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INVITED REVIEW

Cost–benefit analysis of mesophyll conductance: diversities of anatomical, biochemical and environmental determinants

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• **Background** Plants invest photosynthates in construction and maintenance of their structures and functions. Such investments are considered costs. These costs are recovered by the CO_2 assimilation rate (A) in the leaves, and thus A is regarded as the immediate, short-term benefit. In photosynthesizing leaves, CO_2 diffusion from the air to the carboxylation site is hindered by several structural and biochemical barriers. CO_2 diffusion from the intercellular air space to the chloroplast stroma is obstructed by the mesophyll resistance. The inverses is the mesophyll conductance (g_m). Whether various plants realize an optimal g_m, and how much investment is needed for a relevant g_m, remain unsolved.
• **Scope** This review examines relationships among leaf construction costs (CC), leaf maintenance costs (MC) and g_m in various plants under diverse growth conditions. Through a literature survey, we demonstrate a strong linear relationship between leaf mass per area (LMA) and leaf CC. The overall correlation of CC vs. g_m across plant phylogenetic groups is weak, but significant trends are evident within specific groups and/or environments. Investment in CC is necessary for an increase in LMA and mesophyll cell surface area (S_{mes}). This allows the leaf to accommodate more chloroplasts, thus increasing A. However, increases in LMA and/or S_{mes} often accompany other changes, such as cell wall thickening, which diminishes g_m. Such factors that make the correlations of CC and g_m elusive are identified.
• **Conclusions** For evaluation of the contribution of g_m to recover CC, leaf life span is the key factor. The estimation of MC in relation to g_m, especially in terms of costs required to regulate aquaporins, could be essential for efficient control of g_m over the short term. Over the long term, costs are mainly reflected in CC, while benefits also include ultimate fitness attributes in terms of integrated carbon gain over the life of a leaf, plant survival and reproductive output.

Key words: Aquaporin, cooporin, leaf construction cost, leaf maintenance cost, leaf mass per area, mesophyll conductance.

INTRODUCTION: LEAF CO_2 UPTAKE AND ITS CONSTRAINTS IN LAND PLANT

Because land plants face the challenge of using atmospheric CO_2 for photosynthesis at the expense of H_2O loss, photosynthetic carbon (C) gain should be optimally balanced against transpiration. CO_2 uptake and H_2O loss in land plants share the same diffusive pathway through the leaf boundary layer, stomata and sub-stomatal cavities, although the directions of these fluxes are opposite in the light. Additional resistances to CO_2 include the intercellular air space, cell wall, plasma membrane, cytosol, chloroplast envelope and chloroplast stroma (Fig. 1). CO_2 diffusion conductance, the inverse of the resistance imposed by these barriers, changes dynamically in response to environmental variables on short-term scales of minutes to days. Such dynamic changes are attributed to the changes in stomatal aperture, CO_2 permeabilities of the plasma membrane and chloroplast envelope, pH values of the cell wall solution, cytosol and the chloroplast stroma, and positions of chloroplasts. On the other hand, the stomatal density, intercellular diffusion path length and cell wall thickness are established during tissue development and do not change on short-term scales. These static traits, however, can be developmentally adjusted to enhance adaptive function in the growth environment. Similarly, for H_2O diffusion, there are quickly adjustable factors such as stomatal aperture and activity and the abundance of aquaporins, in addition to factors that are not adjustable on short-term scales (e.g. vein density, cuticle thickness, stomatal density and distance from the vascular system to the stomata).

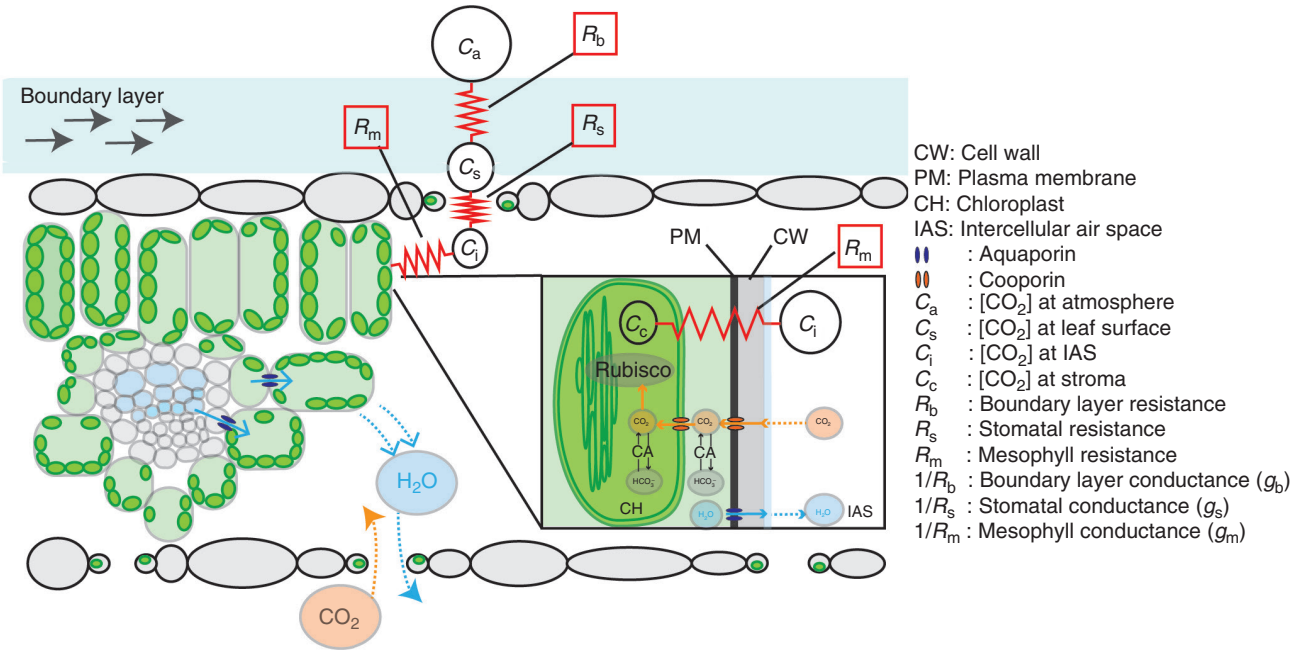

FIG. 1. Schematic CO_2 diffusion pathway from the atmosphere to the chloroplast stroma. C_a decreases to C_c due to R_b, R_s and R_m (see Table 2 for definitions). The inverse of these resistances are the conductance terms g_b, g_s and g_m, respectively. R_m consists of resistances at the intercellular air space ($1/g_{IAS}$), cell wall ($1/g_w$), plasma membrane ($1/g_{pm}$), cytosol ($1/g_{cy}$), chloroplast envelope ($1/g_{env}$) and chloroplast stroma ($1/g_{st}$). CO_2 diffuses through cooporins (CO_2 permeable aquaporins) across the PM and CH envelope. Carbonic anhydrases (CAs) catalyse CO_2 hydration and H_2CO_3 dehydration in the cytosol and chloroplast stroma.

To optimize photosynthetic C gain avoiding severe water stress, plants need to optimally regulate CO_2 and H_2O diffusion, and the C assimilation enzymes on both short- and long-term scales (Chavess, 1991; Fernández-Marín *et al.*, 2020). Important components of these optimal regulations within leaves are the construction costs (CC) and maintenance costs (MC) of the leaves, in additional to their respective functional attributes.

Cost–benefit analysis, originally developed in the field of economics, has been used as a useful tool to evaluate the costs vs. benefits arising from investment in various ecological possibilities. Natural selection is hypothesized to favour optimal investment patterns that maximize the fitness of an individual within a given environment (Bloom *et al.*, 1985), e.g. various resources such as nitrogen (N) are invested into the components of photosynthesis (such as Rubisco, photosystems and the factors contributing to diffusive transport of CO_2 into the leaf) to support C assimilation. When no single process in the leaf limits photosynthesis, the optimal investment pattern is such that the control over C assimilation is equally distributed among all component processes contributing to photosynthesis (Bloom *et al.*, 1985). The assimilated C is then used for the maintenance of the leaf and other organs such as stems and roots, the construction of new organs and the storage of essential fitness functions such as reproduction of the next generation, response to loss such as fire or herbivory, and defense (Table 1). In the case of resource allocation to the components of mesophyll conductance (g_m), too little allocation to leaf diffusive influx could hinder C assimilation by depriving Rubisco of essential CO_2, while too much allocation could divert resources from other essential functions without major enhancement of C assimilation. Worse, too much investment in g_m could have costs in terms of reduced leaf survival should drought occur.

Differences in leaf CC have been studied for almost five decades (Johnson and Tieszen, 1976). However, no reviews have explored the cost–benefit relationships in the context of CO_2 diffusion inside the leaf, i.e. the g_m which refers to the CO_2 diffusion conductance from the sub-stomatal cavity to the carboxylation site of Rubisco (Fig. 1). Because the chloroplast CO_2 concentration (C_c) is well below the CO_2 concentration in the intercellular air spaces (C_i) by 20–50 % (Niinemets *et al.*, 2009; Tosens *et al.*, 2012; Von Caemmerer and Evans, 2015), the value of g_m has a direct effect on the rate of photosynthetic carbon assimilation. More investment in g_m increases C_c and C assimilation, while less g_m reduces C_c. The relative costs required for g_m are discussed, as well as the derived benefits, to the degree to which the literature allows. To apply the cost–benefit analysis to our understanding of g_m, we have to evaluate both the cost required for each of the components determining g_m, and the benefit. Here, the immediate benefit means the increase in g_m and the increase in CO_2 supply to Rubisco, while longer term benefits could be enhanced survival, reproductive output and, ultimately, fitness. In this review, we assume that the net CO_2 assimilation rate at light saturation on a leaf area basis (A_{sat}), which strongly correlates with the leaf daily CO_2 assimilation rate (Hikosaka and Terashima, 1995; Terashima and Hikosaka, 1995; Li *et al.*, 2016), serves as an important proxy for the fitness gain from leaf investment into g_m. Thus, we consider A_{sat} the benefit and shall express it simply as A. With respect to costs, the available information on exacts costs of the components of g_m is lacking. Thus, we complement discussion of specific costs such as aquaporin and carbonic anhydrase (CA) investment with the discussion of CC and MC of leaves. In short, this review analyses relationships between the leaf CC of fixed (unadjustable) leaf traits, the MC related to rapidly adjustable leaf traits and their significance for g_m (Fig. 2). Our cost–benefit analysis on g_m also considers construction costs and g_m in diverse plant taxa under various environmental conditions.

