Title: Mesophyll diffusion conductance to CO$_2$: an unappreciated central player in photosynthesis

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Abstract

Mesophyll diffusion conductance CO$_2$ is a key photosynthetic trait that has been studied intensively in the past years. The intention of the present review is to update the knowledge on $g_m$, and highlight the important unknown and controversial aspects that require future work. The photosynthetic limitation imposed by mesophyll conductance is large, and under certain conditions it can be the most significant photosynthetic limitation. New evidence shows that anatomical traits, such as cell wall thickness and chloroplast distribution are amongst the stronger determinants of mesophyll conductance, although rapid variations in response to environmental changes might be regulated by other factors such as aquaporin conductance.

There are a number of gaps in knowledge that should be major research priorities for the near future include, how different is mesophyll conductance among phylogenetically distant groups and how has it evolved? can it be uncoupled from the water path regulation? what are the main drivers of mesophyll conductance? The need for mechanistic and phenomenological models of mesophyll conductance and its incorporation in process-based photosynthesis models is also highlighted.
1. Introduction

Photosynthesis in plants has been considered for decades to be limited only by two factors: the velocity of diffusion of CO$_2$ through stomata and the capacity of photosynthetic machinery in leaves to convert light energy to biochemical energy and fix CO$_2$ into sugars. While diffusion is a passive physical process, it can be subject to large regulation in plants. According to Fick's law, diffusion depends on substance (e.g. CO$_2$) diffusivity, which differs depending on temperature but also on the nature (mainly viscosity) of the media in which diffusion occurs (e.g. water, air, etc.), and the distance of diffusion. Since the mesophyll pathway consists of a complex array of ‘physical barriers’ to CO$_2$ diffusion, including air, cell walls, lipid membranes and liquid cytoplasm and stroma, differing both in nature and size (i.e. 'distance'), there is a large variation among leaves in diffusion conductance to CO$_2$ in the mesophyll ($g_m$), and the latter can be regulated by changing either the distance or the nature of (part of) the diffusion pathway inside leaves.

Early studies already suggested that the diffusion of CO$_2$ from sub-stomatal cavities to the sites of carboxylation inside chloroplasts could limit photosynthesis (e.g., [1-3]), which has become more evident after several methods for the estimation of $g_m$ have become more available – including a method based on $^{13}$C-discrimination during photosynthesis[4], a method combining chlorophyll fluorescence and gas exchange measurements [5, 6] and model-based methods [6-8]. For details on methods for $g_m$ estimation, the required precautions when using them and specific strategies of adjustment, see refs [9], [10], [11]. Thus, although during decades the majority of researchers considered $g_m$ large enough not to significantly limit photosynthesis, it has become now evident that that $g_m$ is the third major player in the process of photosynthesis, together with stomatal conductance and biochemical capacity.

The current understanding on $g_m$ has been recently reviewed [12]. In addition, specific reviews on the mechanisms regulating $g_m$ [13], and on the ecophysiologival and ecological
significance of $g_m$ [14-16] have been published. These papers are recommended as the best introduction to the importance of $g_m$ in plant physiology. As there has been rapid gain in understanding of $g_m$, the aims of the present paper are: (1) to update information of current knowledge accumulated after the recent reviews; (2) to discuss the most obscure / controversial aspects on $g_m$ function and regulation, such as its response to CO$_2$, or how much it limits photosynthesis; and (3) to highlight the obvious gaps in knowledge on this subject and the future research needs.

2. How different is $g_m$ among phylogenetically distant groups and how have mechanisms controlling $g_m$ evolved?

The rate of diffusion conductance to CO$_2$ in the mesophyll ($g_m$) has now been estimated for more than 100 species, and it is now possible to search for phylogenetic / evolutionary patterns. The vast majority of estimates of $g_m$ are for Spermatophytes [14] (angiosperms and gymnosperms), with only very few data for liverworts and hornworts [17]. Most surprisingly there are no measurements available for phylogenetically intermediate groups such as mosses, lycophytes, equisetophytes, or ferns. This constitutes a serious gap in our knowledge that precludes driving any broad conclusion as for the evolution of mechanisms controlling $g_m$.

