplast envelope (see Evans et al., 1994). The resistance to  $\text{CO}_2$  imposed by plant membranes is unknown, but has been measured for red blood cells ( $167 \text{ s m}^{-1}$ ,  $4.17 \text{ m}^2 \text{ s bar mol}^{-1}$ ; Solomon, 1974). Once inside the chloroplast,  $\text{CO}_2$  diffusion across the chloroplast is facilitated by CA, which rapidly interconverts  $\text{CO}_2$  and bicarbonate so that many more molecules are available for diffusion (at pH 8, the ratio of bicarbonate to  $\text{CO}_2$  is 45). For tobacco, where intercellular air space resistance is probably negligible, the  $\text{CO}_2$  transfer resistance is  $43 \text{ m}^2 \text{ chloroplast s bar mol}^{-1}$ . By subtracting from this the estimated resistances imposed by the cell wall, membranes, and cytosol, we are left with  $16 \text{ m}^2 \text{ chloroplast s bar mol}^{-1}$  (38%) due to the resistance within the chloroplast (Evans et al., 1994).

It has been possible to reduce CA activity to 1% of wild-type activity in transgenic tobacco containing an antisense gene for CA, which resulted in lowering the  $\text{CO}_2$  partial pressure in the chloroplast (Price et al., 1994). This confirmed that CA facilitates  $\text{CO}_2$  diffusion across the chloroplast by reducing diffusion resistance within the chloroplast by one-third. CA plays a similar role in facilitating  $\text{CO}_2$  diffusion and interconversion in red blood cells. Surprisingly, complete inhibition of CA in the blood by specific inhibitors did not noticeably affect  $\text{CO}_2$  transport in the bloodstream (Schmidt-Nielsen, 1991).



**Figure 5.** Light micrographs of cross-sections through *Amaranthus edulis* with centripetally arranged chloroplasts in the bundle sheath (top) and *Z. mays* with centrifugally arranged chloroplasts in the bundle sheath (bottom) (both  $\times 270$ ). Note that bundle-sheath cells are rarely in contact with intercellular air space. The diagram at right summarizes  $\text{C}_4$  photosynthesis and highlights the two key  $\text{CO}_2$  diffusion resistances inside the leaf. 1,  $\text{CO}_2$  exchange between intercellular air spaces and mesophyll cells where it is converted to  $\text{HCO}_3^-$  by CA. 2,  $\text{CO}_2$  exchange between bundle-sheath and mesophyll cells. Normally,  $\text{CO}_2$  leaks out of the bundle sheath, but it can diffuse into the bundle sheath if the external  $\text{CO}_2$  partial pressure is sufficiently high.

## CO<sub>2</sub> DIFFUSION IN C<sub>4</sub> LEAVES

The C<sub>4</sub> photosynthetic pathway is characterized by a CO<sub>2</sub>-concentrating mechanism that involves the coordinated functioning of mesophyll and bundle-sheath cells within a leaf (Fig. 5). CO<sub>2</sub> is initially assimilated into C<sub>4</sub> acids by PEP carboxylase in mesophyll cells. These acids then diffuse to the bundle-sheath cells, where they are decarboxylated. This concentrates CO<sub>2</sub> in the bundle sheath, which enhances ribulose-1,5-bisphosphate carboxylation while inhibiting ribulose-1,5-bisphosphate oxygenation (Hatch and Osmond, 1976). The coordinated functioning of C<sub>4</sub> photosynthesis requires a specialized leaf anatomy in which photosynthetic cells are organized in two concentric cylinders. Thin-walled mesophyll cells adjacent to intercellular air space radiate from thick-walled bundle-sheath cells. The diffusion of CO<sub>2</sub> back out from the bundle sheath limits the efficiency of the CO<sub>2</sub>-concentrating mechanism, and many attempts have been made to quantify the CO<sub>2</sub> diffusion resistance across the bundle sheath (Jenkins et al., 1989; Brown and Byrd, 1993; Hatch et al., 1995). Limitations to CO<sub>2</sub> diffusion from intercellular air spaces to the mesophyll cells have received less attention (Longstreth et al., 1980).

