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Irradiance and phenotype: comparative eco-development of sun and shade leaves in relation to photosynthetic CO_2 diffusion

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Abstract

The subject of this paper, sun leaves are thicker and show higher photosynthetic rates than the shade leaves, is approached in two ways. The first seeks to answer the question: why are sun leaves thicker than shade leaves? To do this, CO_2 diffusion within a leaf is examined first. Because affinity of Rubisco for CO_2 is low, the carboxylation of ribulose 1,5-bisphosphate is competitively inhibited by O_2, and the oxygenation of ribulose 1,5-bisphosphate leads to energy-consuming photorespiration, it is essential for C_3 plants to maintain the CO_2 concentration in the chloroplast as high as possible. Since the internal conductance for CO_2 diffusion from the intercellular space to the chloroplast stroma is finite and relatively small, C_3 leaves should have sufficient mesophyll surfaces occupied by chloroplasts to secure the area for CO_2 dissolution and transport. This explains why sun leaves are thicker. The second approach is mechanistic or 'how-oriented'. Mechanisms are discussed as to how sun leaves become thicker than shade leaves, in particular, the long-distance signal transduction from mature leaves to leaf primordia inducing the periclinal division of the palisade tissue cells. To increase the mesophyll surface area, the leaf can either be thicker or have smaller cells. Issues of cell size are discussed to understand plasticity in leaf thickness.

Key words: Aquaporin, cell wall, chloroplasts, conductance, diffusion, intercellular spaces, mechanical strength, photosynthesis, resistance to CO_2 diffusion, stomata.

Why are sun leaves thicker than shade leaves?

The rate of photosynthesis of C_3 leaves strongly depends on the CO_2 concentration in the chloroplast, because affinity for CO_2 of the primary CO_2-fixing enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), is low (von Caemmerer and Quick, 2000). Moreover, the carboxylation of ribulose 1,5-bisphosphate (RuBP) is competitively inhibited by O_2, and the oxygenation leads to the energy-consuming photorespiration processes. For C_3 plants to perform efficient CO_2 fixation in terms of economical use of energy and resources it is essential, therefore, to increase the conductance for CO_2 diffusion from the ambient air to the chloroplasts. Since the thickness of C_3 leaves is one of the important determinants in conductance for CO_2 diffusion and is a key factor to answering the question why sun leaves are thicker than shade leaves, the path of CO_2 diffusion is first traced from the ambient air to the chloroplast stroma.

Rubisco

Rubisco is a hexadecameric enzyme having eight large subunits encoded in the chloroplast DNA and eight small subunits encoded in the nuclear DNA in higher plants and green algae. Each large subunit has one active site that catalyses the following reaction:

$$\mathrm{RuBP} + \mathrm{CO_2} + \mathrm{H_20} \rightarrow 2\ \text{phosphoglycerate (PGA)}$$

The maximum rate (k_{cat}) at CO_2 saturation at 25 °C is very low and typically 3 mol CO_2 mol active site^{-1} s^{-1} (24 mol CO_2 mol enzyme^{-1} s^{-1}), which is lower than those of other

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Calvin–Benson cycle enzymes by two to three orders of magnitude (Roy and Andrews, 2000). The affinity for CO_2 is low and the Michaelis constant for CO_2, K_c, in the absence of O_2 is about 12 μM. This concentration is near the CO_2 concentration in the water equilibrated with the atmosphere having a CO_2 partial pressure at 37 Pa. Moreover, Rubisco shows high oxygenation activity. Molecular oxygen, O_2, competes with CO_2 for the same substrate, RuBP, in the active site:

$$\text{RuBP} + \text{O}_2 \rightarrow \text{PGA} + \text{phosphoglycolate}$$

In the presence of 21% oxygen, the apparent K_c increases to about 20 μM due to substantial oxygenation. Because phosphoglycolate is an inhibitor of the triose phosphate isomerase of the Calvin–Benson cycle (Leegood, 1990), plants should detoxify this compound and salvage as much carbon as possible. The photorespiration cycle plays these two roles. By this cycle 1.5 C is salvaged and 0.5 C is lost as 0.5 CO_2 per oxygenation. The photorespiration pathway cycles back 0.5 PGA (1.5 C) to the Calvin–Benson cycle at the expense of 1 ATP and 0.5 NADPH (=1 ferredoxin). Thus, 1.5 PGA (4.5 C) is produced per oxygenation. For the regeneration of RuBP (5 C), 0.5 C is lacking. If 1/6 triose-phosphate is used for the 0.5 C, then 5 ATP and 3 NADPH are used altogether because the Calvin–Benson cycle requires 3 ATP and 2 NADPH for 1 carboxylation, RuBP regeneration, and 1/3 triose phosphate production. Thus, the energy requirement and carbon loss by photorespiration are huge (for stoichiometry, see Heldt, 1999; von Caemmerer, 2000). Thus, it is of supreme importance for plants to maintain the CO_2 concentration at the carboxylation site as high as possible for efficient carboxylation, and suppression of photorespiration as has been repeatedly pointed out (for an early review, see Raven, 1970).

Diffusion of CO_2 from the ambient air to the intercellular spaces

In photosynthesizing C_3 leaves, which have no biochemical CO_2 concentrating mechanisms, the CO_2-concentration at the carboxylation site, C_c, is lower than that in the ambient air, C_a, and CO_2 diffuses to the chloroplast stroma along the gradient of CO_2 concentration (Fig. 1).

The CO_2 concentration in the substomatal cavity, C_s, is lower than C_a. C_a can be estimated by the gas exchange technique (Farquhar and Sharkey, 1982):

$$C_s = C_a - 1.6P/g_{leaf,w} \quad (1)$$

where P is the rate of net photosynthetic CO_2 fixation per unit leaf area, $g_{leaf,w}$ is the leaf conductance for water vapour, and 1.6 is a physical conversion factor from the conductance for water vapour to that for CO_2. $g_{leaf,w}$ is expressed as:

$$g_{leaf,w} = g_{s,w} + g_{c,w} \quad (2)$$

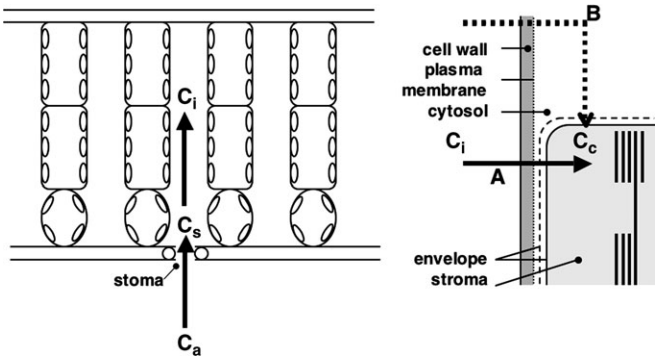

Fig. 1. Diffusion of CO_2 from the ambient air to the chloroplast stroma. C_a, CO_2 concentration in the air; C_s, substomatal CO_2 concentration; C_i, intercellular CO_2 concentration; C_c, CO_2 concentration in the chloroplast stroma. CO_2 in the intercellular spaces is dissolved in the water at the cell wall surface and diffuses to the chloroplast stroma through the cell wall, cell membrane, cytosol, and the chloroplast envelope. Because of the large resistance to CO_2 diffusion in the liquid phase, CO_2 flux via pathway B (dotted line in the right panel) relative to that via pathway A (continuous line) is negligible.

