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Anatomy of CO$_2$ Diffusion in Leaf Photosynthesis

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Introduction

Rubisco is the enzyme that fixes CO$_2$. Affinity of this enzyme to CO$_2$ is low. The K$_m$ at 25°C of this enzyme from C$_3$ plants is around 12 µM and this value is identical to the CO$_2$ concentration in water that is equilibrated with the air containing 360 µmol CO$_2$ mol^{-1} air. Moreover, carboxylation of ribulose 1,5-bisphosphate (RuBP) is competitively inhibited by O$_2$, and oxygenation products such as phosphoglycolate and glycolate are toxic. The photorespiration path metabolizes or detoxifies these compounds and salvages carbon as much as possible from these compounds to synthesize phosphoglycerate. However, the path consumes 3.5 ATP, 2 NADPH, and 1/6 triose phosphate, and releases 0.5 CO$_2$ per one RuBP oxygenation. Thus, the resistance to CO$_2$ diffusion in the leaf lowers efficiency of photosynthesis not only by lowering C$_c$ but also by enhancing photorespiration. Clearly, it is advantageous for C$_3$ plants to maintain CO$_2$ concentration in the chloroplast stroma (C$_c$) as high as possible to suppress RuBP oxygenation and increase velocity of RuBP carboxylation. This is why we are interested in CO$_2$ conductance in the leaf.

I. Conductance for CO$_2$ diffusion in the leaf

In photosynthesizing C$_3$ leaves, CO$_2$ concentration in the substomatal cavity, C$_s$, is lower than that in the ambient air C$_a$, and CO$_2$ diffuses into the leaf along the gradient of
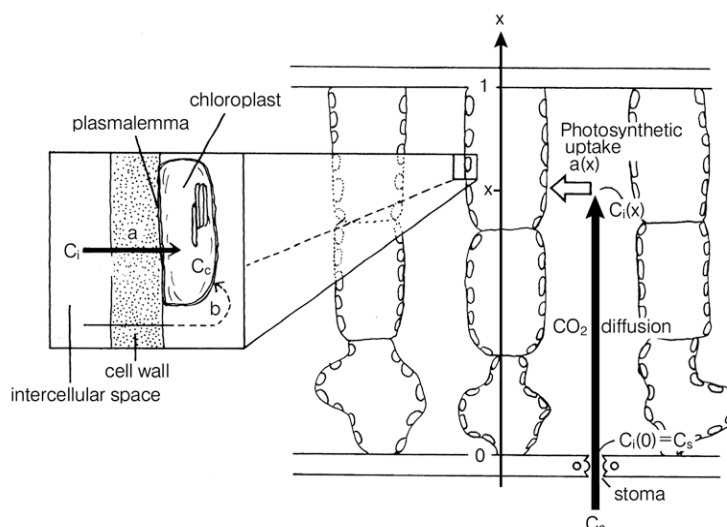

Fig. 1. Diffusion of CO$_2$ in the leaf and the framework of the model described in section III. The CO$_2$ flux by the pathway b is negligible compared with that by the pathway a.

CO$_2$ concentration. C$_s$ can be estimated by the gas exchange techniques (Sharkey *et al*. 1982). In a vigorously photosynthesizing leaf, the bulk CO$_2$ concentration in the intercellular spaces, C$_i$, is lower than C$_s$ due to the resistance to CO$_2$ diffusion in the intercellular spaces (Fig. 1). CO$_2$ concentration in the chloroplast stroma, C$_c$, in C$_3$ plants is lower than C$_i$. Recent technological innovations, including pulse-modulated fluorometry and on-line measurement of carbon isotope discrimination, enable us to estimate C$_c$. For some species, C$_c$ as low as half the C$_a$ was reported (for a review, see Evans and Loreto 2000). This indicates that resistance to CO$_2$ diffusion from the ambient air to the chloroplast stroma is substantial.

Conductance for CO$_2$ diffusion through stomata (g$_s$) has been well studied and the drawdown of CO$_2$ concentration, C$_a$ - C$_i$, is about 60 to 120 µmol mol^{-1} when C$_a$ is 360 µmol mol^{-1} (Evans and Loreto 2000). g$_s$ can be approximated as: g$_s$ = naD/(r+l), where n is stomatal density in m^{-2}, a is stomatal pore area in m^2, D is binary diffusion coefficient of CO$_2$ in the air. In this approximation, a stoma is assumed to be a tube having its radius of r and length of l. When stomata are open, na would be 0.005 to 0.02. Let us assume r is 5 µm and l is 10 µm. At 25°C, D is 1.55 x 10^{-5} m^2 s^{-1}. Then, g$_s$ ranges from 5 to 20 mm s^{-1} or 0.2 to 0.8 mol CO$_2$ m^{-2} s^{-1}. These are comparable to the reported values.