Some valuable information can nevertheless be obtained by comparing the existing data for liverworts and hornwort gametophytes with those for Spermatophytes belonging to different phylogenetic and/or functional groups (Fig.1). At first sight, it is evident that there are variations in the average rate of $g_m$ among different plant groups, and that these variations are more closely correlated with evolutionary advancements than stomatal conductance ($g_s$). The largest values for both conductances are found among non-woody angiosperms, whereas grasses present somewhat higher $g_m$ values than annual dicots (Fig. 1). The lowest values are found in liverworts and
hornworts for which \( g_s \) is set as zero as they lack stomata, and \( \text{CO}_2 \) has to diffuse through the cuticle and epidermis. Among Spermatophytes, conifers show the lowest values. Since angiosperms are evolutionary more recent than gymnosperms and non-Spermatophytes (the earliest fossil records for conifers dating back to 290 Myr as compared to 200 Myr for angiosperms) and grasses represent a relatively recent evolutionary event (ca. 70 Myr), an evolutionary trend towards larger \( g_m \) than \( g_s \) values is plausible. Accordingly, gas exchange capacity of Angiosperms was greatly increased following the Cretaceous period in association with leaf morphological changes [18].

A closer inspection reveals that significant differences also appear among angiosperms as a function of their functional type or growth form. Hence, herbaceous plants show the highest values, followed by semi-deciduous and deciduous shrubs and trees, while the lowest values are found in evergreen shrubs and trees, similar to those displayed by gymnosperms. Therefore, at least, part of the observed variation may not reflect evolutionary trends but is simply the result of adaptations to particular growth forms and/or environments, e.g., thicker leaves having lower \( g_m \) (see next section). Conversely, in some of the groups displayed in Fig. 1, the number of species included is low and biased. For instance, there are only three genera within grasses (monocots) for which \( g_m \) has been determined (\textit{Triticum, Oryza} and \textit{Phragmites}), while herbaceous genera are dicots. Hence, currently available data do not allow distinguishing patterns between monocots and dicots, or separating the effects of life form and phylogenetic position.

In the case of hornworts and liverworts, estimates of \( g_m \) are five orders of magnitude smaller than for Spermatophytes (Fig. 1 inset). Despite the fact that hornworts and liverworts lack stomata and show a low degree of cuticularization, they may contain some kind of cuticle or procuticle whose conductance could affect \( g_m \) estimates. However, the conductance to \( \text{CO}_2 \) of the thicker and more developed cuticle of higher plants is already around 2.5 \( 10^{-4} \) mol m\(^{-2} \) s\(^{-1} \) [19], i.e., still an order of magnitude larger than \( g_m \) estimated in Bryophytes [17]. This evidence suggests
that hornworts and liverworts present a truly restricted $g_m$, which may explain their slow growth. Again, this strongly suggests the occurrence of an evolutionary trend towards higher $g_m$, although knowledge of $g_m$ values of intermediate groups – i.e. mosses and ferns – may be necessary to confirm such a trend. It is remarkable that values found for bryophytes are similar to the lowest values of CO$_2$ permeabilities reported for biological membranes [13]. It is not known whether this means that internal CO$_2$ diffusion in tissues of early land plant forms depends on simple diffusion through membranes without facilitating agents such as aquaporins. Of the genes encoding for proteins possibly facilitating $g_m$ in higher plants (see section 3), no aquaporin (searched as putative PIP1 family - i.e. Plasma membrane Integral Protein - gene) or carbonic anhydrase has been described for hornworts, liverworts, lycophytes, equisetophytes or ferns, and only two putative genes for PIP1 but up to 13 for carbonic anhydrase have been described in mosses (www.ncbi.nlm.nih.gov/genbank/). However, this does not necessarily mean that these genes/proteins do exist is these groups, as very few data are available in databases for the genome sequences of these species groups. Moreover, in these groups the importance of carbon concentrating mechanisms involving carbonic anhydrases, pyrenoids, carboxysomes, etc. probably mask the importance of $g_m$-related components.