where $g_{s,w}$ is the stomatal conductance for water vapour and $g_{c,w}$ is cuticular conductance for water vapour. Because CO_2 diffuses almost exclusively through stomata and not across the epidermis, the use of $g_{s,w}$ instead of $g_{leaf,w}$ is more appropriate. However, due to technical difficulty, $g_{leaf,w}$ is routinely used for $g_{s,w}$. When $g_{c,w}$ is much smaller than $g_{s,w}$, $g_{leaf,w}$ can be used for $g_{s,w}$. In vigorously photosynthesizing C_3 leaves with widely open stomata, C_s/C_a would range from 0.6 to 0.9.

The value of $g_{c,w}$ is relatively constant, irrespective of $g_{s,w}$ values, and thereby the contribution of g_c would be significant when $g_{s,w}$ is small. Thus, when some stress factor induces the closure of stomata, calculated C_s using $g_{leaf,w}$ tends to be overestimated (for a review, see Evans *et al.*, 2004). When stomata tend to close, photosynthesis can be non-uniform over the leaf. This also often causes overestimation of C_s (for reviews, see Terashima, 1992; Evans *et al.*, 2004).

When the C_3 leaf is vigorously photosynthesizing, 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, r_{ias} (Parkhurst, 1994). However, this resistance is usually much smaller than the stomatal resistance, r_s. Except for very thick hypostomatous leaves (Parkhurst and Mott, 1990) or succulent leaves with small intercellular spaces (Maxwell *et al.*, 1997), C_i/C_s is >0.9 for hypostomatous leaves (Terashima *et al.*, 2001). The resistance to CO_2 diffusion in the intercellular spaces in the amphistomatous leaves is one-third to one-quarter of that in the hypostomatous leaves having the same thickness (Parkhurst *et al.*, 1988; Terashima *et al*, 2001). Thus, it is unlikely that r_{ias} is a major limiting factor of leaf photosynthesis, particularly in the amphistomatous leaves. From here, therefore, C_s will not be distinguished from C_i and only C_i will be used.

CO_2 concentration at the carboxylation site in the chloroplast stroma, C_c, in C_3 plants is even lower than C_i (Evans and von Caemmerer, 1996; Evans and Loreto, 2000). Two different methods have mainly been used to estimate C_c. One method is based on the comparison of the electron transport rate estimated by the fluorescence method and the gas exchange rate measured simultaneously. This method is simpler but relies on several assumptions. The other one, the concurrent measurement of gas exchange and carbon isotope discrimination, is more complex but gives a more accurate estimation. C_c/C_i, thus obtained for vigorously photosynthesizing C_3 leaves, ranges from 0.5 to 0.75.

From the intercellular spaces to the chloroplast stroma

The data of conductance for CO_2 diffusion from the substomatal cavity to the chloroplast stroma, g_i, are plotted against the cumulated chloroplast surface area that faces the intercellular spaces on a leaf area basis, S_c (Fig. 2). The resistance to CO_2 diffusion in the liquid phase for a given distance is 10^4 of that in the gas phase for the same distance. When pH is greater than 6, the ratio of the cumulated concentration of the inorganic carbon species relative to that of CO_2 ($\Sigma C/CO_2$) is >1 (Nobel, 1999). Then, the diffusion of CO_2 will be faster with the increase in $\Sigma C/CO_2$ if the interconversion among these inorganic carbon species is fast enough. Because cytoplasmic carbonic anhydrase activity would be sufficient (for a review, see Coleman 2000), $\Sigma C/CO_2$ in the cytosol at pH 7 may be >7. Still, the flux of inorganic carbon species via the pathway like B (dotted line) in the right panel of Fig. 1 should be negligible compared with that of pathway A (continuous line) because of the large liquid phase resistance. Thus, S_c is important as the active area for CO_2 diffusion to the chloroplast stroma (Laisk *et al.*, 1970; Raven, 1970, 1977; Nobel, 1977; Evans and Loreto, 2000; Evans *et al.*, 2004).

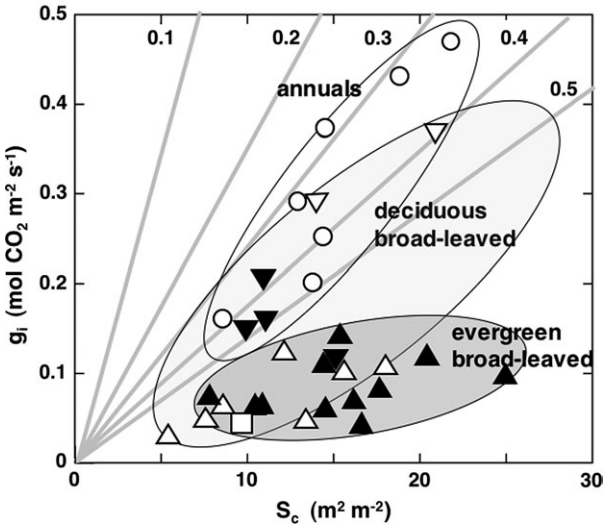

Fig. 2. Internal conductance (g_i) plotted against chloroplast surface area, S_c. Open circles, annual herbaceous plants; open triangles, deciduous broad-leaved trees; solid triangles, evergreen broad-leaved trees; an open square, *Kalanchoë daigremontiana*. Faint lines denote wall conductance per unit leaf area. g_w is drawn assuming that p/τ of the cell wall is 0.1 for wall thicknesses of 0.1, 0.2, 0.3, 0.4, and 0.5 μm, respectively. See Table 1 for details of the samples.

The data in Fig. 2 that include plants of various functional types (Table 1) are, however, scattered. Among them, g_i values for annual herbs such as crop species are greatest when compared at a given S_c and range from 0.2 to 0.5 mol CO_2 m^{-2} s^{-1}. On the other hand, those of evergreen broad-leaved trees are much lower and range from 0.03 to 0.2 mol CO_2 m^{-2} s^{-1}.