Similarly, the conductance in the intercellular spaces is expressed as, g$_{ias}$ = Dp/τδ, where p is porosity, τ is tortuosity of mesophyll, and δ is distance for CO$_2$ diffusion. p usually ranges from 0.1 to 0.5. Let us assume τ is 1.5. Given that δ is 100 µm (for the leaf having mesophyll of 200 µm thickness), g$_{ias}$ would range from 10 to 50 mm s^{-1} or from 0.4 to 2.0 mol CO$_2$ m^{-2} s^{-1}. These values are larger than the maximum stomatal conductance. For amphistomatous leaves, g$_{ias}$ further increases by three to four-folds. Thus, it is unlikely that g$_{ias}$ is a major limiting factor of leaf photosynthesis, in particular in amphistomatous leaves.

The internal conductance for CO$_2$ diffusion from the surface of mesophyll cell walls to the chloroplast stroma, via plasma membrane, cytoplasm and chloroplast envelope, g$_i$, is not large. Evans and Loreto (2000) summarized the data of g$_i$. g$_i$ ranges from very small value such as 0.03 to about 0.6 mol CO$_2$ m^{-2} s^{-1} bar^{-1}(Fig. 2). g$_i$ values of mature leaves differ depending on plant functional types. g$_i$ values for annual herbs such as crop species are greatest and range from 0.2 to 0.6 mol CO$_2$ m^{-2} s^{-1} bar^{-1}. On the other hand, the values of evergreen trees were very low ranging from 0.03 to 0.2 mol CO$_2$ m^{-2} s^{-1} bar^{-1}. In mesic deciduous trees, g$_i$ values are intermediate between those of annual herbs and evergreen
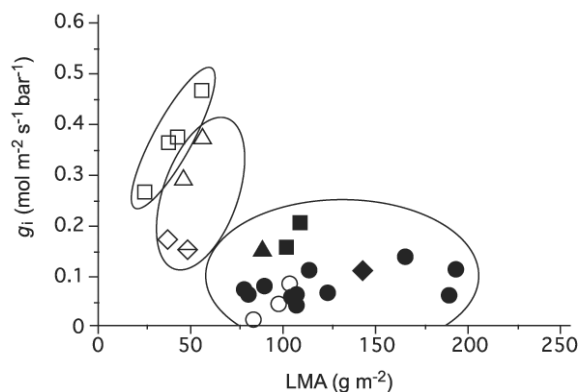
Fig. 2. The relationships between g$_i$ and leaf mass per area. Open square, tobacco (annual herb); closed triangle, peach; open diamond, *Alnus japonica;* open diamond with bar, *Acer mono;* open diamond with bar, *Castanea sativa*; closed square, grapefruit; closed triangle, lemon; closed diamond, macadamia; closed circle, Japanese evergreen trees (*Castanopsis sieboldii, Camellia japonica, Ligustrum lucidum, Qeurcus glauca*, and *Q. phillyraeoides*).

trees. g_i of the leaves in tree species that develop leaves successibly may be greater that that for flush type species (Hanba et al. 2001). *Castanea sativa*, a Mediterranean deciduous chestnut, had g_i of 0.1 mol CO_2 m^{-2} s^{-1} bar^{-1}(Lauteri et al. 1997), which is the lowest record for the deciduous trees and is comparable to those of evergreen trees.

A Hawaiian tree species, *Meterosideros polymorpha* (Vitousek et al. 1990), and a pioneer clonal plant, *Polygonum cuspidatum* (= *Reynoutria japonica*, Kogami et al. 2001) both showed decrease in g_i with growth altitude. Under the apline conditions, these plants develop leaves with low g_i. In *P. cuspidatum*, g_i for the plants at the altitude of 10 m was 0.2 mol CO_2 m^{-2} s^{-1} bar^{-1}, while that for the plants at 2500 m was 0.075 mol CO_2 m^{-2} s^{-1} bar^{-1} (Kogami et al. 2001). This effect of the altitude on g_i would explain a general trend of changes in carbon isotope composition with altitude (Körner et al. 1988).