TABLE 1. *The key concepts and terms used in this review*

Key concepts and terms	Definition in this review
Investment	Total carbon used to gain benefit.
Resource	Materials used for generating carbon gain and constructing the plant body (e.g. light, water, CO_2, nutrients, etc).
Cost	Carbon expenses used for maintenance and construction of present and new tissues.
Benefit	Carbon gain by photosynthesis (CO_2 assimilation rate).
Construction cost (CC)	Carbon expenses used for producing new tissues expressed as grams of glucose per unit area. Data derived from the literature were determined via chemical composition and heat of combustion methods.
Maintenance cost (MC)	Energy expenses per unit leaf area for maintaining existing tissues such as cellular homeostasis and protein turnover.
Leaf mass per area (LMA)	A morphological trait determined by leaf thickness and density related to anatomical tissue and chemical composition expressed as g m^{-2}.
Instantaneous cost–benefit relationship (*A*/CC)	CO_2 assimilation rate divided by CC as an indicator of how much carbon expenses are recovered on a short-term basis.

TABLE 2. *The key symbols and abbreviations used in the text*

Symbols and abbreviations	Definition
CA	Carbonic anhydrase
CC	Construction cost
C_c	CO_2 concentration at chloroplast stroma
δ_w	Mesophyll cell wall thickness
g_b	CO_2 diffusion conductance from air to the leaf surface. The inverse of resistance at the leaf boundary layer (R_b).
g_s	CO_2 diffusion conductance from the leaf surface to the intercellular air space. The inverse of resistance at the stomata (R_s).
g_m	CO_2 diffusion conductance from the intercellular air space to the chloroplast stroma. The inverse of resistance at the mesophyll (R_m).
g_w	CO_2 diffusion conductance in the cell wall. The inverse of cell wall resistance (R_w).
g_{pm}	CO_2 diffusion conductance across the plasma membrane. The inverse of plasma membrane resistance (R_{pm}).
g_{cy}	CO_2 diffusion conductance in the cytosol. The inverse of cytosolic resistance (R_{cy}).
g_{env}	CO_2 diffusion conductance across the chloroplast envelope. The inverse of chloroplast envelope resistance (R_{env}).
g_{st}	CO_2 diffusion conductance in the chloroplast stroma. The inverse of chloroplast stroma resistance (R_{env}).
LMA	Leaf mass per area
MC	Maintenance cost
PIP	Plasma membrane intrinsic protein (aquaporin)
S_c	Chloroplast surface area facing the intercellular air spaces per leaf area.
S_{mes}	Mesophyll cell surface area facing the intercellular air spaces per leaf area.

The contents of this review are as follows: (1) the concept of g_m and its CO_2 diffusion processes; (2) the concept of cost–benefit analysis used in this study; (3) cost–benefit analyses of long-term responses of g_m in response to environmental stress; (4) cost–benefit analyses of short-term responses of g_m including possible mechanisms of rapid g_m changes; (5) other factors affecting and/or masking the cost–benefit relationship of g_m; and (6) phylogenetic variation in CC and g_m.

WHAT IS MESOPHYLL CONDUCTANCE?

Mesophyll conductance, g_m, is the conductance for CO_2 diffusion from the sub-stomatal cavity to the chloroplast stroma, which affects CO_2 concentration in the chloroplast stroma (C_c), thus constraining A. CO_2 diffuses through the intercellular air space to the cell wall surface, where it dissolves in the cell wall water and diffuses through the cell wall, membrane, cytosol and chloroplast envelope, to finally enter the stroma where it is fixed by Rubisco (Fig. 1). To analyse the cost of each of the CO_2 diffusion processes, we summarize characteristics of the processes.

CO_2 and HCO_3^- concentrations in the liquid phase

From the cell wall to Rubisco, CO_2 diffuses in the liquid phase, and the resistance to CO_2 diffusion in the liquid phase is four orders of magnitude greater than that in the gas phase. According to Henry's law, CO_2 concentration in the liquid phase, $[CO_2]$, is proportional to the partial pressure of CO_2 in the adjacent gas phase:

$$H = \frac{[CO_2]}{P \cdot C_g} \quad (1)$$

where H is Henry's constant in mol m^{-3} Pa^{-1}, $[CO_2]$ is the CO_2 concentration in the liquid phase in mol m^{-3}, P is the atmospheric pressure in Pa and C_g is the CO_2 concentration in the gas phase in mol CO_2 mol^{-1}. Thus, $P{\cdot}C_g$ denotes the partial pressure of CO_2 in Pa. Assuming that Henry's constant is 0.33 mmol m^{-3} Pa^{-1} at 25 °C, $[CO_2]$ in an aqueous solution equilibrated with the air containing CO_2 at 41 Pa is 13.5 μM. Taking account of the effects of cellular solutes on chemical constants and coefficients, the chemical values used in this review are not for pure water but for a solution containing 5 g of sea salt kg^{-1}, which roughly corresponds to a 90 mM NaCl solution (Fig. 3).

$[CO_2]$ *per se* does not change with pH, whereas $[HCO_3^-]$ increases with the increase in pH. Assuming that $[HCO_3^-]$ is in equilibrium with $[CO_2]$, the ratio $[HCO_3^-]/[CO_2]$ is expressed by the Henderson–Hasselbalch equation:

$$\log \frac{[HCO_3^-]}{[CO_2]} = pH - pK_a, \quad (2)$$

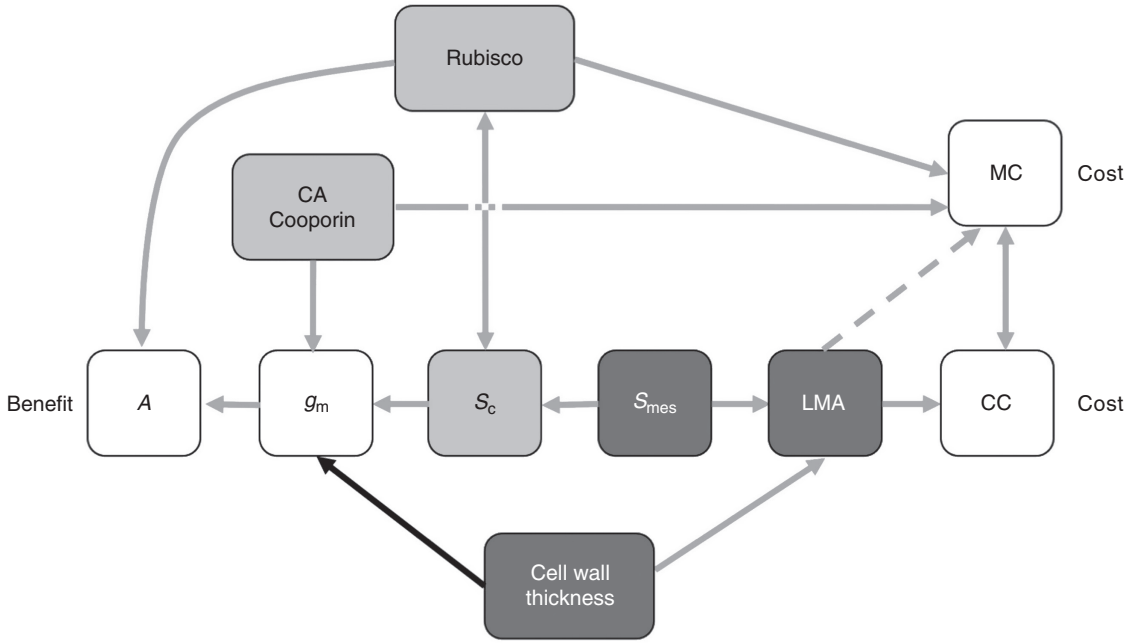

FIG. 2. The relationship between traits contributing to the benefit and costs of mesophyll conductance in C_3 plant leaves. Blue solid arrows indicate known positive effects; a blue dashed arrow indicates a possible positive effect; a red arrow indicate negative effects. Orange coloured variables are short-term responsive traits; blue coloured variables are long-term responsive traits. *A*, net CO_2 assimilation rate; g_m, mesophyll conductance; S_c, chloroplast surface area exposed to the intercellular air space; S_{mes}, mesophyll surface area exposed to the intercellular air space; LMA. leaf mass per area; MC, maintenance cost; CC, construction cost; CA, carbonic anhydrase.

where, K_a is the acidity constant expressed as:

$$K_a = \frac{[HCO_3^-]\cdot[H^+]}{[CO_2]}. \quad (3)$$

Cell wall conductance

CO_2 diffusion occurs preferentially along the shortest pathway from the cell wall surface to chloroplasts. Thus, it is useful to express the conductance for CO_2 diffusion across the cell wall (g_w) divided by the cumulated chloroplast surface area facing the intercellular air spaces per leaf area, S_c:

$$\frac{g_w}{S_c} = \frac{p \cdot D \cdot (1 + \alpha \cdot \kappa) \cdot H \cdot P}{\tau \cdot \delta_w} \quad (4)$$

where p is the porosity of the cell wall ($m^3\ m^{-3}$), D is the diffusion coefficient of CO_2 (see Fig. 3), α is the ratio of the diffusion coefficient of HCO_3^- to that for CO_2 (0.56), κ is the ratio $[HCO_3^-]/[CO_2]$, τ is the tortuosity of the path through the cell wall ($m\ m^{-1}$) and δ_w is the cell wall thickness (m). p expresses how much space in the cell wall is used for CO_2 diffusion, while the tortuosity, τ, expresses how much the path is lengthened or convoluted in the cell wall. The pH of the apoplast liquid is typically 5 (Aloni *et al.*, 1988). Since the pK_a of carbonic acid is 6.06, the corresponding κ for the cell wall solution is 0.087. p/τ may range from 0.02 to 0.05 (calculated by the authors based on the data in fig. 5 in Evans *et al.* (2009) and fig. 2 in Terashima *et al.* (2011); see also Evans (2021) and the section below: 'Cost–benefit of short-term responses of g_m'). Typically, in annual herbs, such as *Helianthus annuus* and *Phaseolus vulgaris*, and broad-leaved trees, *Acer negundo* and *Quercus ilex*, the contributions of the wall resistance ($1/g_w$) to overall mesophyll resistance ($1/g_m$) ranges from 5 % to 70 % (Tomás *et al.*, 2013; Carriquí *et al.*, 2021). The contribution of $1/g_w$ is greater in thick leaves than in thin leaves due largely to wall thickness and tortuosity. Thicker and denser walls increase CC while reducing g_w and, in turn, C_c and C assimilation.

Cell membrane

It was once believed that CO_2 permeates lipid bilayers rather easily (Gutknecht *et al.*, 1977). However, since the plasma membrane contains considerable amounts of proteins and sterols (Yoshida and Uemura, 1986), the conductance is much lower than that of the artificial lipid bilayer. Although the data are not abundant, at least some aquaporins transport CO_2 across membranes and thus enhance g_m (Groszmann *et al.*, 2017; see 'Cost–benefit of short-term responses of g_m'). According to Tomás *et al.* (2013) and Carriquí *et al.* (2021), the contribution of the resistance across the plasma membrane ($1/g_{pm}$) to $1/g_m$ is around 6 %, comparable with that of the chloroplast envelope ($1/g_{env}$). These values increase when the membrane porins are downregulated. For further considerations of these calculations, see 'Cost–benefit of short-term responses of g_m'.