In summary, there is a significant gap in knowledge concerning phylogenetic/evolutionary trends in $g_m$. To overcome this gap it may be necessary to compile data for monocot species of families other than Poaceae as well as for lower forms such as lycophytes, equisetophytes, ferns and mosses. These data should be measured under the same environmental and developmental conditions to derive phylogenetic implications from a trait like $g_m$, which is under strong environmental control. This knowledge will help understanding the evolution of $g_m$, the mechanisms involved and the extent of co-variation of $g_m$ and $g_s$. 
3. New evidence as for the main determinants of $g_m$

3.1. Changing the nature of the diffusing molecule: carbonic anhydrases

CO$_2$ molecules passing from sub-stomatal cavities to chloroplasts diffuse through gas-phase in leaf intercellular air spaces, liquid phase in cell walls, cytosol and chloroplast stroma and lipid phase in plasmalemma and chloroplast envelope membranes (Fig. 2). The rate of diffusion through the composite segments of the diffusion pathway depends on the effective thickness and diffusivity of each component section [16]. “Effective” denotes the circumstance that the diffusion path length is generally longer than the linear distance from sub-stomatal cavities to chloroplast due to tortuosity and/or limited porosity of the diffusion pathway [20]. Diffusion coefficients for CO$_2$ in free water, tabulated in physical chemistry handbooks cannot be directly applied to leaves due to presence of solutes and macromolecules in liquid-phase components of the diffusion pathway, changes in pH, temperature, etc. [20]. Moreover, CO$_2$ can interconvert with HCO$_3^-$ inside leaf cells in a reversible reaction catalyzed by carbonic anhydrases. Since the diffusivities of CO$_2$ and HCO$_3^-$ as well as their pH and temperature dependencies differ [21] carbonic anhydrases could play a role on the regulation of $g_m$ by means of changing the nature of the diffusing molecule.

Early work [22, 23] showed that extremely high reduction in carbonic anhydrase activity did not result in major photosynthetic limitation. However, it has been also shown that the contribution of carbonic anhydrase to $g_m$ is species dependent, and their role may become more important when $g_m$ is low as in sclerophyllous species [24]. Moreover many different carbonic anhydrases, with different cellular locations have been characterized [25] and it has been suggested that carbonic anhydrases can account of 1% of total protein, and those located in chloroplasts (βCA1 and βCA5) could potentially contribute to increase $g_m$. Up to now, however, genetic modification of different forms of carbonic anhydrases in Arabidopsis, either chloroplastic or not, have resulted in differently phenotypes differing in growth but with no measurable difference in $g_m$ (Genty, personal communication). Despite of these results, a role of carbonic
anhydrases in the regulation of $g_m$ in some species and/or under certain conditions cannot be ruled out.

3.2. Changing the nature of the diffusing medium: aquaporins

Besides the nature of the diffusing molecule (i.e., $\text{CO}_2$ or $\text{HCO}_3^-$), carbon dioxide diffusion can be altered either by the nature or the distance of the diffusion media. Concerning the nature of the diffusion media, the lipid phase is presumably more limiting for CO$_2$ diffusion than the aqueous phase and this in turn more limiting than the air phase. Membranes (cell, chloroplast) constitute the physical components of the lipid phase. Although the rate of diffusion of CO$_2$ through membranes has been often considered very large and the lipid phase assumed to have a negligible effect on CO$_2$ diffusion, there is still a debate over the degree to which biological membranes are permeable to CO$_2$, and estimated permeation coefficients vary over several orders of magnitude [26-28]. With the discovery of aquaporins, it has been suggested that apart from their function in facilitating water diffusion across membranes they constitute a key means for regulating CO$_2$ diffusion through membranes [29-31]. Altered expression of aquaporins has been shown to result in changes in membrane permeability to CO$_2$ in plants [32, 33] and in animals [30, 34]. Experimental evidence in favor of an important role for aquaporins in CO$_2$ diffusion in intact leaves comes from studies demonstrating enhanced photosynthesis in plants over-expressing aquaporins, and reduced photosynthesis in genetically modified plants with impaired aquaporin function (Fig. 3). These changes are driven at least in part by changes in $g_m$ [32-36]. Similarly, reduced photosynthesis and $g_m$ are observed in plants in which aquaporins have been inhibited by HgCl$_2$ [37-39]. However, genetic transformation to change aquaporin expression and HgCl$_2$ inhibition also lead to simultaneous changes in net CO$_2$ assimilation rates ($A_n$) and stomatal conductance ($g_s$) (Fig. 3, 4) [40]. Indeed, the relative diffusion limitation of photosynthesis due to $g_m$ is directly related to the CO$_2$ drawdown from sub-stomatal cavities to chloroplasts ($C_r-C_c = A_n/g_m$) that depends on $g_m$, $A_n$.
and \( g_s (C_i = f(A_n, g_s)) \) [41, 42]. Therefore, demonstrating changes in \( g_m \) is not sufficient to confirm an alteration in the degree to which mesophyll diffusion limits photosynthesis.