It has been shown that the internal conductance increases with the increase in cumulated surface areas of chloroplasts that face the intercellular spaces (S_c, Evans and Loreto 2000). This indicates that increasing the effective area for CO_2 diffusion increases internal conductance. In this respect, thick sun leaves have larger S_c than thin shade leaves. We will discuss this problem separately (see section III). g_i decreases with the increase in thickness of mesophyll cell walls (Terashima et al. 1995). The decrease in distance from the mesophyll surface to the plasma membrane by having thin cell walls should be effective in increasing internal conductance because diffusion of CO_2 in the water is slower than that in the air by 10^{-4}. We have attributed low g_i values in evergreen tree leaves (Hanba et al. 1999, Miyazawa and Terashima 2001) and an alpine plant (Kogami et al. 2001) to thick mesophyll cell walls. Effects of S_c and wall thickness are clearly physical.
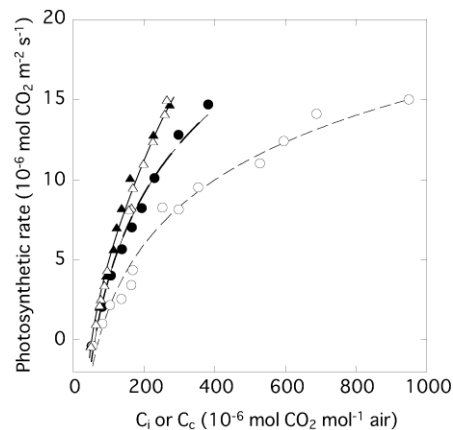
II. Are water channels in the plasma membrane involved in CO_2 diffusion? (Terashima I, submitted to *Plant and Cell Physiology*)

g_i is strongly correlated with S_c and mesophyll cell wall thickness. However, g_i also changes drastically without marked changes in S_c and/or cell wall thickness. For example, g_i changes in the courses of leaf development (Miyazawa and Terashima 2001) and of senescence (Loreto et al. 1994), and during salt stress (Delfine et al. 1998, 1999). These studies indicate that CO_2 permeability of membranes would change.

Water channels, one of the most abundant proteins in plant plasma membranes, mainly transfer water molecules according to the gradient of water potential (Kjellbom et al. 1999). Very recently, it was shown that animal aquaporin 1 transports CO_2 as well as water (Cooper and Boron 1998, Ramesh Prasad et al. 1998). In these studies, *Xenopus* oocytes and/or liposomes were used and the CO_2 permeability was monitored as changes in pH. So far, there are no studies examining possibility whether water channels transfer CO_2 in plant cells. Because plant mesophyll cells has an efficient intrinsic CO_2 probe, the chloroplast, we examined effects of $HgCl_2$, a potent inhibitor of water channel, on CO_2 responses of leaf photosynthesis.

We have conducted two series of experiments to examine whether mercury sensitive water channels are involved in photosynthetic CO_2 diffusion across the plasma membrane. $HgCl_2$ was fed to the leaf of *Phaseolus vulgaris* via the transpiration stream. After the leaf was fed with 1 mM $HgCl_2$, the rate of photosynthesis on leaf area basis (A) at a given CO_2 concentration in the intercellular spaces (C_i), measured at PPFD of 630 μmol m^{-2} s^{-1} and at a leaf temperature of 25 °C decreased by about 30% (Fig. 3). However, when A was plotted against CO_2 concentration in the chloroplast stroma (C_c), which was calculated from fluorescence data and kinetics of rubisco, relationships between A and C_c did not change. More directly, *Vicia faba* leaflets without abaxial epidermes were treated with $HgCl_2$ solution.

Fig. 3. Effects of 1 mM $HgCl_2$ feeding via the transpiration stream on dependence of the rate of photosynthesis (A) on C_i or C_c in a leaf of *Phaseolus vulgaris*.Closed circle, A-C_i relationships before $HgCl_2$ feeding; open circle, A-C_i after $HgCl_2$ feeding; closed triangle, A-C_c before $HgCl_2$ feeding; and, open triangle, A-C_c after $HgCl_2$ feeding

After the $HgCl_2$ treatment, dependence of A on the ambient CO_2 concentration changed in a similar way in which A-C_i relationships changed. Given that the primary site of $HgCl_2$ inhibition in the present feeding methods is the water channel, the results would indicate that CO_2 diffusion in the untreated leaves is greatly facilitated by the water channel. Further studies in this area are needed.