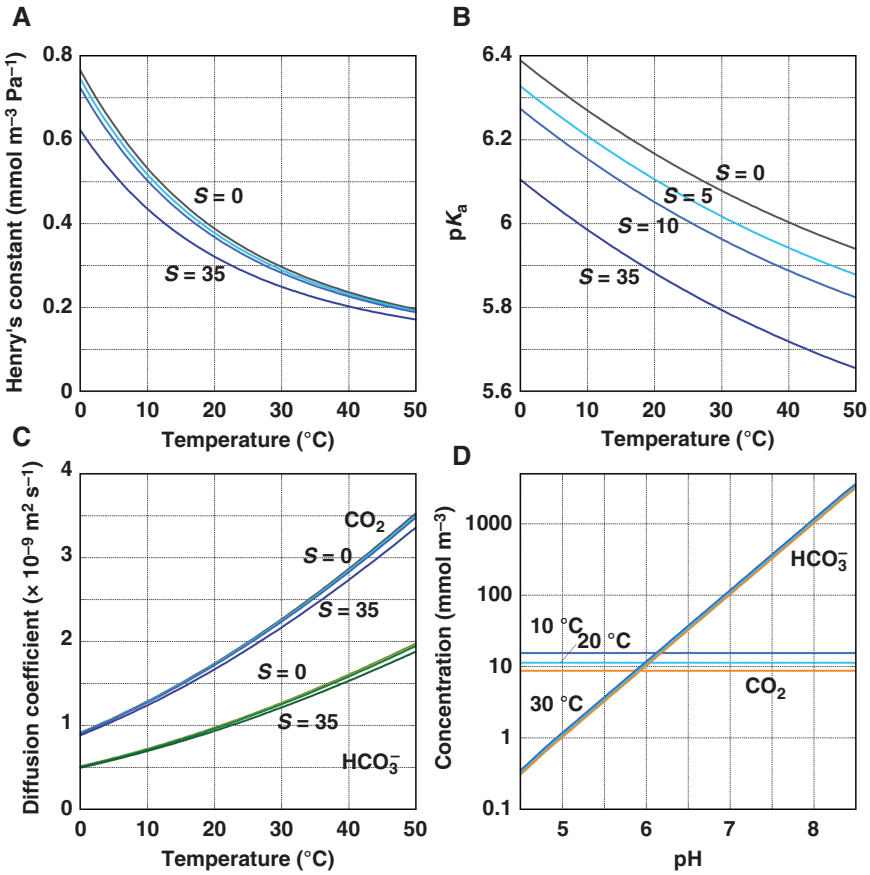

FIG. 3. Effects of temperature and solute concentration on Henry's constant for CO_2 (A), pK_a of carbonic acid (B), diffusion coefficients of CO_2 and HCO_3^- (C) and effects of temperature and pH on CO_2 and HCO_3^- concentrations (D). The average seawater contains 35 g of salts kg^{-1}, which is designated as 35 *S* and roughly corresponds to 0.6 M NaCl solution. In (A), (B) and (C), the values for 0 *S*, 5 *S*, 10 *S* and 35 *S* are shown, while the values for 5 *S* are shown in (D). The values used in the main text are those for 5 *S* containing roughly 90 mM NaCl. Salinity relationships are from the following oceanography studies: (A) Teng *et al.* (1996); (B) Mojica Prieto and Millero (2002); (C) Ratcliff and Holdcroft (1963) and Sharqawy *et al.* (2010), and (D) Zeebe (2011). For (A), the density of 35 *S* seawater was assumed to be 1.024. In (D), the values for 5 *S* in (A) and (B) were used assuming the air containing 30 Pa CO_2 (as a proxy of intercellular CO_2 partial pressure) equilibrated with the 5 *S* solution. These relationships using seawater demonstrate how solutes in living tissue can affect CO_2 and HCO_3^- availability.

Cytosol and chloroplast stroma

Diffusion of CO_2 and HCO_3^- in the cytosol and chloroplast stroma is also described by eqn (4). The thickness of the cytosol varies from 0 up to 0.5 µm. In the cytosol, p/τ approaches 1, while D is somewhat lowered because the solute concentration and viscosity of the cytosol are greater than those of the apoplast water (see Fig. 3). The typical pH of the cytosol is 7.4. Then, κ is 21.9, and $(1 + \alpha\kappa)$ is $(1 + 0.56 \times 21.9) = 13.3$.

Chloroplast thickness ranges from 1 to 2 µm and the Rubisco content per chloroplast surface area ranges from 0.1 to 0.4 µmol m^{-2} (Terashima *et al.*, 2011). The volume of Rubisco protein would then be 5–30 % of the stroma volume. Thus, p/τ is considerably smaller than 1 and may be as low as 0.6. At the typical pH of the stroma in the light (i.e. pH = 8.0), κ is 87.2 and $(1 + \alpha\kappa)$ is $(1 + 0.56 \times 87.2) = 49.8$. D is also lower than that in water and comparable with that in the cytosol. The conductance for CO_2 diffusion in the stroma would not limit photosynthesis greatly because the sum of CO_2 and HCO_3^- concentrations is large. For the effects of viscosity of the stroma due to concentrated Rubisco, the pH of the cytosol and chloroplasts stroma, and the roles of CAs, see 'Cost–benefit of short-term responses of g_m'.

As recent studies employing limitation analyses confirmed, the most important anatomical factors that limit g_m are cell wall thickness (δ_w) and chloroplast surface area exposed to the intercellular air space (S_c) (Peguero-Pina *et al.*, 2017; Carriquí *et al.*, 2019). δ_w is a typical fixed factor largely determined during tissue development, S_c is also often regarded as being fixed but changes considerably on the short-term scale due to chloroplast movement (Tholen *et al.*, 2008). Some biochemical factors such as pH of the compartments and membrane permeability are also adjustable on the short-term scale during the day.

CONSTRUCTION COST AND MAINTENANCE COST RELATED TO LEAF ANATOMICAL TRAITS

The CC of a leaf is about 160 and 420 times larger than the MC per day in *Quercus prinus* (deciduous tree) and *Eucalyptus tetrodonta* (evergreen tree), respectively (Eamus and Prichard, 1998; Nagel *et al.*, 2002). However, when both are expressed on the basis of leaf life span, the respective costs may be

comparable (Deans *et al.*, 2020). To express CC, Williams *et al.* (1987) proposed the use of glucose as the energy equivalence unit. This is now widely used, including in this review, because it is directly comparable with the benefit defined as C gain. The ratio of CC to the daily C assimilation rate has been used as an important parameter for estimation of the payback time for any investment possibility (Eamus and Prichard, 1998; Nagel *et al.*, 2002). Increasing MC through fine adjustment of cellular functions is necessary for the leaf to quickly cope with environmental variation and thereby maximize daily C gain. For example, leaves with high N contents pay a high MC to maintain protein function, as would be needed for repair of photosystem complexes damaged by high light (Eamus and Prichard, 1998). Traits such as A, dark respiration rate and leaf life span vary widely among plant species, and tend to be intercorrelated, reflecting the underlying costs as measured by CC and MC (Reich *et al.*, 1997; Nagel *et al.*, 2002; Wright *et al.*, 2003).

One of the main anatomical factors determining g_m is the mesophyll surface area facing the intercellular air space, S_{mes}, which strongly correlates with leaf mass per area (LMA), the most frequently measured leaf trait. The LMA differs across species and varies in response to environmental factors such as light intensity, water availability, temperature and CO_2 concentration (Niinemets, 2001; Poorter *et al.*, 2009). To begin our analysis of costs associated with g_m, we first searched the literature for studies reporting leaf CC as grams of glucose per unit area as well as photosynthetic and leaf morphological traits; however, papers reporting both CC and A are scarce. In our review of these papers, we observed a strong positive correlation between CC and LMA across plant functional types (Supplementary data Figs S1 and S2; Table S1). Thus, we can estimate CC from LMA. Since LMA is often reported together with A and g_m, there are many examples to exploit for analysing the relationships between g_m and CC.

Adaptative strategies to recover the investment are diverse in land plants depending on their functional types and/or phenological groups, and enhancement of A by increasing g_m may be a vital strategy to recover the investment in the leaf (Flexas *et al.*, 2015). How much should plants invest in CC and MC to realize an optimal g_m to maximize A? In the following sections, we investigate these costs of g_m and discuss how plants adjust g_m in various environments.

COST–BENEFIT ANALYSIS OF LONG-TERM RESPONSES OF G_M UNDER DIFFERENT ENVIRONMENTS

Plants often adjust leaf thickness in response to varying environmental conditions, such as light intensity, temperature, water availability and nutrient availability (Björkman, 1981; Boese and Huner, 1990; Poorter *et al.*, 2009; Oguchi *et al.*, 2018), each of which can affect g_m and CC. Since acclimation of leaf structure to the growth light intensity is particularly well documented (Terashima *et al.*, 2001, 2006; Yano and Terashima, 2001; Hoshino *et al.*, 2019), we start our discussion with the effects of light, then proceed to those of water and CO_2.

Light

In response to high light, leaves are thickened and thus exhibit high LMA, often accompanied by increases in S_c, g_m and A (Björkman, 1981; Hanba *et al.*, 2002; Poorter *et al.*, 2019). It has been proposed that sun leaves are thicker than shade leaves to accommodate more chloroplasts over the mesophyll cell surface, which is needed for the higher A per leaf area (Terashima *et al.*, 2001). Having a higher amount of Rubisco per leaf area without increasing S_c would diminish the photosynthetic efficiency by decreasing the CO_2 concentration in the chloroplast stroma (C_c) (Terashima *et al.*, 2011). Therefore, the leaf responses to light environments reflect strong correlations between Rubisco content, S_c and A (Oguchi *et al.*, 2003, 2005). If the mesophyll cell surface area is already occupied by chloroplasts ($S_c/S_{mes} \cong 1$), S_{mes} needs to be greater to allow for more chloroplasts, which then increase A. To increase S_{mes}, plants need to increase total cell wall volume, which also directly enhances leaf CC (Fig. 4 of the CC–g_m or CC–A relationship). In general, S_{mes} is strongly correlated with LMA for leaves of the same species from various light environments (e.g. Oguchi *et al.*, 2008). It is theoretically possible to increase S_{mes} without additional CC through thinning cell walls, but such a response has not been reported, probably because a thinning cell wall reduces leaf mechanical strength and herbivory tolerance. Leaves, particularly in herbaceous and deciduous woody species in high light, often show high S_c/S_{mes}, which would be adaptive for achieving the highest g_m for a given CC. This is because complete coverage of S_{mes} by chloroplasts would realize the shortest and widest CO_2 diffusion pathway to Rubisco and thus the highest g_m and C_c (Hanba *et al.*, 2001; Oguchi *et al.*, 2003, 2005; Onoda *et al.*, 2017). In some species, such as *Acer rufinerve*, *A. mono* and *A. japonicum*, even fully expanded leaves with high S_c/S_{mes} developed in low light increased leaf thickeness, S_{mes} and S_c upon transfer to high light (Oguchi *et al.*, 2005, 2006), which clearly indicates that incurring CC for increasing S_{mes} is necessary to increase g_m in the new, high-light environment.