### From Intercellular Airspace to the Mesophyll

The necessity for metabolite transport between mesophyll and bundle-sheath cells requires intimate contact between these cells and limits the amount of mesophyll tissue that can be functionally associated with bundle-sheath tissue. For example, the number of chlorenchymatous mesophyll cells between adjacent bundle sheaths is usually between two and four. Mesophyll surface area exposed to intercellular air space per unit of leaf area is slightly less in C<sub>4</sub> than in C<sub>3</sub> species (Longstreth et al., 1980; Dengler et al., 1994), and thus the surface area available for CO<sub>2</sub> diffusion is also less.

No techniques are available at present that allow the estimation of conductance to CO<sub>2</sub> diffusion from intercellular air space to sites of PEP carboxylation (Fig. 5, arrow 1). Carbon isotope discrimination cannot be used because of the low discrimination factor of PEP carboxylase and the confounding effects of CO<sub>2</sub> leakage from the bundle sheath (Henderson et al., 1992). Similarly, Chl fluorescence signals are not suitable, because bundle-sheath and mesophyll cells have different chloroplast populations.

For both C<sub>3</sub> and C<sub>4</sub> species, one can calculate a minimum CO<sub>2</sub> transfer conductance. This limit is reached when  $p_c$  is reduced to the CO<sub>2</sub> compensation point ( $\Gamma$ ;  $g_{w\min} = A/[p_i - \Gamma]$ ), where  $g_{w\min}$  is the minimum value of  $g_w$ . At 25°C, high light, and ambient CO<sub>2</sub>, intercellular CO<sub>2</sub> is approximately 100  $\mu$ bar in C<sub>4</sub> species versus 250  $\mu$ bar for C<sub>3</sub> species, and the compensation point is close to 0 in C<sub>4</sub> versus approximately 50  $\mu$ bar in C<sub>3</sub> species. Thus, for the same A value,  $g_{w\min}$  of C<sub>4</sub> species needs to be approximately twice that of C<sub>3</sub> species. Given that there is less mesophyll surface exposed in C<sub>4</sub> species, conductance across the cell wall through to the cytosol in C<sub>4</sub> species must be more than double that in C<sub>3</sub>

species. There are several possible contributing factors. First, mesophyll cell walls of C<sub>4</sub> species may be thinner than those of C<sub>3</sub> species (0.07  $\mu$ m in *Amaranthus retroflexus* [Longstreth et al., 1980]; cf 0.3  $\mu$ m in *Nicotiana tabacum* [Evans et al., 1994]), although a general survey is needed to confirm this. Second, in contrast to C<sub>3</sub> plants, where CA is located mainly in the chloroplast to facilitate CO<sub>2</sub> diffusion, in C<sub>4</sub> plants CA is found in the cytosol alongside PEP carboxylase (Hatch and Burnell, 1990). Recently, 20 to 60% of CA activity in *Z. mays* has been localized to the plasmalemma, compared to 1 to 3% in wheat (Utsunomiya and Muto, 1993), which may facilitate CO<sub>2</sub> movement across membranes, perhaps even delivering HCO<sub>3</sub><sup>-</sup> into the cytosol. Third, the initial carboxylation reaction by PEP carboxylase, which utilizes HCO<sub>3</sub><sup>-</sup>, occurs in the cytosol, so the liquid diffusion path in C<sub>4</sub> plants may be considerably shorter and does not have to cross the chloroplast envelope. It is likely that A will be sensitive to reduction in CA activity in C<sub>4</sub> plants because PEP carboxylase would have to rely on the uncatalyzed rate of conversion of CO<sub>2</sub> to bicarbonate, which is 10,000 times slower than the catalyzed rate. Generation of C<sub>4</sub> plants containing antisense genes to CA will soon answer this question directly.