Among the evergreen broad-leaved trees, *Citrus* spp. showed greater g_i than *Macadamia integrifolia* and the evergreen broad-leaved trees native to Japanese laurel forests (for scientific names and their authorship, see Table 1 and legend to Fig. 3). In mesic deciduous broad-leaved trees, g_i values are intermediate between those of annual herbs and evergreen trees. Among them, g_i for the tree species that develop leaves successively (*Alnus japonica*, *Populus maximowiczii*, and *Prunus persica*) tends to be greater than those for *Acer* spp. that flush their leaves in the spring. When the data points are grouped into annuals, including wheat and rice, deciduous broad-leaved trees, and evergreen broad-leaved trees, a tendency that g_i increases with the increase in S_c may be apparent.

The main difference among these groups is the thickness of the mesophyll cell wall (δ_w). The δ_w in typical annuals, deciduous broad-leaved species, and evergreen broad-leaved species range from 0.1 to 0.2, 0.2 to 0.3, and 0.3 to 0.5 μm, respectively. Within the deciduous tree species, the flush-type species tend to show greater δ_w than in the successive-type species (Hanba *et al.*, 2001, 2002). *Acer rufinerve* (100% light) showed a large S_c but a low g_i, which is probably explained by its very large δ_w for a deciduous tree species, 0.25 μm for the palisade tissue cells and 0.46 μm for the spongy tissue cells.

A clonal herbaceous perennial, *Polygonum cuspidatum* (synonymous to *Reynoutria japonica* Houttuyn), is a well-known pioneer plant as the first colonizer of volcanic deserts (Adachi *et al.*, 1996). The plants grown at 2500 m a.s.l. in a volcanic desert on Mt Fuji-san, Japan, had a thick δ_w of 0.35–0.42 μm, while the same species at 10 m a.s.l. had a δ_w of 0.22–0.29 μm. In agreement with the difference in wall thickness, g_i for the plants from 2500 m was 0.076 mol m^{-2} s^{-1} while that for the plants from 10 m was 0.2 mol m^{-2} s^{-1} (Kogami *et al.*, 2001; see Fig. 3). When autotetraploidy was artificially induced in *Phlox drummondii*, the leaves became thicker and S_c increased. However, at the same time, cell wall thickness increased considerably (from 0.12 to 0.24 μm), which would partly explain the absence of the increase in g_i with the increase in S_c (P Vyas, unpublished observation; see Fig. 3).

Table 1. *Mesophyll surface area, chloroplast surface area and internal conductance of species of various functional types*

Species	S_{mes} (m^2 m^{-2})	S_c (m^2 m^{-2})	g_i (mol Co_2 m^{-2} s^{-1})	Source
Annuals				
Nicotiana tabacum L. (sun)	23.7	21.8	0.470	Evans *et al.*, 1994
N. tabacum (shade)	17.0	14.5	0.370	Evans *et al.*, 1994
Triticum aestivum L.	23.5	18.8	0.430	Evans and Vallen, 1996
Oryza sativa L.	19.0	14.0	0.200	Hanba *et al.*, 2004
Phaseolus vulgaris L.	14.3	13.0	0.290	Hanba *et al.*, 2003
P. vulgaris	16.0	14.5	0.250	Hanba *et al.*, 2003
Phlox drummondii Hook.	14.6	8.5	0.160	P Vyas *et al.*, unpublished
Deciduous broadleaved trees				
Acer mono Maxim. (17% sun)	14.0	12.0	0.125	Hanba *et al.*, 2001
Ac. mono (full sun)	12.0	8.5	0.065	Hanba *et al.*, 2001
Acer palmatum Thunb.(17% sun)	8.0	5.5	0.030	Hanba *et al.*, 2001
Ac. palmatum (full sun)	10.0	7.5	0.052	Hanba *et al.*, 2001
Acer rufinerve Sieb. et Zucc. (17% sun)	8.5	5	0.047	Hanba *et al.*, 2001
Ac. rufinerve (full sun)	18.0	13.5	0.047	Hanba *et al.*, 2001
Alnus japonica (Thunb.) Steud.	20.0	18.0	0.110	Hanba *et al.*, 2001
Populus maximowiczii A. Henry	78.0	15.5	0.100	Hanba *et al.*, 2001
Prunus persica L. Batsch(sun)	30.1	21.0	0.370	Syvetsen *et al.*, 1995
Pr. persica (shade)	26.6	14.0	0.291	Syvetsen *et al.*, 1995
Evergreen trees				
Quercus glauca Thunb. ex Murray	17.0	10.8	0.066	Hanba *et al.*, 1999
Q. glauca	11.2	7.8	0.076	Hanba *et al.*, 1999
Q. phillyraeoides A. Gray	31.4	15.4	0.143	Hanba *et al.*, 1999
Cinnamomum camphora (L.) J. Presl	19.6	14.5	0.061	Hanba *et al.*, 1999
Castanopsis sieboldii (Makino) Hatus.	20.7	16.7	0.044	Hanba *et al.*, 1999
Cas. sieboldii	22.1	17.8	0.082	Hanba *et al.*, 1999
Cas. sieboldii	25.0	25.0	0.100	Miyazawa and Terashima, 2001
Ligustrum lucidum Aiton	20.3	10.5	0.067	Hanba *et al.*, 1999
L. lucidum	28.0	14.6	0.113	Hanba *et al.*, 1999
Camellia japonica L.	41.6	20.5	0.119	Hanba *et al.*, 1999
Cam. japonica	32.9	16.2	0.069	Hanba *et al.*, 1999
Citrus paradisi Macfad	26.2	11.0	0.208	Syvetsen *et al.*, 1995
Cit. paradisi	24.7	11.0	0.157	Syvetsen *et al.*, 1995
Citrus limon (L.) Burm.f.	26.4	10.0	0.149	Syvetsen *et al.*, 1995
Macadamia integrifolia Maiden et Betche	24.4	15.0	0.114	Syvetsen *et al.*, 1995
CAM				
Kalanchoë daigremontiana Hamet et Perr.	25.4	9.8	0.050	Maxwell *et al.*, 1997

S_c represents the area for CO_2 dissolution and δ_w represents the path length for CO_2 diffusion in the liquid phase. Thus, it is understandable, that g_i is proportional to S_c and inversely related to δ_w.

The role of aquaporins

In addition to the two physical factors dealt with above, the role of aquaporins in CO_2 diffusion will be highlighted. Changes in g_i without marked changes in S_c and/or cell δ_w have been reported. For example, Evans and Vellen (1996) followed senescing wheat leaves and observed the drastic decrease in g_i without marked changes in S_c (Fig. 3). Perhaps δ_w did not increase greatly either. Decreases in g_i have been reported for plants under water stress (Flexas *et al.*, 2002) and salt stress (Delfine *et al.*, 1998, 1999). These studies indicate that CO_2 permeability of the plasma membrane or chloroplast envelope might have changed.