The instantaneous cost–benefit ratios (A/CC) are similar between leaves grown at high light and low light in deciduous trees (Fig. 5, filled symbols), which suggests that the δ_w does not change much with changing light (Hanba *et al.*, 2002; Tosens *et al.*, 2012). However, this is not true for herbaceous plants (Fig. 5, open symbols). For example, high-light-grown *Nicotiana tabacum* (tobacco) leaves have about 2.1 times higher g_m than those grown in low light, but CC increased by only 1.7-fold from low to high light (Yamori *et al.*, 2010). On the other hand, high-light-grown *Arabidopsis thaliana* showed about 1.8 times higher g_m than those grown in low light, but CC increased 3-fold in both mature and young leaves in high light (Carriquí *et al.*, 2021). Although g_m tends to increase with the increase in CC, the proportions are different in herbaceous plants. In the case of *A. thaliana*, cell walls were thickened in high light: mesophyll cell wall thicknesses in young and mature leaves were 0.137 μm and 0.197 μm in low light, and 0.208 μm and 0.260 μm in high light, respectively. This would be one of the causes of the decrease in A/CC with the increase in growth light level. Overall, how much of the CC allocated to increase S_c, S_{mes} and cell wall thickness in response to light intensity

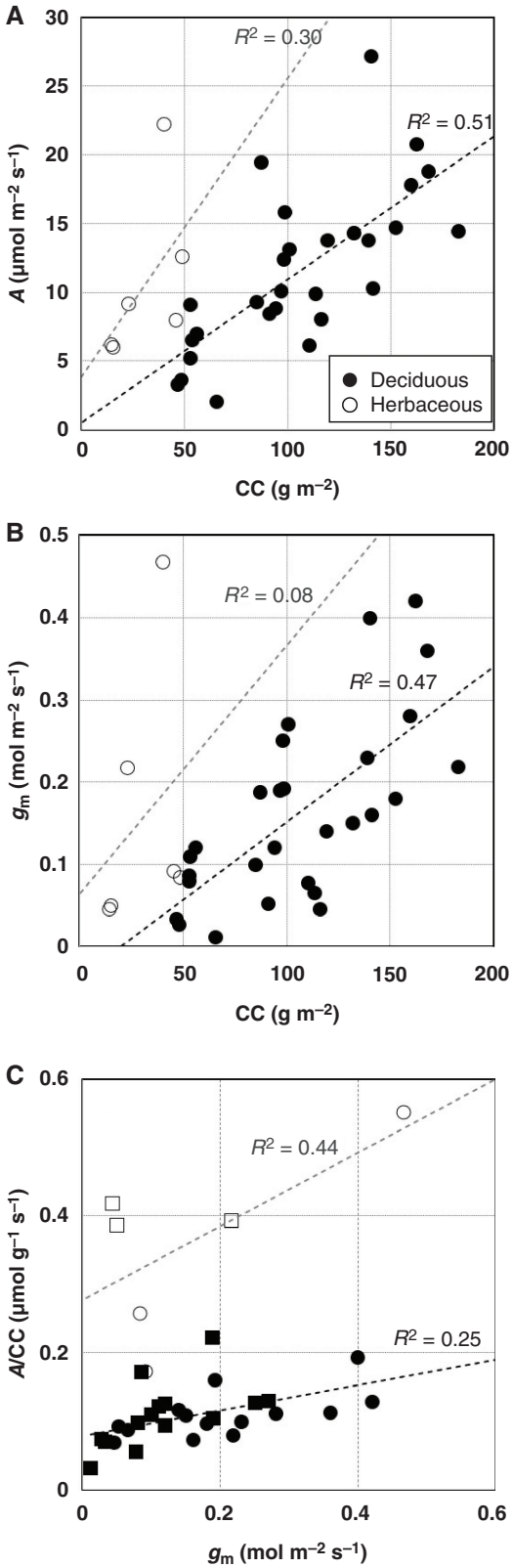

FIG. 4. Correlations among A, g_m, CC and the instantaneous cost–benefit relationship (A/CC) in plants cultivated under different light levels. (A, B) Filled and blank circles indicate deciduous and herbaceous species, respectively. (C) Circles and squares indicate high- and low-light-grown plants, respectively. Datasets are compiled from Hanba *et al.* (2002), Piel *et al.* (2002), Yamori *et al.* (2010), Cano *et al.* (2011), Tosens *et al.* (2012), Cano *et al.* (2013) and Carriquí *et al.* (2021). All datasets are listed in Supplementary data file S1. Parts of the datasets were extracted from the figures using Web Plot Digitizer (Rohatgi, 2021).

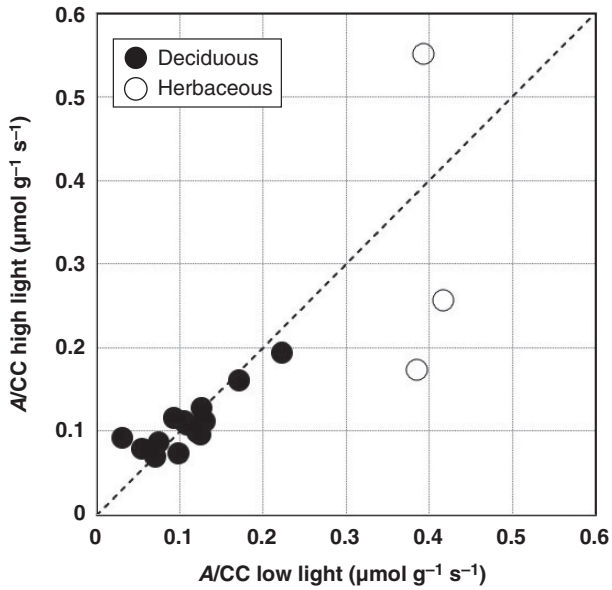

FIG. 5. The relationship between the A/CC of plants grown at high and low light levels. Datasets are the same as Fig. 4. Supplementary data file S1.

determines whether plants can realize appropriate g_m in order to gain sufficient benefit. While the thickened cell wall apparently has negative effects on g_m and thus lowers the immediate benefits in terms of C gain, it might also be part of a wider strategy to recover CC by extending leaf life span and pest resistance through producing tougher leaves (see ‘ Other factors affecting and/or masking the cost–benefit relationship of g_m’).

Water

Plants acclimated to water stress usually develop leaves of high LMA due to greater leaf mesophyll density and/or thickness (Niinemets, 2001; Medrano *et al.*, 2009; Niinemets *et al.*, 2009; Galmés *et al.*, 2011). The LMA also increases due to progressive accumulation of non-structural carbohydrates (NSCs), typically starch (Virgona and Barlow, 1991). Water stress usually decreases g_m in trees and herbaceous plants over both the short and long term (Galmés *et al.*, 2007; Miyazawa *et al.*, 2008; Warren, 2008; Cano *et al.*, 2013; Mizokami *et al.*, 2015) but increases CC, unlike high light where both g_m and CC increase. How would these contrasting drought responses of g_m and CC be advantageous to the plant? To clarify this point, we further analyse detailed mechanisms of drought responses of key components affecting g_m, although the mechanisms for g_m reduction in droughted leaves are still controversial, especially for short-term responses. The decrease in g_m in response to long-term drought stress is often accompanied by increases in δ_w and declines in S_c (Tosens *et al.*, 2012; Galmés *et al.*, 2013; Ouyang *et al.*, 2017). The thickened cell wall and the increased mesophyll density increase CC, while reduction in S_c lowers g_m and A. The decrease in S_c could be linked with decreases in the chloroplast number in a mesophyll cell, which could lower MC; however, reduction in MC is probably small. Tosens *et al.* (2012) showed that water stress induced by polyethylene glycol application decreased g_m by 43 % and increased CC by 9 % in *Populus*

tremula grown in high light. As *A*/CC decreased by 36 %, there were no apparent benefits of these physiological responses to water stress. In *Triticum aestivum* (wheat), g_m decreased and S_c increased in response to severe water stress (Ouyang *et al.*, 2017). The increase in S_c would typically enhance g_m, but in wheat it was not enough to maintain g_m and *A* under water stress conditions. Cell wall composition can also change in response to water stress. For example, cellulose content in *Vitis vinifera* (grapevines) and lignin content in *Helianthus annuus* (sunflower) leaves both increase under water stress conditions (Roig-Oliver *et al.*, 2020*a*, 2020*b*). The increase of these cell wall components is costly, but it is ambiguous whether the increase is beneficial in the short term. At least, the increase in lignin content has been considered to decrease g_m (see 'Cost–benefit analysis of short-term responses of g_m').

Overall, it appears that plants invest considerable CC and MC in response to long-term water stress. However, they cannot gain enough short-term benefit through increasing g_m, because the cell wall thickening under water stress is relatively more expensive than the benefits of greater g_m. From a wider point of view, the ultimate benefit to the plants of thicker walls could be improved drought survival, which could be realized at the expense of other functions such as g_m and CC.

CO_2

Growing C_3 herbaceous plants in elevated CO_2 usually increases LMA and CC (Mizokami *et al.*, 2019*a*, *b*; Jahan *et al.*, 2021). *A* generally increases in elevated growth CO_2, unless photosynthesis is not downregulated by sink limitation or nutrient deficiency (Ellsworth *et al.*, 2004; Mizokami *et al.*, 2019*b*). *A*/CC is increased by elevated CO_2 in species with no photosynthetic downregulation such as wheat (Jahan *et al.*, 2021) and *Eucalyptus tereticornis* (Crous *et al.*, 2020). On the contrary, in *A. thaliana*, *A*/CC was decreased by photosynthetic downregulation due to the increases in cell wall thickness and NSC. These contrasting responses of photosynthetic downregulation that affect *A* and CC need to be considered to discuss *A*/CC in elevated growth CO_2. The former, cell wall thickening, has a substantial negative impact on g_m and *A*/*CC*. The latter, high NSC, suppressed *A* through reducing Rubisco content and/or activity (Drake *et al.*, 1997; Moore *et al.*, 1999; Rogers and Ellsworth, 2002). The high NSC, however, would exert no direct effect on g_m (Mizokami *et al.*, 2019*b*). Although these two responses both cause photosynthetic downregulation, the effect on leaf costs and g_m, and potential reversibility after the elevated CO_2 treatment, are different between the two responses. When the CO_2 level decreases, thickened cell walls are not easily reversed, while accumulated NSC rapidly declines, indicating that the cell wall thickening represents irreversible CC, but NSC accumulation may be regarded as a reversible cost affecting MC.