### Across the Bundle Sheath

A low conductance to CO<sub>2</sub> diffusion across the bundle sheath is an essential feature of the C<sub>4</sub> pathway (Fig. 5, dashed arrow 2). It effectively limits CO<sub>2</sub> exchange with the normal atmosphere, and C<sub>4</sub> acid decarboxylation is the major source of CO<sub>2</sub> in this compartment. Inhibiting PEP carboxylase activity reduced CO<sub>2</sub> assimilation rate by 80 to 98% (Jenkins, 1989). The conductance to CO<sub>2</sub> diffusion across bundle-sheath walls is considerably less than that across mesophyll cell walls, with estimates ranging from 0.6 to 2.4 mmol m<sup>-2</sup> leaf s<sup>-1</sup> or 0.5 to 0.9 mmol m<sup>-2</sup> bundle sheath s<sup>-1</sup> for different C<sub>4</sub> species (Jenkins et al., 1989; Brown and Byrd, 1993) compared to 25 mmol m<sup>-2</sup> chloroplast s<sup>-1</sup> for tobacco (Evans et al., 1994). The absence of CA in the bundle sheath prevents the rapid conversion of CO<sub>2</sub> to bicarbonate, which would increase the diffusion of CO<sub>2</sub> out of the bundle sheath, similar to the way it facilitates CO<sub>2</sub> diffusion across chloroplasts in C<sub>3</sub> plants. The liquid path length is also long, imposing a considerable resistance. Furthermore, in C<sub>4</sub> species that have either centrifugally arranged chloroplasts (NADP grasses, e.g. *Zea*; Fig. 5, bottom) or bundle sheaths with uneven cell outlines, the bundle-sheath cell wall is lined by a suberized lamella, which may also help reduce conductance to CO<sub>2</sub>.

The C<sub>4</sub> cycle consumes energy and so leakage of CO<sub>2</sub> from the bundle sheath is an energy cost to the leaf that represents a compromise between keeping CO<sub>2</sub> in, letting O<sub>2</sub> out, and letting metabolites diffuse in and out at rates fast enough for the rate of CO<sub>2</sub> fixation. The leakage depends on the balance between the rates of PEP carboxylation and Rubisco activity and the conductance of the bundle sheath to CO<sub>2</sub>. Various estimates have been made of what proportion of CO<sub>2</sub> fixed by PEP carboxylase subsequently leaks out of the bundle sheath. This has been termed leakiness, and estimates have ranged from 8 to 50% (see Henderson et al., 1992; Hatch et al., 1995). Despite earlier suggestions that leakiness differed between C<sub>4</sub>

decarboxylation types (being less in species with a suberized lamella; Hattersley, 1982), recent measurements of leakiness have not confirmed this (Henderson et al., 1992; 8–12%, Hatch et al., 1995). Extensive concurrent measurements of  $\Delta$  with gas exchange have revealed little variation in leakiness either among species, or with variation in photosynthetic rate due to short-term changes in  $\text{CO}_2$ , irradiance, and temperature, or with longer-term changes in leaf nitrogen content (average 21%, Henderson et al., 1992, 1994). Since leakiness is determined not only by the physical conductance of the bundle sheath but also by the balance of the capacities of the  $\text{C}_4$  and  $\text{C}_3$  cycles, this suggests that the biochemistry of  $\text{C}_4$  photosynthesis is highly regulated.

### CONCLUSIONS

Although initial experiments have revealed correlations between  $\text{CO}_2$  transfer conductance and photosynthetic capacity in  $\text{C}_3$  species (Fig. 4) and chloroplast surface exposed to intercellular air space, we need a better understanding of where the major limitations reside. Now that techniques are available to measure the  $\text{CO}_2$  gradient within leaves, it will be possible to examine the effects of, for example, temperature, stress, age, and anatomy on  $\text{CO}_2$  diffusion within  $\text{C}_3$  leaves. Further insight into diffusional limitations in  $\text{C}_4$  leaves are likely to come from experiments with transgenic plants.

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