Aquaporins, the most abundant proteins in plant plasma membranes, transport water molecules according to the gradient of the water potential (Maurel, 1997; Kjellbom *et al.*, 1999). However, there are reports indicating that some animal aquaporins transport CO_2 (Cooper and Boron, 1998; Nakhoul *et al.*, 1998; Yang *et al.*, 2000).

Terashima and Ono (2002) estimated g_i using the concurrent measurements of gas exchange and fluorescence in the leaves of *Vicia faba* L. and *Phaseolus vulgaris* L. before and after the application of $HgCl_2$, an inhibitor of most of the aquaporins, to the petiole. Because g_i and hydraulic conductivity of the mesophyll cells decreased at the same concentration range of $HgCl_2$, it is proposed that aquaporins are involved in diffusion of CO_2 across the plasma membrane. Application of chloromercuribenzene sulphonate that would not permeate membranes easily gave similar results (I Terashima, Y Tazoe, V Oja, A Laisk, unpublished results). Bernacchi *et al.* (2002) observed a clear peak at 35–37 °C in the temperature dependence of g_i measured by the fluorescence method in tobacco, which suggests the involvement of protein(s) in CO_2 diffusion across the plasma membrane.

Recently, it was shown that the aquaporin 1 of *Nicotiana tabacum* L., expressed in *Xenopus* oocytes, transfers CO_2

(Uehlein *et al.*, 2003). In this study, tobacco aquaporin 1 (NtAQP1) was expressed in *Xenopus* oocytes and the CO_2 permeability was monitored as the decrease in pH with a pH micro-electrode. Hanba *et al.* (2004) attempted to over-express an aquaporin of *Hordeum vulgare* L. (HvPIP2; 1) in rice (*Oryza sativa* L.). By contrast to their original expectation, they obtained plants with varying aquaporin contents. The concurrent measurement of the gas exchange and carbon isotope discrimination of these leaves revealed that g_i clearly increases with the increase in aquaporin abundance.

Besides the abundance of aquaporins, conductance of CO_2 through aquaporins could also be regulated, for example, by pH and/or protein phosphorylation, because water permeability has been reported to be regulated by pH (Tournaire-Roux *et al.*, 2003), and by protein phosphorylation (Maurel, 1997; Kjellbom *et al.*, 1999). These possibilities are to be studied.

Components of the internal conductance

The internal conductance (or resistance) can be dissected further into several components (Fig. 4). The resistance to CO_2 diffusion from the cell wall surface to the carboxylation site per unit chloroplast surface area (R_i) may be expressed as:

$$R_i = R_w + \frac{1}{\frac{1}{R_{aq}} + \frac{1}{R_{bm}}} + R_{cytosol} + R_{env} + R_{stroma} \qquad (3)$$

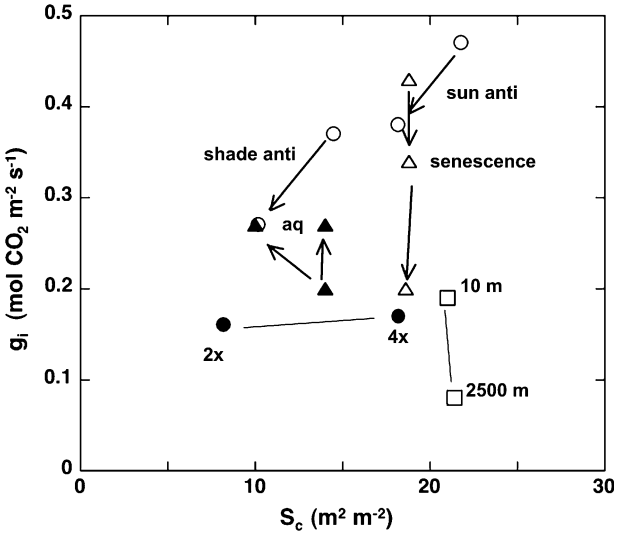

Fig. 3. Effects of environmental manipulations, genetic manipulations, and senescence on the S_c versus g_i relationship. Open circles, tobacco plants expressing antisense Rubisco small subunits (Evans *et al.*, 1994); solid triangles, rice plants over-expressing barley aquaporin (Hanba *et al.*, 2004); open triangles, senescing wheat leaves (Evans and Vellen, 1996); solid circles, diploid and artificially induced autotetraploid of *Phlox drummondii* Hook. (P Vyas *et al.*, unpublished observation); open squares, alpine and low land *Polygonum cuspidatum* Sieb. et Zucc. (Kogami *et al.*, 2001).

where R_w, R_{aq}, R_{bm}, $R_{cytosol}$, R_{env}, and R_{stroma} are resistances to CO_2 diffusion across the cell wall, aquaporin, bulk plasma membrane, cytosol, chloroplast envelope, and chloroplast stroma to the carboxylation site expressed on a unit chloroplast surface area, respectively. The corresponding conductances are G_{aq}, G_{bm}, $G_{cytosol}$, G_{env}, and G_{stroma}. Note that lower-case letters, r and g, are used for the resistance and conductance, respectively, on a leaf area basis.

For the moment, let us neglect $R_{cytosol}$, because the distance between the plasma membrane and chloroplast envelope is very small in many wild plants, and the abundance of inorganic carbon species, mostly HCO_3^- and CO_2, relative to CO_2 ($\Sigma C/CO_2$) may be >7 at pH 7 if the carbonic anhydrase activity in the cytosolic is sufficient (Evans *et al.*, 1994; Nobel, 1999; Coleman, 2000). R_{env} is also neglected. In the light, pH in the chloroplast stroma increases and there is carbonic anhydrase (Coleman, 2000). Thus the abundance of HCO_3^- relative to CO_2 would be >50 (Nobel, 1999). Because HCO_3^- also diffuses together with CO_2, R_{stroma} would be very small (Raven and Glidewell, 1981; Cowan, 1986).