These contrasting CO_2 responses depending on the species might be due to a trade-off between CO_2 and other resources or the degree of sink limitation. When the sink activities of *Glycine max* and *Phaseolus vulgaris* were suppressed by defoliation of young leaves, *A* was downregulated due to a thickened cell wall and decreased g_m in a manner similar to that occurring in *A. thaliana* in elevated CO_2 (Sugiura *et al.*, 2020). The sink strength is a key to determine whether high CO_2 is beneficial to plants by bringing about the increase in *A* without decreasing g_m, especially in the long term.

COST–BENEFIT OF SHORT-TERM RESPONSES OF G_M

When a plant kept in the dark is illuminated, g_m is initially low and increases gradually over 20–30 min (Sakoda *et al.*, 2021). Conversely, in response to various stresses, g_m can decrease within a few minutes (e.g. Mizokami *et al.*, 2015, 2019*a*). In this section, we first examine the CO_2 diffusion processes. Then, we consider factors potentially responsible for short- to medium-term changes of g_m, and the costs and benefits associated with these dynamic changes.

Cell walls

The diffusion coefficient of CO_2 in water (D) is 1.97×10^{-9} m^2 s^{-1} at 25 °C, much slower than that in air, where the value is 1.56×10^{-5} m^2 s^{-1}. Because HCO_3^- is bulkier than CO_2, the diffusion coefficient of HCO_3^- in water is 56 % of the coefficient for CO_2 (Kigoshi and Hashitani, 1963). Thus, the diffusion coefficient for the total inorganic C is expressed as $D \times (1 + 0.56\ \kappa)$, where κ is $[HCO_3^-]/[CO_2]$. When the pH in the apoplastic solution is acidic, e.g. 5.0, κ is 0.087 and we can assume that inorganic C species in the cell wall is virtually all CO_2. However, when nitrate is abundant in the xylem sap or the soil is dry, the pH of the apoplast might increase to neutral levels (Jia and Davies, 2007). When the pH of the apoplast solution is increased to 7.0, then κ equals 8.7, and the wall conductance would increase by $(1 + 0.56 \times 8.7)$ or 5.9-fold. The presence of CA in mesophyll cell walls has not been reported (Tiwari *et al.*, 2005; DiMario *et al.*, 2017; Ignatova *et al.*, 2019), and hydration of CO_2 in the absence of CA is slow (see below). Thus, even when the pH in the apoplast is 7, κ might be effectively lower than 8.7. Still, g_m would increase slightly due to higher pH. Values of tortuosity (τ) and/or porosity (p) would also change with pH due to ionization/protonation of various residues and side groups. Because the pK_a for galacturonic acid is 3.2 (Haas *et al.*, 2021), carboxyl groups in pectin (see below) are ionized at physiological pH. However, carboxyl groups of cell wall proteins would be ionized only at high pH, and cross-linked by divalent cations, which might decrease p (Tazawa *et al.*, 2022). Unfortunately, there have been no experimental studies that examine the effects of apoplast pH or of ionic composition on g_m using artificial xylem sap.

Cell walls mainly consist of cellulose microfibrils, hemicellulose (xyloglucans and/or rhamnoglucans) and pectin (polyuronates such as polygalacturonate) (Cosgrove, 2016), which may correspond, respectively, to reinforcing bars, cross-linkers of the reinforcing bars and 'plastic' cement of a reinforced concrete wall. Cell walls of tracheary elements and fibres in leaf veins are lignified, and those of bundle sheath cells and bundle sheath extension cells are often lignified (Ohtsuka *et al.*, 2018). Recently, Flexas *et al.* (2021) reviewed studies examining the relationships between cell wall composition and g_m, where cell wall compositions were changed genetically or by growth environments. Lignin contents in the leaves of sunflower, altered by various water stress conditions during growth,

were negatively related to g_m (Roig-Oliver *et al.*, 2020*a*; Flexas *et al.*, 2021). When lignin is deposited, p could decline due to the formation of cross-linkages with small molecules such as ferulate (Grabber, 2005). However, in these studies, whether mesophyll cell walls were lignified was not evaluated. The content of pectin could also be negatively related to g_m (Clemente-Moreno *et al.*, 2019), because pectin would lower p (Flexas *et al.*, 2021). However, pectin is most abundant in the middle lamellae of the cell walls between adjacent mesophyll cells. Thus, increases in pectin content could result from the greater cell–cell contact in the mesophyll, which would reduce g_m by decreasing S_{mes}. The overall reduction in g_m from pectin itself could be low, unless pectin also accumulates in the cell wall facing the air spaces. The abundance of pectin in the cell walls directly exposed to the intercellular space should thus be evaluated. Cytochemical examinations should be useful.

Cytosol and chloroplast stroma

Equation (4) can also evaluate inorganic C diffusion into the cytosol and chloroplast stroma. In these compartments, the viscosity of the solution is thought to be important. However, it should be noted that diffusion of small molecules, such as CO_2, is not affected much by the bulk viscosity increased by macromolecules such as proteins (Ratcliff and Holdcroft, 1963; Hoshino and Sato,1967). The diffusion is only affected by the viscosity brought about by small solutes. Tholen and Zhu (2011) assumed that viscosities of the cytosol and chloroplast stroma are two and ten times that of pure water (0.89×10^{-3} Pa s), respectively, and that the diffusion coefficient of CO_2 is inversely proportional to the viscosity of the solution as indicated by the Stokes–Einstein relationship (Einstein, 1905; Ratcliff and Holdcroft, 1963). However, the viscosities assumed by them would be the bulk viscosities. We propose that the presence of Rubisco in the stroma in the order of 10 % (v/v) is regarded as a factor affecting p/τ in eqn (4). Diffusion coefficients of CO_2 and HCO_3^- *per se* would not be lowered very much by the viscosity caused by small solutes in the stroma (for the effects of concentration of small solutes on CO_2 and HCO_3^- diffusion, see Fig. 3C).

One potential cost of g_m in cells pertains to the size and positioning of chloroplasts in C_3 mesophyll cells. In the mesophyll tissue of C_3 plants, chloroplasts tend to be relatively small and thin, and occur in high numbers appressed against the plasma membrane, forming a sheath of chloroplast that can cover >90 % of the cell periphery facing the intercellular spaces (Stata *et al.*, 2014, 2016). This arrangement minimizes the diffusion distance from the intercellular air spaces to the stroma, which is important given that the diffusion through the liquid of the cytosol is 10 000 times slower than that through air. Maintaining this chloroplast position could result in substantial MC, especially under fluctuating light conditions which could require repositioning of the chloroplast to track light exposure. As an example of the costs of the C_3 arrangement, consider the pattern in C_4 plants. Here, mesophyll chloroplasts lack Rubisco, and carboxylation occurs in the cytosol. Instead of a near continuous sheath of chloroplasts covering the mesophyll cell periphery, C_4 plants have fewer chloroplasts than C_3 plants, by around 50 % on average, and they are often larger and can be recessed from the mesophyll cell periphery (Stata *et al.*, 2014, 2016). The relaxed form of C_4 chloroplasts appears to be a more economical arrangement than in C_3 plants, reflecting a functional imperative where light harvesting is the priority rather than chloroplast position to maximize S_c and g_m.

Aquaporin, cooporin and aqua-cooporin

Aquaporins in the cell membrane are called PIPs (plasma membrane intrinsic proteins) and are divided into two subfamilies, PIP1 and PIP2 (Luang and Hrmova, 2017; see reviews by Maurel *et al.*, 2015, Groszmann *et al.*, 2017, Kapilan *et al.*, 2018, and a comprehensive monograph edited by Chaumont and Tyerman, 2017). Their monomers are 28–30 kDa and form not only homotetramers but also heterotetramers (Jozefkowicz *et al.*, 2017). *Nicotiana tabacum* AQP1 (= NtPIP1;2) is also reported to be expressed in the chloroplast envelope (Uehlein *et al.*, 2008). PIPs are not merely the pores for H_2O, but may also transport CO_2, and thus it is probable that their gating is responsible for rapid changes in g_m. We previously proposed to call aquaporins that facilitate CO_2 diffusion, cooporins (Terashima *et al.*, 2006). The meaning of this term is two-fold: first, coo (=CO_2) + porin and second, the channel can co-operate with CA to move inorganic C into the cell. Here, we propose the use of cooporins for PIPs that are channels for CO_2 but channel little to no H_2O, and aqua-cooporins for PIPs that are permeable to both H_2O and CO_2. Most PIPs that have been reported to transport CO_2 are aqua-cooporins (Groszmann *et al.*, 2017). There are some candidates for cooporins: PIP1;2 of *A. thaliana*, PIP1;2 of *N. tabacum* and PIP1;1 of *Helianthemum almeriense* (Groszmann *et al.*, 2017). However, in recent studies in which AtPIP1;2 was knocked out in *A. thaliana* (Kromdijk *et al.*, 2020) or overexpressed in *N. tabacum* (Clarke *et al.*, 2022), g_m and A were unaffected, implying that AtPIP1;2 may not be a cooporin. If there are cooporins with nil activity of H_2O permeation, the CO_2 permeation can be regulated independently of the regulation of water permeation in other PIPs. Also, CO_2 permeation of aqua-cooporins may be regulated differentially from water permeation.

A theoretical study employing dynamic simulations of the membrane-embedded bovine AQP1 homotetramer and a ligand sampling protocol indicates that CO_2 passes through the central pore of the tetramer or side pores located between AQP1 monomers, while H_2O passes through the central pore of each monomer (Wang *et al.*, 2007). Thus, in aqua-cooporins, CO_2 permeation could be regulated differently from H_2O permeation. In *Hordeum vulgare* (barley) PIP2s, Ile254 has been identified to be responsible for CO_2 permeation. The native PIP2;4 (Met254) did not transport CO_2, while a site-directed mutant of PIP2;4 (Met254Ile) did transport CO_2 (Mori *et al.*, 2014). Because this site-directed mutagenesis modifies the shape of a central pore of the monomer, CO_2 would pass through the central pore of the monomer in HvPIP2s. If the CO_2 pathway is only the central pore of the monomer in these barley aqua-cooporins, regulation of CO_2 permeation independent of that of H_2O would not be possible. However, as mentioned above, CO_2 permeation through different pathways might be possible in the barley aqua-cooporins as well.