III. Why are sun leaves are thicker than shade leaves?

When expressed on leaf area basis, the light-saturated rate of leaf photosynthesis in C_3 plants strongly depends on structural parameters such as leaf thickness, leaf mass per area, mesophyll surface area (S_{mes}) and chloroplast surface area (S_c). Because diffusion of CO_2 in the water is slower than that in the air by 10^{-4}, the flux via the pathway like "b" in Fig. 1 is negligibly important compared with that of pathway "a." Then, it is useless to have mesophyll cell surfaces without chloroplasts. Actually, when grown with sufficient nutrients, most of the mesophyll surfaces facing the intercellular spaces are occupied by chloroplasts. Thickness of chloroplasts is also important. The drawdown of CO_2 concentration from the intercellular spaces to the stroma, C_i - C_c, is proportional to the flux of CO_2 across the liquid phase per unit chloroplast surface area and the resistance to CO_2 diffusion per unit chloroplast surface area. With the increase in the amount of Rubisco per unit chloroplast surface area, photosynthetic rate per unit chloroplast surface area increases. However, the photosynthetic rate per Rubisco decreases because C_c decreases. From the viewpoint of efficiency of Rubisco use, thicker leaves with greater S_c, to a given extent, would be advantageous because the amount of Rubisco per unit chloroplast surface area becomes smaller and thereby Rubisco would operate at higher C_c. On the other hand, g_{ias} decreases with leaf thickness, which causes a decrease in the bulk C_i. Moreover, the construction and
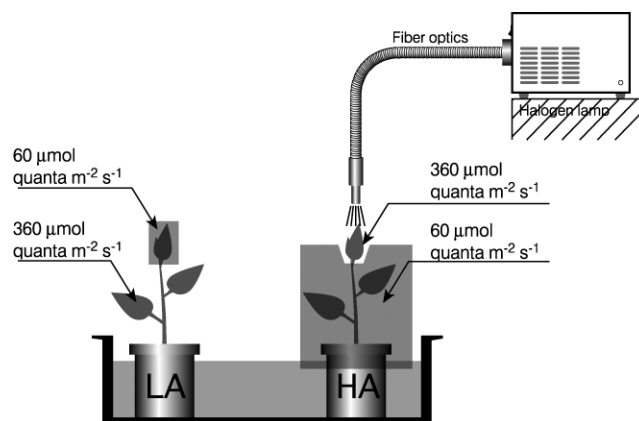
maintenance costs of thick leaves are more expensive than the costs of thin leaves in terms of carbon economy. Thick leaves are not advantageous in these respects.

To evaluate effects of various aspects of mesophyll structure on photosynthesis, we constructed a one-dimensional model of CO_2 diffusion in the leaf (for detail, see Terashima et al. 2001). When mesophyll thickness of the leaf is changed without changing Rubisco content per unit leaf area, the maximum rate of photosynthesis occurs at an almost identical mesophyll thickness irrespective of the Rubisco contents per leaf area. On the other hand, with an increase in Rubisco content per leaf area, the mesophyll thickness that realizes a given photosynthetic gain per mesophyll thickness or per leaf carbon cost increases. This probably explains the strong relationships between the maximum rate of photosynthesis and leaf parameters such as mesophyll thickness.

In these simulations, the increase in mesophyll thickness simultaneously means a decrease in g_{ias}, an increase in S_c, and an increase in construction and maintenance carbon costs of the leaf. Alternatively, the leaf can increase S_c and g_{ias} by decreasing cell size. The leaf with smaller cells are also mechanically stronger. However, actual leaves do not have very small cells. This could be because leaves exhibiting considerable rates of leaf area expansion, adequate heat capacitance, high efficiency of resource use, etc are favored by natural selection. Further studies are needed for evaluating exchange rates for such influential factors of different natures.

IV. Development of sun and shade leaves
(Yano S, Terashima I, submitted to *Plant and Cell Physiology*)

Plant development is characterized by flexibility. Because of this flexibility, plants can finely acclimate to their environment. We are analyzing differentiation processes of sun and shade leaves using *Chenopodium album*, an annual herb. We hypothesized that mature leaves sense the light environment and this information determines anatomy of new leaves, because young developing leaves are not directly exposed to growth light. To examine this hypothesis, we shaded plant partially. For the

Fig. 4. Diagrams explaining HA and LA treatments. Light environments of the shoot apex and the mature leaves were differentiated.