Wall

The wall conductance for the unit chloroplast surface area can be expressed as:

$$G_w = \frac{1}{R_w} = \frac{p \cdot D_C \cdot K_{CO_2}}{\tau \cdot \delta_w} \qquad (4)$$

where p is porosity, D_C is the weighed diffusion coefficient of inorganic carbon species in water, K_{CO_2} is the

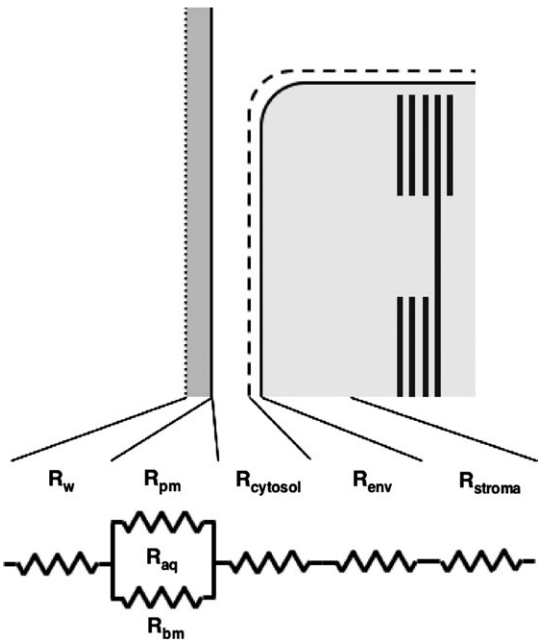

Fig. 4. Components of the internal resistance.

partitioning coefficient, the ratio of the concentrations of the inorganic carbon species in the liquid phase to that of CO_2 in gas phase (Nobel, 1999), τ is tortuosity, and δ_w is cell wall thickness.

Nobel (1999) assumed that the p/τ of the mesophyll cell wall is around 0.3. Because D_C at 20 °C is not very different from the diffusion coefficient of CO_2 (see below), 1.7×10^{-9} m^2 s^{-1}, he used pD_C/τ of 5×10^{-10} m^2 s^{-1}. K_{CO_2} used was 1 because apoplast pH is somewhat acidic (Nobel, 1999; Stahlberg and Van Volkenburgh, 1999; but see Raven and Farquhar, 1989) and the inorganic carbon species is almost exclusively CO_2 and δ_w was 0.3×10^{-6} m, then G_w was calculated to be 1.7×10^{-3} m s^{-1}. Assuming S_c is 20 m^2 m^{-2}, the wall conductance on the leaf area basis, g_w, is 0.03 m s^{-1}, which corresponds to 1.2 mol m^{-2} s^{-1}. Based on such calculations, Nobel claimed that wall conductance is very large and thus the cell wall is not limiting photosynthesis. However, the actual p/τ is not known for mesophyll cell walls of higher plants.

Although permeability of CO_2 across the cell wall has not been measured, permeability of water across the cell wall was measured. Kamiya *et al.* (1962) measured water permeability of the cell wall in *Nitella flexilis* (L.) Ag., a characean plant having giant internodal cells, using the pressurizing method. They found that the cell wall, which is10 μm thick, showed water permeability of 5×10^{-7} m s^{-1} atm^{-1}. They also found that the permeability is inversely proportional to the cell wall thickness. Because 1 atm corresponds to the concentration difference of 40.4 mol m^{-3} at 25 °C, p/τ of the cell wall can be calculated from:

$$\frac{5\times10^{-7}}{V_w} = \frac{p\cdot D_{H_2O}}{\tau\cdot\delta_w}\cdot 40.4 \qquad (5)$$

where V_w is the molar volume of water (18×10^{-6} m^3) and D_{H_2O} is the diffusion coefficient of water in water (2.3×10^{-9} m^2 s^{-1} at 25 °C). When δ_w is 10×10^{-6} m, p/τ is calculated to be 3.

It is known that the permeability obtained by the pressurizing technique is greater than that by the diffusion method if the material has bulky holes or pipes (Gutknecht, 1967). The p/τ value of 3, therefore, indicates that there are holes or pipes in the cell wall.

Gutknecht (1967) measured the wall conductance (P_d) in the cell wall of a giant green alga *Valonia ventricosa* J. Agardh by the diffusion method using tritiated water and obtained 2.5×10^{-6} m s^{-1}. Because δ_w of these algae would be around 10 μm (not given by Gutknecht, 1967; but see Okuda *et al.*, 1997),

$$2.5\times10^{-6} = (p\cdot D_{H_2O})/(\tau\cdot\delta_w) \qquad (6)$$

p/τ thus obtained is 0.011. If this p/τ value, δ_w of 0.3 μm, and S_c of 20 m^2 m^{-2} are used, then a very low g_w of 0.045 mol m^{-2} s^{-1} is obtained. This value is too low, even when the lowest g_i values in Fig. 2 are considered.

Accurate measurements of wall resistance to diffusion of water and CO_2 with higher plants are needed. It should be noted that one cannot use the pressure probe method in the pressurizing mode to determine wall resistance.

Assuming p/τ =0.1, g_w (see the grey lines in Fig. 2) was calculated. If p/τ is less than about 0.1, then wall conductance is an important factor determining internal conductance. Of course, p/τ values would vary considerably, because of the variation in cell wall constituents across species. However, it is proposed that the p/τ value is around 0.1 or lower, because the difference in g_i among leaves having a given S_c, but from different functional groups, is roughly explained by δ_w.

The resistance to CO_2 diffusion in the liquid phase is 10^4 of that in the gas phase. If p/τ is 0.1, then the wall thickness of 0.3 μm will correspond to an air layer 30 mm thick. Thus, wall resistance would be an important limiting factor of photosynthesis.

Plasma membrane

When $HgCl_2$ was applied to the leaflets of *Phaseolus vulgaris* L., g_i decreased by up to 60% depending on the concentration of $HgCl_2$ (Terashima and Ono, 2002). Some aquaporins are known to be insensitive to $HgCl_2$ (Kjellbom *et al.*, 1999) and, therefore, a part of the residual conductance could be attributed to such insensitive aquaporins. As already mentioned above, the tobacco aquaporin (NtAQP1) has been shown to transport CO_2 across the plasma membrane of *Xenopus* oocytes (Uehlein *et al.*, 2003)

Hanba *et al.* (2004) have reported that rice leaves expressing various amounts of barley aquaporin (HvPIP2,1) showed differences in g_i, the range of which corresponds to 50% of the g_i in the control plants. g_i in tobacco plants expressing antisense aquaporin 1 was lower than that of the control plants by 40%, while the plants over-expressing aquaporin 1 showed g_i greater than that in the control plants by 30% (Flexas *et al.*, 2004; International Photosynthesis Congress, 2004, Montreal; J Flexas, personal communication).

There are >10 different aquaporin species that are expressed in the plasma membrane of plants (Kjellbom *et al.*, 1999). It is not known how many of these are responsible for the transport of CO_2. Thus, CO_2 transport activity in these aquaporins should be examined on a one by one basis. Then, the contribution of aquaporins to the overall conductance of the plasma membrane should be studied.