Because aqua-cooporins and cooporins can control g_m, then they would directly determine the costs and benefits of g_m control. Greater investment in PIPs could increase g_m, but at what cost? Here, we present a simple exercise to assess protein costs of PIP investment in membranes, which in turn could influence both CC and MC. We evaluate the cost of all PIPs given the uncertainty in the relative abundance of cooporins; however, the analysis indicates low costs overall. Our reasoning is as follows: about 40–50 % of the weight of the plant cell membrane is proteins and, in spinach leaves, 15–20 % of the cell membrane proteins are PIPs (Johansson *et al.*, 1996). We can suppose that two-thirds of the area of the cell membrane is occupied by membrane lipids with a specific density of 0.9 g cm^{-3} (Noureddini *et al.*, 1992). Assuming that the thickness of the lipid bilayer is 6 nm, then the weight of membrane lipids is 3.6 mg m^{-2}. This roughly agrees with the value, 2.8 mg m^{-2}, calculated assuming that the membrane area occupied by a diacylphosphatidylcholine molecule (750 g mol^{-1}) is about 0.6 nm^{-2} (Petrache *et al.*, 2000). We further assume that the proteins occupy a third of the plasma membrane area and their average thickness is 8 nm, considering the large protein complexes protruding from the membrane surfaces, and that the specific density of the protein is 1.37 g cm^{-3} (Erickson, 2009). The membrane protein content is then estimated to be 3.7 mg m^{-2}. If 15 % of the cell membrane proteins is PIPs, it would be 0.56 mg m^{-2}. The density of the PIP tetramer (28 kDa × 4) is calculated to be 3000 μm^{-2} of the plasma membrane. Considering the total area of cell membranes is around 30 times that of the leaf area in sun leaves (Terashima *et al.*, 2001), the weight of PIPs per leaf area amounts to about 17 mg m^{-2}. Well-fertilized spinach leaves have about 2–3 g Rubisco m^{-2}, which typically accounts for 20–30 % of total leaf protein in C_3 leaves (Terashima and Evans, 1988; Hikosaka and Terashima, 1995). Thus, PIPs comprise about 0.6–0.9 % of the Rubisco fraction, and just a few-tenths of a percentage of leaf N. As already mentioned, *N. tabacum* AQP1 (= NtPIP1;2) is reported to be expressed in the chloroplast envelope (Uehlein *et al.*, 2008). There have been some biochemical/proteome studies reporting the presence of PIPs in chloroplast envelopes and tonoplast intrinsic proteins (TIPs) in the envelope and thylakoids (Gao *et al.*, 2018). If chloroplasts have abundant PIPS/TIPS, the total cost of the porin function could be greater than the above calculation that only took account of PIPs in the plasma membrane.

Estimation of the CC and MC of PIPs

As outlined above, the activities of PIPs are controlled in various ways. It is understandable that water movement through membranes is strictly regulated to be able to direct water flow and to prevent localized desiccation of mesophyll cells. It is not enough to tightly shut the stomata in order to save water given the consequences for loss of C acquisition, particularly if intercellular air spaces are undersaturated, as discussed by Rockwell *et al.* (2022) in this issue. However, if the CO_2 flux into mesophyll cells is regulated independently from that of H_2O, there would be no reason for suppression of CO_2 permeability, and thus one might envisage high constitutive expression of cooporins, unless cooporins are particularly expensive to build and maintain. To address this issue, it is necessary to first identify cooporins and aqua-cooporins and determine their contents in leaf tissues. However, the results of CO_2 permeation assays are often controversial depending on the assay systems.

For the first step, we consider the cost of PIPs in a leaf over the course of a day. As we have already calculated, the abundance of plasma membrane PIPs may be >10 mg m^{-2} of leaf area, which corresponds to 2.2×10^{17} PIP monomers. Each monomer has about 280 amino acids. Synthesis of a protein needs about ten ATPs per amino acid (Noguchi *et al.*, 2001). Thus, 1 mmol of ATP is needed for *de novo* synthesis of 10 mg of PIPs. Given that 30 mol of ATP is produced when 1 mol of glucose is respired, in a leaf respiring 1 μmol CO_2 m^{-2} s^{-1}, the ATP production rate is about 5 μmol of ATP m^{-2} s^{-1}. Thus, a complete turnover of plasma membrane PIPs could require the entire leaf respiratory ATP production for 200 s (3.3 min). Assuming chloroplast PIPs turn over at the same rate, the respiratory costs could be higher in direct proportion to PIP content in the chloroplast membranes. Although there are no quantitative data of abundance of PIPs in the chloroplast envelope, the density of immunogold labelling of NtPIP1;2 for the envelope was comparable with that for the plasma membrane (Uehlein *et al.*, 2008). Since the presence of other PIPs in the envelope has not been confirmed, the respiratory costs for the envelope PIP may not be very large. While these energy costs of PIPs in the plasma membrane and chloroplast envelope may not seem high, there are many proteins which also turn over during the day and require ATP to do so. Thus, the relative cost of PIP turnover could come at the expense of other essential functions and, by doing so, may require mechanisms to economically regulate PIP expression. Temporal internalization of PIPs could be one means to reduce synthesis costs, because PIPs could be internally stored rather than resynthesized (see below). PIP internalization has been observed in roots responding to salinity stress (e.g. Kaneko *et al.*, 2015). However, in relation to g_m, the abundance of cooporins and aqua-cooporins and the associated CC and MC would be much smaller than that of total PIPs, so the overall PIP cost associated with CO_2 permeation may be relatively low unless their gating function also has significant metabolic costs, as discussed next.

Gating of PIPs would be responsible for g_m *downregulation*

Drought treatment decreases g_s and g_m. The abscisic acid (ABA) synthesis mutant with a constitutively low ABA level, *aba1* of *Nicotiana plumbaginifolia*, showed no decline in g_s and g_m in response to drought, whereas addition of ABA to the petiole xylem rapidly decreased g_s and g_m (Mizokami *et al.*, 2015). In leaves of *Spinacia oleracea* (spinach), SoPIP2;1 is deactivated by dephosphorylation under drought conditions (Johansson *et al.*, 1996). X-ray structures of phosphorylated and dephosphorylated SoPIP2;1 have been analysed to clarify the detailed gating mechanisms (Törnroth-Horsefiel *et al.*, 2006). Although whether SoPIP2;1 is an aqua-cooporin has not been known, if it is and if CO_2 is transported through the pore in each monomer, the downregulation of g_m could be due to its dephosphorylation. Because ABA triggers drought

responses, we hypothesize its mode of action for g_m decline is via downregulation of activities of cooporins and/or aqua-cooporins. This has not been shown for any mesophyll PIPs, but a close example in support of the hypothesis can be found in stomata. In the cell membrane of stomatal guard cells, AtPIP2;1 is phosphorylated in response to application of ABA by Open Stomata 1 protein kinase (OST1). Upon phosphorylation, water permeability of the guard cell membrane is enhanced and facilitates water efflux from the guard cell, leading to rapid stomatal closure (Grondin *et al.*, 2015). Furthermore, Wang *et al.* (2015) showed that AtPIP2;1 is an aqua-cooporin and responsible for enhanced SLAC1 anion channel activity in response to high CO_2. In this function, βCA4, closely associated with PIP2;1, plays an important role. To monitor the CO_2 level, CO_2 transport activity of AtPIP2;1 should be always on, while the water transport activity is activated by phosphorylation. This could be the first example of differential regulation by ABA/phosphorylation of CO_2 and H_2O transport activities of an aqua-cooporin. Regulation modes of PIPs by phosphorylation/dephosphorylation could be different depending on the tissue. Further, it is also probable that each of the PIPs in the same cell is regulated differently, for example by different kinases. Taken all these together, it is probable that the rapid regulation mechanisms of aqua-cooporins by dephosphorylation in the mesophyll cell membrane contribute to rapid decreases in g_m in response to ABA/drought as seen in SoPIP2;1. Since phosphorylation/dephosphorylation is a central mechanism of regulation of PIP activity, the ATP cost of PIP phosphorylation could substantially increase MC over the course of a day.

Internalization of PIPs

In leaves, g_m could be regulated by internalization of cooporins and/or aqua-cooporins as indicated by results observed in salt-stressed roots. Kaneko *et al.* (2015) examined porin behavior in barley roots in response to NaCl stress. Although there were significant differences in the salt tolerance among the varieties, short-term NaCl stress for a few hours caused internalization of PIPs, with the PIPs moving from the plasma membrane to the intracellular space as vesicle-like structures. When NaCl was replaced with water, the PIPs returned to the plasma membrane. When the NaCl treatments were prolonged, the PIPs were degraded by the ubiquitin system. Indeed, internalization of AtPIP2;1–green fluorescent protein (GFP) was observed in the leaves of *A. thaliana* in response to an NaCl treatment, whereas application of ABA did not induce internalization (M. Carriquí and Y. Sakai, unpubl. obs.). If such a mechanism exists in the mesophyll cells, plants can reduce MC by internalization of aquaporins rather than turnover of aquaporins in response to environmental variables.

Here, we conduct a simple simulation to estimate how much impact the downregulation of cooporins/aqua-cooporins might have on g_m, and in turn CO_2 concentration in the chloroplast (C_c). Based on the dataset of Carriquí *et al.* (2021), the contributions of the plasma membrane and chloroplast envelope resistances ($1/g_{pm}$ and $1/g_{en}$) to overall mesophyll resistance ($1/g_m$) are both 6 %. When cooporins/aqua-cooporins located at the plasma membrane and chloroplast envelope are downregulated, these membrane resistances become larger by about three times according to Terashima *et al.* (2006), in which, based on the inhibition of g_m by the $HgCl_2$ treatment, they estimated that two-thirds of CO_2 passing through the membrane was via cooporins and/or aqua-cooporins. The downregulation of cooporins results in an 18 % decrease in g_m and then a 7 % decrease in C_c and A. Note that we assume constant g_s in this case. Because Carriquí *et al.* (2021) assumed that the viscosity in the chloroplast stroma is three times that of pure water, and that the increase in $[HCO_3^-]$ in the stroma is negligible, the contribution of the chloroplast stroma resistance ($1/g_{st}$) to $1/g_m$ is as high as 70 %. Given that the diffusion coefficient of CO_2 is little affected by the bulk viscosity brought about by abundant proteins, and that $[HCO_3^-]/[CO_2]$ approaches >80 at the stromal pH in the light, the share of $1/g_{st}$ would be much smaller than 70 % and the contribution of other components such as $1/g_{pm}$ and $1/g_{en}$ to $1/g_m$ would be greater than in the present estimation. If so, the impact of the downregulation of cooporins/aqua-cooporins on g_m and A would be more substantial. Since the impact of g_m decline on C_c can be large, the rapid regulation mechanisms of g_m need to be clarified if a comprehensive cost–benefit accounting of g_m is to become possible.