low-light apex treatment (LA), the shoot apex with developing leaves was covered by a cap made of shading screens so as to receive photosynthetically active photon flux density (PPFD) of 60 μmol m^{-2} s^{-1} and the other mature leaves were exposed to 360 μmol m^{-2} s^{-1}. In the high-light apex treatment (HA), the apex was exposed and the other mature leaves were covered by shade screen (Fig.4). After these treatments for six days, we analyzed leaf anatomy and chloroplast ultrastructure of the leaves. Anatomy of LA

leaves with the two-layered palisade tissue was similar to that of sun leaves, while their chloroplasts were shade-type with thick grana. Anatomies of HA leaves and shade leaves were similar and both had one-layered palisade tissues, while chloroplasts of HA leaves were sun-type having thin grana. These results clearly showed that anatomy of new leaves is determined by light environment of mature leaves while chloroplasts differentiate depending on local light environment.

Because, development of sun-type leaves is induced not only by bright light but also by high atmospheric CO_2 concentration, phytochromes and blue light receptors may not be involved in differentiation of sun and shade leaves. Our working hypothesis is that anatomy of the leaf is regulated by the concentration of photosynthates. When photosynthates are abundant, leaves would develop into sun leaves. Stomatal frequency could be also regulated by the same mechanism (Lake et al. 2001).

V. Comparative leaf development across functional types

It is widely believed that, in the course of leaf development/senescence, the rate of photosynthesis peaks just before or at full leaf expansion. However, in many evergreen tree leaves, photosynthetic rate on leaf area basis attains its maximum well after the full expansion of the leaf (Miyazawa et al. 1998). We compared leaf development in *Castanopsis sieboldii* and *Quercus glauca*, evergreen trees, and *Phaseolus vulgaris*, a control annual herb. In *C. siebildii* and *Q. glauca*, chloroplasts development proceeded more slowly than mesophyll cell expansion, whereas, in *P.vulgaris*, these processes proceeded synchronously and were completed by the full leaf expansion (Fig 5). After full leaf expansion, photosynthesis in leaves of *C. sieboldii* was markedly limited by low g_i. These suggest that, in the evergreen broad-leaves trees, the mechanical protection of mesophyll cells would have priority over the efficient CO_2 transfer and quick construction of the chloroplasts.
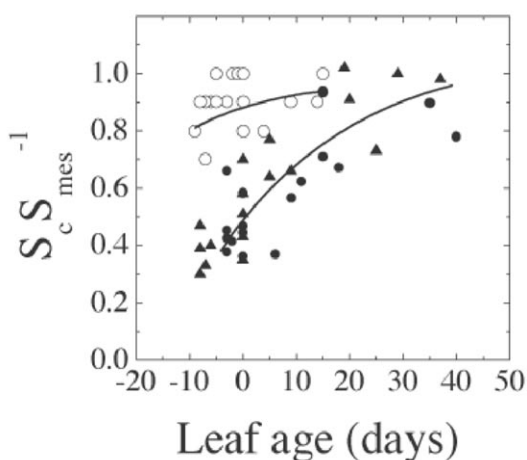
Fig. 5. Changes in the ratio of chloroplast surface area to mesophyll surface area directly exposed to the intercellular air spaces (Sc/Smes) during leaf development. Open circle; *Phaseolus vulgaris*, closed triangle; *Castanopsis sieboldii* and closed circle; *Qerucus glauca*

Hanba et al. (2001) conducted a similar study with deciduous trees. She compared two species: *Alnus japonica* , a pioneer, which has successive type leaf phenology (leaves develop one after another) and *Acer mono*, a climax species with flush type leaf phenology. Clearly, development of the leaf photosynthetic apparatus relative to leaf expansion was

faster in *Alnus japonica* than in *Acer mono*. This probably reflects the differences in phenological characteristics and in leaf longevity between these species. Leaves of *Alnus japonica*, with shorter longevity, develop and senesce faster than those of *Acer mono*.

Conclusion

In ecophysiological studies, properties of mature leaves have been studied in detail. From now, we have to pay more attention to developmental / senescence processes of photosynthetic function. Responses of leaves to environmental factors can be more deeply understood when we know the regulation mechanisms of "functional" leaf development and senescence. It is also important to study leaf behavior in the context of fitness of the whole plant. Probably a leaf can sense its status within a plant through monitoring the demand for its photosynthates by other plant parts (Ono et al. 2001). In this respect, organ to organ interaction will be one of the key subjects of ecophysiological studies.

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