At this stage, only a rough estimation can be made. Let us assume that the CO_2 diffusion through aquaporins in a *P. vulgaris* leaf is completely suppressed by $HgCl_2$ and g_i decreased from 0.3 mol m^{-2} s^{-1} to 0.12 mol m^{-2} s^{-1}. If p/τ, cell wall thickness, and S_c are 0.1, 0.1 μm, and 13 m^2 m^{-2}, respectively, then g_w will be 0.9 mol m^{-2} s^{-1}. If it is further assumed that internal resistance is the sum of

wall resistance and membrane resistance only, the membrane conductance values before and after the $HgCl_2$ treatment are 0.45 and 0.138 mol m^{-2} s^{-1}. The corresponding conductance for the unit chloroplast surface area, G_{aq} and G_{bm} is calculated to be 0.024 and 0.011 mol m^{-2} s^{-1}, or 5.86×10^{-4} and 2.69×10^{-4} m s^{-1}, respectively. These calculations may indicate that more than two-thirds of CO_2 molecules transported across the plasma membrane are via aquaporins.

It should also be pointed out that the effects of changes in abundance or conductivity of aquaporins on total internal conductance is largest in annual plants with thin cell walls but smallest in the evergreen broad-leaved tree species.

In summary, g_i is determined by S_c, wall thickness, and by abundance and/or conductivity of aquaporins. These aquaporins that transport CO_2 may be called 'cooporins' to highlight CO_2-porins that are co-operating with other photosynthetic components such as carbonic anhydrase.

Why are sun leaves thicker than shade leaves?

The light-saturated rate of leaf photosynthesis per unit area (P_{max}) in C_3 plants strongly depends on leaf nitrogen content and photosynthetic components such as Rubisco, cytochrome *f*, H^+-ATPase, and reaction centres. P_{max} is also strongly correlated with structural parameters such as leaf thickness, leaf mass per area, mesophyll surface area (S_{mes}), and chloroplast surface area (S_c). Importance of S_c as an area for CO_2 dissolution and for the CO_2 diffusion pathway has been already discussed.

Let us consider the drawdown of CO_2 concentration from the intercellular spaces to the stroma ($\Delta C=C_i-C_c$) for a unit chloroplast surface area (Fig. 5). The drawdown is proportional to the flux of CO_2 across the cell wall, plasma membrane, cytosol, and chloroplast envelope per unit chloroplast surface area and to the internal resistance, R_i. With the increase in the amount of Rubisco per unit chloroplast surface area, the CO_2 flux increases. However, the photosynthetic rate per Rubisco decreases because C_c decreases. From the viewpoint of efficiency of Rubisco use (or nitrogen use), thicker leaves with greater S_c would be advantageous because the amount of Rubisco per S_c becomes smaller and thereby C_c would increase. On the other hand, the construction and maintenance costs of thick leaves are expensive. Also, the drawdown of CO_2 concentration during the diffusion in the intercellular spaces, C_s-C_i, becomes greater with the increase in leaf thickness, which thus causes a decrease in the bulk C_i. In the latter two aspects, thick leaves are not at all advantageous.

Effects of various aspects of mesophyll structure, in particular mesophyll thickness on photosynthesis, were evaluated using a one-dimensional model of CO_2 diffusion in the C_3 leaf (Terashima *et al.*, 2001). In this model, mesophyll is composed of columnar cells, the lateral surfaces of which are fully occupied by chloroplasts. When

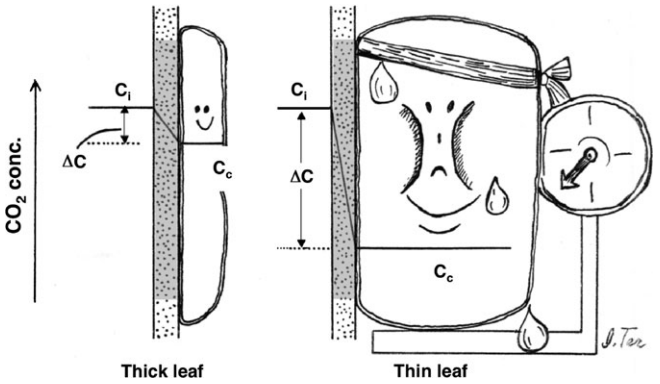

Fig. 5. Consequences of the difference in Rubisco content per unit chloroplast surface area. Given that the Rubisco content per leaf area is identical, thinner leaves having a smaller S_c should have more Rubisco per S_c. Then Rubisco in the thinner leaves would operate at a lower CO_2 concentration.

mesophyll thickness was increased, keeping the Rubisco content per leaf area constant, S_c increases and Rubisco content per S_c decreases. The model leaves are either hypostomatous or amphistomatous. The main results can be summarized as follows:

(i) When mesophyll thickness was increased keeping the Rubisco content per leaf area constant, the rate of photosynthesis per leaf area increased at first due to the increase in C_c, attained the peak value, and then gradually decreased. The gradual decrease was due to the gradual decrease in C_i due to the increased r_{ias}. The thickness that gives the peak value was identical for the leaves with various Rubisco contents per leaf area.

(ii) The thickness that gives the maximum photosynthetic rate for the amphistomatous leaves was greater than that for the hypostomatous leaves because r_{ias} in the former were one-quarter to one-third of that in the latter leaves.

(iii) The mesophyll thickness that realizes a given photosynthetic rate per unit mesophyll thickness increased with the increase in the Rubisco content per leaf area.

Obviously, neither (i) nor (ii) explains the strong relationship between P_{max} and S_c. Thus (iii) or constraints of this kind explain the strong relationships between P_{max} and leaf morphological parameters such as mesophyll thickness and S_c. In other words, leaf thickness is determined as a compromise between the increase in chloroplast surface area for CO_2 dissolution and the decrease in the construction and maintenance costs of the leaf. If such the economical optimum is strongly favoured, S_c/S_{mes} can be expected to be very high. Moreover, thickness of chloroplasts or Rubisco/S_c would not vary much. Both are roughly true in nature (see below). Thus, this would be the answer to the question: why are sun leaves thicker than shade leaves?