Carbonic anhydrases

Along the diffusion path from the cell wall surface facing the intercellular space to the stroma, the pH changes several times. When the inorganic C species passes across the cell membrane and chloroplast inner envelopes, irrespective of whether the path is through cooporins, aqua-cooporins or the lipid bilayer, the inorganic C species that permeates these membranes would be exclusively CO_2. When the apoplast is acidic, there is little conversion of inorganic C species at the outer surface of the plasma membrane. However, at the inner surface of the plasma membrane, conversion of CO_2 to HCO_3^- occurs. At the outer surface and the inner surface of the chloroplast envelope, conversions of HCO_3^- to CO_2 and CO_2 to HCO_3^- occur. In the absence of CA, these processes are slow (Stumm and Morgan, 1995). Thus, for rapid conversions, CAs are indispensable. By using interconversion of CO_2 and HCO_3^-, plants can create a second, complementary pathway for C influx into the cell and chloroplast, enhancing g_m, but at the cost of CA synthesis (Evans and von Caemmerer, 1996).

Carbonic anhydrase is the second most abundant cellular protein in green leaves after Rubisco, comprising 1–2 % of total soluble protein in spinach (Okabe *et al.*, 1984). It has been shown that the plasma membrane, cytosol, and chloroplast stroma and thylakoids have CAs (for reviews, see Tiwari *et al.*, 2005; DiMario *et al.*, 2017; Ignatova *et al.*, 2019). For example, the presence of βCA at the cytosolic face was shown in several species including spinach and wheat (Utsunomiya and Muto 1993). In *A. thaliana*, AtβCA2 and AtβCA4 are in the plasma membrane (Fabre *et al.*, 2007). In tobacco, the orthologue of AtβCA2 and aquaporins are concentrated in the lipid rafts (Mongrand *et al.*, 2004).

These CAs including βCA1 in the stroma had been thought to facilitate hydration of CO_2 and dehydration of H_2CO_3 (HCO_3^- + OH^-) near Rubisco. The rate constants of hydration of CO_2 and dehydration of H_2CO_3 in pure water at 25 °C are 0.025–0.04 s^{-1} and 10–20 s^{-1} (Stumm and Morgan, 1995).

$$CO_2 + H_2O \underset{k_{H_2CO_3}}{\overset{k_{CO_2}}{\rightleftharpoons}} H_2CO_3 \overset{\text{very fast}}{\rightleftharpoons} H^+ + HCO_3$$

Let us assume C_c is 8 μM. When the pH in the stroma is 8, $[HCO_3^-]/[CO_2]$ is 87. Then, the corresponding inorganic C would be 700 μM and the leaf with S_c of 30 and stroma thickness 2 μm contains 42 μmol inorganic C m^{-2} leaf area. This much inorganic C is photosynthesized in 1–2 s. In the absence of CA, the hydration occurs only slowly and thus the CO_2 concentration gradient between the stroma near the envelope and the carboxylation site of Rubisco would be large and the stroma would impose considerable resistance to inorganic C diffusion. Thus, at least some CAs are needed in the stroma, particularly just inside the envelope. Considering the hydration and dehydration rate constants of CA (both $>10^5$ s^{-1} at physiological pH; Pocker and Ng, 1973; Pocker and Miksch, 1978), only some CA activity could be sufficient for CO_2 hydration and H_2CO_3 dehydration in most situations. The dehydration process is fast even in the absence of CAs, but the conversion is greatly accelerated by CAs.

Wild-type *N. tabacum* plants were transformed with antisense constructs directed against transcripts encoding βCA1 (Majeau *et al.*, 1994; Price *et al.*, 1994). Primary transformants with 1–2 % of the CA activity of the wild plants showed no significant differences in *A*, Rubisco activity and chlorophyll content relative to wild-type plants. This was confirmed by a similar study using an antisense line of chloroplastic CA in *N. tabacum* (Williams *et al.*, 1996). Although the knockout lines of βCA1 of *A. thaliana* showed some defects at the seedlings stage, the mature plants showed no phenotypic differences (Ferreira *et al.*, 2008). This has been also confirmed using the double knockout lines of βCA1 and βCA5 by CRISPR/Cas9 in *N. tabacum* (Hines *et al.*, 2021). However, these results do not mean that CA activity is not needed. The stroma αCA1 and thylakoid CAs would contribute to both hydration and dehydration (Ignatova *et al.*, 2019). The role of abundant βCA1 in mature leaves is not known, although several functions including pH regulation have been suggested (Ignatova *et al.*, 2019). The turnover of CAs has also not been well studied, although *in vitro* experiments show that CA activity is stable (Tiwari *et al.*, 2005). Expression of CA is affected by environmental conditions. For example, it is enhanced in high light, and thus can potentially contribute to higher g_m following increased light during growth (DiMario *et al.*, 2017; Ignatova *et al.*, 2019).

Because the abundance of CA, i.e. 1–2 % of leaf soluble proteins in spinach leaves (Okabe *et al.*, 1984), is one order of magnitude greater than that of PIPs, more energy is needed to construct and maintain CAs. Again, given that only a part of CA activity contributes CO_2 hydration and H_2CO_3 dehydration, the costs in relation to g_m should be low. These considerations indicate that the benefits well outweigh the costs of increasing g_m, but the costs associated with the large reduction in CO_2 concentration in the stroma relative to the intercellular air spaces remain uncertain.

Future studies

At this stage, it is not possible to precisely estimate the benefit to g_m by short-term regulation of PIPs and other biochemical factors such as CA, pH and membrane trafficking. Short-term decreases in g_m have been observed, indicating that biochemical control probably exists at the level of PIPs and other g_m contributors (e.g. Xiong *et al.*, 2015; Mizokami *et al.*, 2019*a*; Sakoda *et al.*, 2021). To date, the mechanisms of g_m downregulation have not been comprehensively addressed, although the gating and/or internalization of PIPs are plausible as the candidates of downregulation. We envisage that future techniques in cell biology will enable estimation of cooporin/aqua-cooporin roles in g_m regulation, as well as other potential controls such as CA, thereby leading to improved assessments of the associated costs and benefits (Evans, 2021).

OTHER FACTORS AFFECTING AND/OR MASKING THE COST–BENEFIT RELATIONSHIP OF G_M

The positive correlation between CC and g_m tends to disappear when datasets for different species and those for different growth conditions are combined. Plants face the trade-off problem between CO_2 gain and acquisition or maintenance of other resources depending on the growth conditions, e.g. severe drought conditions. Looking at g_m from the cost–benefit viewpoint, the maintenance of higher g_m should provide the obvious benefits. However, most plants emphasize drought survival above C gain on the long-term scale, reflecting that the ultimate cost–benefit ratio of importance is the fitness benefits passed onto the next generation. For example, previous studies have shown that in response to drought, plants decrease stomatal density and stomatal size to reduce transpiration rate and, by doing so, increase water use efficiency (WUE*: A/E or A/*g_s) (Xu and Zhou, 2008; Doheny-Adams *et al.*, 2012; Bertolino *et al.*, 2019). In this situation, where saving water is more impactful for fitness than C gain, plants adjust cost–benefit trade-offs to reduce water consumption. In such situations, g_m could also decrease, particularly when the strategy for water saving leads to thicker walls and other features which increase internal resistance to diffusion. As an example, consider investment in leaf venation, whose costs can be considerable. The increase in the vein length per unit leaf area (VLA) scales with leaf hydraulic conductance (K_{leaf}), and often enhances g_s and *A* (Brodribb *et al.*, 2007; Brodribb and Feild, 2010). Having high VLA, or vascular redundancy in the sense of Sack *et al.* (2008), would be an effective strategy to optimize leaf investments for drought tolerance, but at the expense of greater CC. These traits are closely linked to g_m. In detail, K_{leaf} consists of hydraulic conductance inside the xylem (K_x) and outside the xylem, notably in the apoplast and symplast along the mesophyll and bundle sheath tissue (K_{ox}). VLA is often associated with K_x, while g_m is closely associated with K_{ox}, because the CO_2 diffusion pathway into a leaf is similar to the water diffusion pathway outside the xylem (Nardini *et al.*, 2012; Flexas *et al.*, 2013; Xiong *et al.*, 2017; Sonawane *et al.*, 2021). This close relationship between K_{ox} and g_m might indicate that an increase in K_{ox} is indispensable for improving g_m. A key factor for this relationship could be the PIPs, which play similar roles in balancing CO_2 gain against H_2O loss, as evident in stomatal guard cells (Wang *et al.*, 2007; Berry *et al.*, 2010). High investment in PIPs, however, could be a challenge for maintaining leaf water status. In addition to the slight N cost of high PIP investment, overexpression of PIPs could disrupt the

ability to regulate internal leaf water status, particularly if the intercellular air spaces are undersaturated (see Rockwell *et al.*, 2022 and Šantrůček, 2022 in this issue for a discussion of undersaturation). As understanding of PIP types and their investment increases, we should be in a stronger position to properly integrate PIP investment with other aspects of leaf structure to better understand costs vs. benefits as they pertain to g_m.

From the responses of plants to long-term elevated CO_2, we can judge whether plants favour less H_2O loss or more CO_2 gain. In general, in response to elevated CO_2, plants close stomata on the short-term scale and reduce stomatal density of leaves through leaf development on the long-term scale; both responses reduce water loss and increase WUE (Ainsworth and Rogers, 2007). For responses of g_m to elevated CO_2, aquaporins and cooporins/aqua-cooporins may be the main players to determine whether plants will emphasize less H_2O loss or more CO_2 gain in changing CO_2 conditions. However, there are few studies to guide our understanding of the possibilities. In *Solanum lycopersicum* (tomato), expression levels of most PIPs were downregulated upon exposure to elevated CO_2, which corresponded to decreases in g_s and K_{leaf} (Fang *et al.*, 2019; Li *et al.*, 2021). These responses indicate that tomato plants at elevated CO_2 emphasize less H_2O loss. We may speculate that WUE increases due to the enhanced A at elevated CO_2, even when g_m decreases; however, this may require reconsidering assumptions of water vapour saturation in the leaf interior. On the other hand, Xu *et al.* (2019) demonstrated that the expression level of *OsPIP1;2* was upregulated under elevated CO_2 in *Oryza sativa* (rice) and that overexpression of this PIP gene increased g_m and A. Unfortunately, the authors only showed g_m at ambient CO_2 and did not show g_m at elevated CO_2 in the wild type and the overexpression lines. However, judging from previous reports showing a rapid decrease in g_m in response to high CO_2 (Xiong *et al.*, 2015), g_m should have declined at elevated CO_2 in rice. Despite the higher expression of *OsPIP1;2*, the g_m decline could be due to other mechanisms such as a decrease in S_c or alterations in the apoplastic environment on short-term scales (pH or porosity changes, for example), and increasing wall thickness on long-term scales.