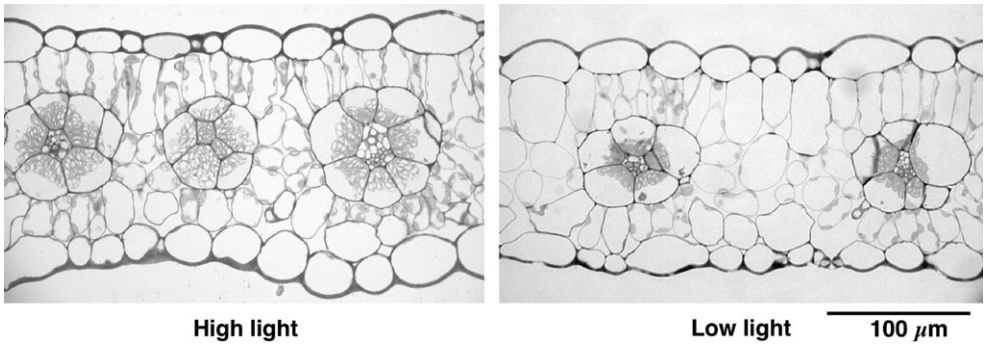

Fig. 6. Sun and shade leaves of *Amaranthus cruentus* L. The plants were grown in a glasshouse. The midday PPFD for high-light plants was 1600 μmol m^{-2} s^{-1}, while that for low-light plants was around 500 μmol m^{-2} s^{-1}. High-light and low-light leaves showed a P_{max} of about 30 and 15 μmol CO_2 m^{-2} s^{-1}, respectively (Y Tazoe, unpublished micrographs).

Because C_4 plants have CO_2 concentration mechanisms, and the CO_2 fixation enzyme is located in the cytosol of mesophyll cells, the arguments above are exclusively for C_3 plants. The changes in leaf thickness or mesophyll cell surface area have not been intensively studied for C_4 plants. It was found recently that the thickness of the leaves of *Amaranthus cruentus* L., an NAD-ME-type C_4 dicotyledonous plant, did not respond to growth in the light (Fig. 6; Tazoe *et al.*, 2005). For responses of C_4 photosynthesis to growth photosynthetic photon flux densities (PPFD), see the review by Sage (2006).

How do C_3 sun leaves have greater S_c than shade leaves?

In many deciduous broad-leaved tree species, sun leaves have a greater S_c than shade leaves. In sun leaves, the height of the palisade tissue is greater than in shade leaves. In some cases, thickening of the palisade tissue is accompanied by periclinal cell division as well as cell elongation of the palisade tissue cells.

Some ecotypes of Japanese beech (*Fagus crenata* Blume), including those on the Pacific side, differentiate sun leaves with thick palisade tissue comprising two cell layers (T Koike, personal communication; Fig. 7). In such ecotypes, not only the number of leaves (Kozlowski and Clausen, 1966) but also the number of cell layers in the palisade tissue of these leaves is determined in the winter buds, by early winter of the year prior to leaf unfolding (Eschrich *et al.*, 1989). When the sun-exposed branches with young expanding leaves of *F. crenata* were shaded by shade cloths, the resultant leaves showed intermediate characteristics: they had the palisade tissue that comprised two cell layers but the height of the palisade tissue and P_{max} were lower than those in the fully exposed sun leaves (Uemura *et al.*, 2000; A Uemura and A Ishida, personal communication). These strongly indicate that several different signals are used for the determination of characteristics of sun leaves, including multi-layered palisade tissue, greater cell height, larger S_c, higher contents of photosynthetic enzymes, and higher stomatal frequency and conductance. At least, the current-year PPFD and previous-year PPFD play different roles (Kimura *et al.*, 1998; Uemura *et al.*, 2000).

Using *Chenopodium album* L., an annual herb, Yano and Terashima (2001) established a more sophisticated experimental system and examined the differentiation processes of sun and shade leaves. The plants were shaded in various ways and the effects of these shade treatments on the properties of the developing leaves were examined. When mature leaves were exposed to high light, the developing leaves, irrespective of their light environments, formed palisade tissue with two cell layers. On the other hand, when mature leaves were shaded, palisade tissue with one cell layer was formed. These results clearly showed that the light environment of mature leaves determined the number of cell layers in the palisade tissue of new leaves. There must be a signal transduction system that conveys the signal(s) from the mature leaves to the developing leaves (Yano and Terashima, 2001).

Yano and Terashima (2004) also conducted a detailed developmental study of sun and shade leaves of *C. album*. Whether the plants were grown under typical sun or shade conditions, the number of cells in the palisade tissue per leaf was almost identical. Moreover, in sun leaves, anticlinal and periclinal divisions occur almost synchronously. Thus, the signal from the mature leaves regulates the direction of cell division. In the future sun leaves, the signal probably induces periclinal division in addition to anticlinal division, while the signal from the shaded mature leaves only allows the cells to divide anticlinally (Yano and Terashima, 2004).

Yano and Terashima (2001, 2004) hypothesized that the signal is the abundance of photosynthates; when the photosynthates from mature leaves are abundant, leaves would develop into sun leaves. Currently, the effects of the sucrose content of the agar on leaf development in *Arabidopsis thaliana* (L.) Heynh. are being examined. There is a clear