For plants with long leaf life span, leaf mechanical strength and herbivory tolerance can be more important. Increase in δ_w can also contribute to leaf mechanical strength via an increase in tissue density and LMA (Onoda *et al.*, 2017). Therefore, species with a long leaf life span tend to have thicker cell walls at the expense of g_m and the negative correlation between LMA and g_m can be seen in the interspecific variation of leaf traits (Onoda *et al.*, 2017). However, these negative correlations between LMA and g_m do not mean that the CC for increasing

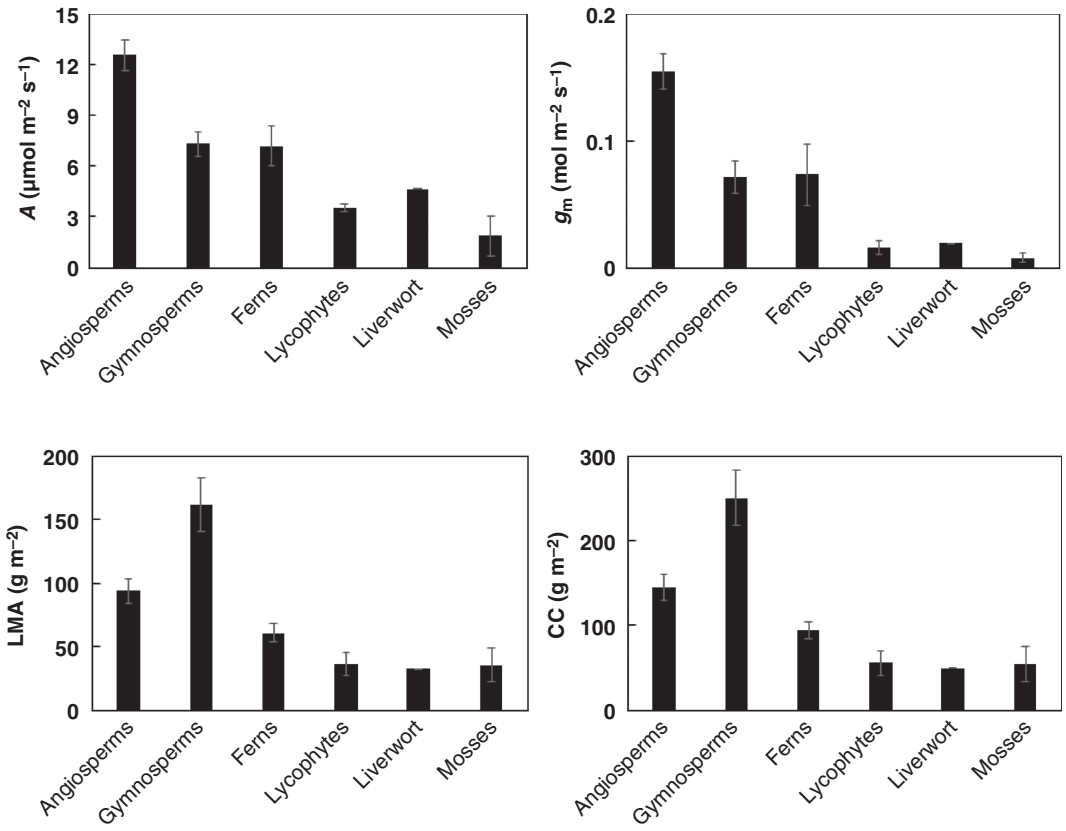

FIG. 6. Comparative analyses of A, g_m, LMA and CC in different phylogenetic groups using data from Maxwell *et al.* (1997), Hanba *et al.* (1999, 2001, 2002), De Lucia *et al.* (2003), Flexas *et al.* (2006), Priault *et al.* (2006), Galmés *et al.* (2007), Miyazawa *et al.* (2008), Tholen *et al.* (2008), Perez-Martin *et al.* (2009), Hassiotou *et al.* (2009), Tosens *et al.* (2012), Tomás *et al.* (2013), Carriquí *et al.* (2015, 2019, 2020), Xiong *et al.* (2015, 2018) and Mizokami *et al.* (2019*a*, *b*). Parts of the datasets were extracted from the figures using Web Plot Digitizer (Rohatgi, 2021). Data include the following phylogenetic types; angiosperms (n = 90), gymnosperms (n = 12), ferns (n = 9), lycophytes (n = 3), liverwort (n = 1) and moss (n = 6). Data represent the average ± s.e. All datasets are listed in Supplementary data file S1.

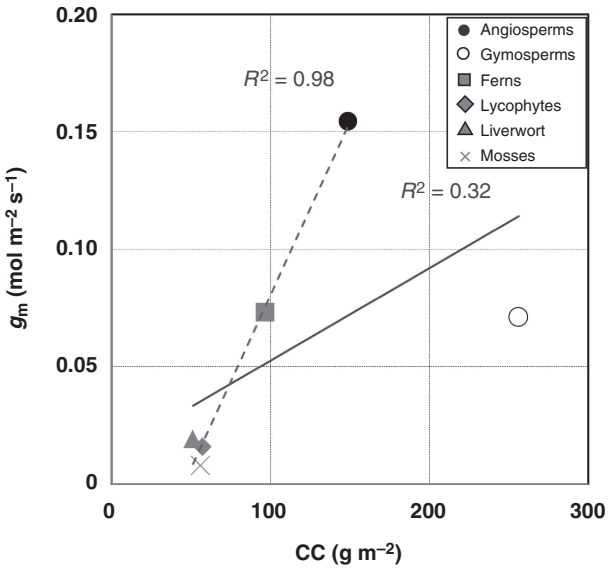

FIG. 7. Correlation between CC and g_m in different phylogenetic groups. Data points represent averages of each group. The solid line is a linear regression line for all groups, and a dotted line is a linear regression line for groups except a data point of gymnosperms. Data include the following phylogenetic types: angiosperms (n = 90), gymnosperms (n = 12), ferns (n = 9), lycophytes (n = 3), liverwort (n = 1) and mosses (n = 6).

g_m is unnecessary. As already discussed, the increase in S_{mes} is definitely needed for increasing g_m, while plants also increase S_{mes} for reasons other than increasing g_m. For example, some evergreen and vine species have leaves with very low S_c/S_{mes} (Oguchi *et al.*, 2006; Onoda *et al.*, 2017), which is considered to enhance light scattering inside the leaves leading to more light absorption in shaded environments. As such, the increase in S_{mes} would have multiple functions and thereby the higher S_{mes} does not necessarily lead to the higher g_m, especially when S_{mes} of various species are compared. This example demonstrates how a comprehensive understanding of fitness costs and benefits in general can facilitate understanding of the relative contribution of g_m investment to the overall fitness landscape on which natural selection operates.

To increase total chloroplast volume for the increase in S_c, plants need to invest N (Oguchi *et al.*, 2003; Zhu *et al.*, 2020), because photosynthetic proteins require high N costs (Mooney and Gulmon, 1979; Farquhar *et al.*, 1980; Hikosaka and Terashima, 1995) Also, a certain amount of C cost is needed simultaneously to increase S_{mes}, which would lead to an increase in S_c and thus g_m. The importance of C cost relative to N cost changes depending on the growth conditions: N cost would limit plant growth under high light and low N availability, while C cost would limit plant growth under low light and high N availability (Sugiura and Tateno, 2011). In both cases, it is possible that g_m investment cannot be sufficient to realize the maximize benefit for A. Sugiura *et al.* (2020) reported that low N availability decreases S_c/S_{mes} in leaves of soybean and French bean, which leads to a decrease in g_m per CC. This means that severe N conditions can mask the positive correlation between CC and g_m. Under severe conditions where other resources such as phosphorus and potassium are limited, the cost of these resources becomes important (Verhoeven *et al.*, 1996; Wright *et al.*, 2011). For example, g_m increases with increases in leaf P concentration in *Gossypium hirsutum* (cotton) (Singh *et al.*, 2013; Singh and Reddy, 2016), although the underlying mechanisms still remains unclear. Further research is needed to carry out a cost–benefit analysis of g_m that takes phosphorus and potassium into account in addition to N.

PHYLOGENETIC DIFFERENCES IN CC AND G_M

There have been several reports showing the trends of g_m across plant life forms, functional groups and phylogenetic groups (Flexas *et al.*, 2008, 2012; Carriquí *et al.*, 2015). In this review, we observed more obvious trends of g_m across the phylogenetical groups than those across the plant life forms and functional groups (Fig. 6; Supplementary data Figs S2 and S3). Although CC and g_m across phylogenetical groups are not strongly correlated, when the data of gymnosperms are excluded, the correlation becomes much stronger (Fig. 7). Gymnosperms showed higher LMA relative to the other phylogenetic groups due to their higher leaf density and thickness on average (Veromann-Jürgenson *et al.*, 2017). These characteristics are due to exceptionally thick cell walls, and the low S_c/S_{mes} ratios. Therefore, if the cell walls constitute a major part of the leaf dry mass, CC would be large and the benefit would be low due to a low g_m. Instead of their small short-term benefit in terms of C assimilation potential, gymnosperms generally have longer leaf life span, except for some deciduous conifers such as *Larix* and *Taxodium* (Kikuzawa, 1991; Reich *et al.*, 1999). Long-lived leaves indicate that gymnosperms could recover costs of their greater leaf investment over time, such that they retain a favourable cost–benefit ratio (Reich *et al.*, 1995, 2007). According to our dataset, gymnosperm leaves show roughly doubled CC and half the potential A compared with angiosperm leaves. Thus, by simply extending the leaf life span by four times, the initial investment could be recovered. Although further investigation is needed, higher g_m due to thinner cell walls and lower leaf CC might be one of the major strategies in angiosperms, allowing for greater A, yet lower initial investment. In terms of overall fitness, this strategy would be beneficial in favourable environments; however, where environmental stress is high, higher LMA may be necessary to minimize leaf injury, but at the cost of reduced g_m.

CONCLUDING REMARKS

We reviewed the leaf cost–benefit relationships focusing on the g_m. The LMA, the most measured leaf trait, is the main determinant of leaf CC, and it varies within plant species and in response to environmental variables through changing mesophyll cell density and cell wall thickness. g_m is one of the factors determining A as the benefit and is significantly affected by leaf morphological traits that determine leaf CC. It would seem that further analyses are needed to answer the question of whether g_m attains a reasonable value to recover CC. Studies on leaf life span and leaf ageing processes are necessary for the complete evaluation. Relationships between leaf MC and g_m are fairly complex, but a detailed estimation or simulation of MC of pH

homeostasis and of regulation of protein functions, especially those of PIPs in a different time scals (e.g. day/night, per day, leaf life span), could be a key to understand how plants optimize trade-offs between H_2O and CO_2 fluxes, and the numerous resistances in their respective diffusion pathways.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: results of ANOVA to clarify the similarity of two regression lines between two different CC estimation methods. Figure S1: relationship between leaf mass per area and leaf construction cost. Figure S2: comparison of two different CC estimation methods, the chemical composition method and heat of combustion method. Figure S3: comparative analyses of A, g_m, LMA and CC in different functional types. Figure S4: comparative analyses of A, g_m, LMA and CC in different life forms. Data file S1: Excel file containing a list of references and datasets used for the analyses of LMA and CC, and photosynthetic characteristics and CC.

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