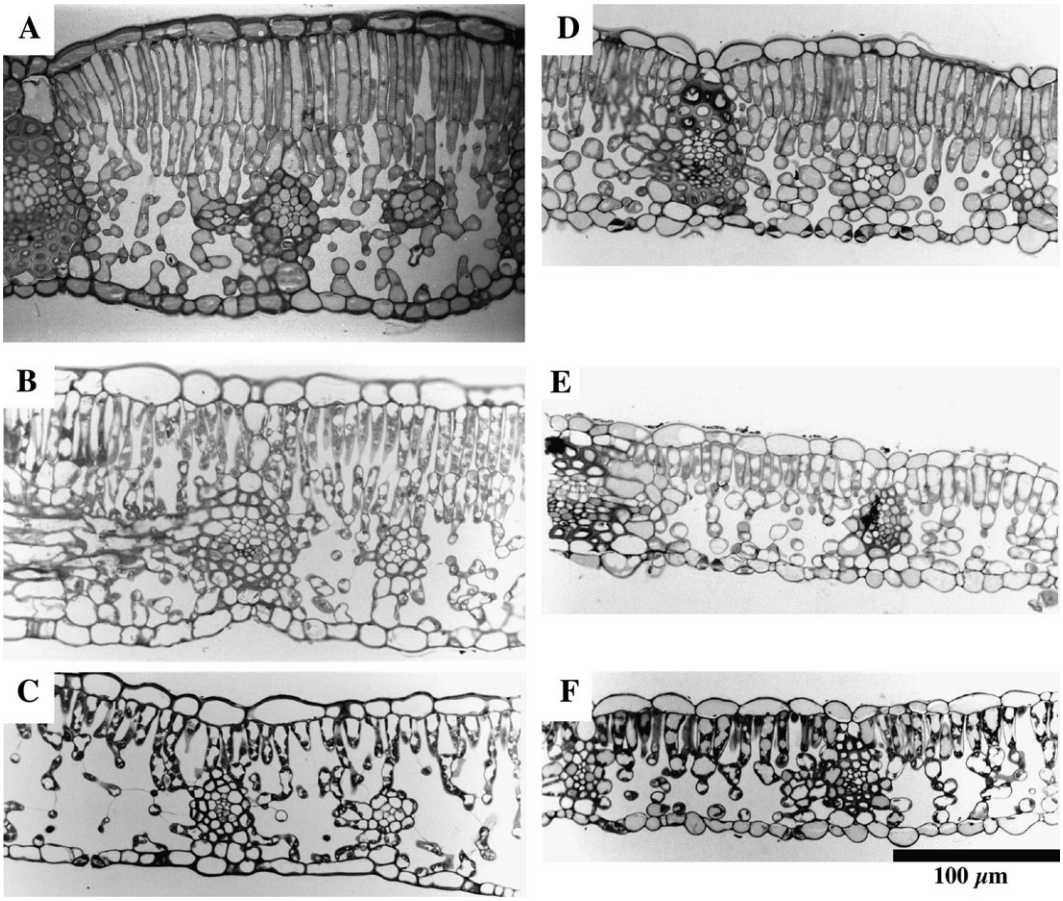

Fig. 7. Sun and shade leaves of *Fagus crenata* Blume (A–C) and *Fagus japonica* Maxim. (D–F). (A) and (D) were from fully exposed shoots; (B) and (E) were from the shoots that had been exposed but were shaded from early spring until sampling in mid-summer; (C) and (F) were from the lower shaded shoots. Note that the palisade tissues of *F. japonica* had only one cell layer irrespective the light conditions (Uemura *et al.*, 2000). Original micrographs of I Terashima.

indication that the number of cell layers in the palisade tissue increases with increasing sucrose content (S Yano, H Tsukaya, personal communication).

Stomatal frequency and morphology of epidermal cells in young leaves are also regulated by the environment of the mature leaves (Lake *et al.*, 2001; Thomas *et al.*, 2003; Coupe *et al.*, 2006), although the signal transduction mechanisms involved may be different from those that regulate the direction of cell division. For the palisade tissue cells to elongate, the local light environment of the developing leaves appears to be essential, although detailed studies have not been conducted.

On the other hand, chloroplast properties are mostly determined by the local light environment of the developing leaves (Terashima and Hikosaka, 1995; Yano and Terashima, 2001). It is known that there is a gradient in light environment within a leaf (Terashima and Saeki, 1983, 1985) and that chloroplasts within the leaf acclimate to their respective light environments (Terashima and Inoue, 1985*a*, *b*). When the leaves were inverted or irradiated from the bottom after their unfolding, the properties of chloroplasts changed and acclimated to their new light environments (Terashima and Takenaka, 1986; Terashima *et al.*, 1986). Thus, acclimation of chloroplast properties to their local light environments is very plastic.

After maturation, leaves of some species respond to changes in the light environment. When grown with sufficient nutrients, most of the mesophyll cell surfaces facing the intercellular spaces are occupied by chloroplasts. However, it was shown that, for the shade leaves of *Chenopodium album*, unoccupied spaces are indispensable for re-acclimating to brighter light environments (Oguchi *et al.*, 2003). When the shade leaves of *C. album* were exposed to a higher light for growth, an increase in S_c was observed. This increase in S_c was not accompanied by an increase in mesophyll surface area. Oguchi *et al.* (2005) examined shade leaves of three deciduous tree species, *Betula ermanii* Cam., *Fagus crenata* Blume, and *Acer rufinerve* Sieb. et Zucc. In *F. crenata* mesophyll surfaces were fully occupied by chloroplasts and did not increase P_{max} when the plants were exposed to a higher growth PPFD. On the other hand, shade leaves of *B. ermanii* that

Table 2. *Possible effects of cell size on leaf characteristics*

	Small cells	Large cells
Leaf thickness for the same S_c	Thin	Thick
Mechanical strength	Strong	Weak
Maintenance of leaf morphology	Cell walls (armour)	Turgor (balloon)
Longevity	Long	Short
Heat capacity	Small	Large
Intercellular resistance to CO_2 diffusion	Small	Large
Stomatal occurrence	Hypostomatous	Amphistomatous
Area expansion	Slow	Fast
Informational cost (nuclear N and P/chloroplast N and P)	High	Low

had unoccupied mesophyll surfaces increased P_{max} in response to the exposure. Shade leaves of *A. rufinerve* elongated palisade tissue cells and increased P_{max} in response to the increase in growth PPFD.

In *Hedera helix* L., the mature palisade tissue cells divided periclinally in response to the increase in growth PPFD (Bauer and Thoni, 1988). Such division of the palisade tissue cells has not been described for other species. However, for the evergreen leaves of extended longevity, such plastic adjustment may be important. Recently, mechanisms of the sun and shade leaf differentiation were reviewed elsewhere (Terashima *et al.*, 2005).

Plasticity of leaf structure: importance of cell size

In this review, attention was first drawn to the importance of having sufficient S_c for efficient photosynthesis. Mechanisms responsible for the regulation of S_c are also discussed. These arguments are based on an assumption that cell diameter would not change. However, in nature, the size of mesophyll cells varies greatly and the leaf can increase S_c and decrease resistance to CO_2 diffusion in the intercellular spaces by decreasing cell size (Terashima *et al.*, 2001, 2005; Miyazawa *et al.*, 2003). The leaf with smaller cells is also mechanically tougher (Terashima *et al.*, 2001). Actually, mesophyll cell size differs considerably across species (Terashima *et al.*, 2001). However, 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. have been favoured by natural selection (Terashima *et al.*, 2001). The merits or demerits of having large and small mesophyll cells are summarized in Table 2. If the leaves have large S_c with large cells, as is found in annual herbs, the leaves should be thick. Such leaves would expand quicker, keep their shape by turgor, and have stomata on both leaf surfaces. The leaves with extended longevity tend to have small cells (Terashima *et al.*, 2001). We mentioned previously that alpine plants tend to have smaller mesophyll cells (Terashima *et al.*, 2005). However, their cells are not necessarily smaller than those of related lowland species (Körner, 1999). It is also worth mentioning that the mesophyll cell sizes of bonsai plants are comparable with those of plants grown normally (Körner *et al.*, 1989). Although such leaves with small cells are mechanically tougher, area expansion would be slower. Moreover, the volume ratio of chloroplasts/nucleus in small cells might be smaller. Because N and P are important constituents of nucleic acids or proteins in nuclei as well, the cost, which may be called 'information cost,' would be much greater in leaves of small cells. If this informational cost is large, the nitrogen use efficiency of photosynthetic production would decrease. If this is very important, cell size would be greater in leaves under low availability of P and/or N,. There are other features such as water relationships with respect to cell size. Comprehensive studies approaching the diversity in leaf thickness based on causes and consequences of cell size should be made.

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