Inherent limitation of drawing broad conclusions from such studies is the lack of biological replication, even if multiple transformed lines have been used in specific studies. So far, no study with transformants has looked into the changes in quantitative limitations between biochemistry, stomata and mesophyll diffusion. We calculated the limitations of photosynthesis due to stomata \( (l_s) \), mesophyll diffusion conductance \( (l_m) \) and biochemistry \( (l_b) \) according to Grassi and Magnani[43]:

\[
\begin{align*}
l_s &= \frac{g_{s,\text{CO}_2}k}{g_{s,\text{CO}_2}(g_{\text{tot}} + k)} \\
l_m &= \frac{g_mk}{g_m(g_{\text{tot}} + k)} \\
l_b &= \frac{g_{\text{tot}}}{g_{\text{tot}} + k}
\end{align*}
\]

(1)

where \( g_{s,\text{CO}_2} \) is the stomatal conductance to \( \text{CO}_2 \), \( g_{\text{tot}} \) is the total diffusion conductance for \( \text{CO}_2 \) determined as:

\[
g_{\text{tot}} = \frac{1}{\frac{1}{g_{s,\text{CO}_2}} + \frac{1}{g_m}},
\]

(2)

and \( k \) is the first derivative of net assimilation rate \( A_n \) with respect to chloroplastic \( \text{CO}_2 \) and is given as [44]:

\[
k = \frac{V_{c_{\text{max}}} (\Gamma^* + K_m)}{(C_c + K_m)^2},
\]

(3)

where \( V_{c_{\text{max}}} \) is the maximum carboxylase activity of Rubisco, \( \Gamma^* \) is the hypothetical \( \text{CO}_2 \) compensation point in the absence of dark respiration, and \( K_m \) is the effective Michaelis-Menten constant for \( \text{CO}_2 \) that considers the competitive inhibition by \( \text{O}_2 \). Rubisco kinetic characteristics were taken from Niinemets and Tenhunen [45] and \( C_c \) was the value reported in the given study.
Due to lack of biological replication, we had to pool different aquaporins atpip1;2-1, atpip2;3-1, Nt AQP1, HvPIP2;1 in the quantitative limitation analysis. Nevertheless, the analysis with pooled data for photosynthesis, stomatal conductance and mesophyll conductance broadly agreed with individual published studies, i.e., that overexpressed lines generally had higher values of these traits, while antisense transformants had lower values (Fig. 3). Analysis of the quantitative limitations further demonstrated that the degree of $g_m$-limitation of photosynthesis is larger in plants with genetically reduced aquaporin levels (Fig. 3). However, in modified lines with over-expressed aquaporins, $g_m$ limited photosynthesis to a similar degree as in control plants due to the parallel occurrence of reduced stomatal limitation (Fig. 3). In addition, genetic modifications in aquaporins also led to alterations in the degree of the limitation by photosynthetic capacity per se (Fig. 3) and also can lead to changes in foliage anatomy and photosynthesis [32-36].

Therefore, there is evidence for the involvement of aquaporins in limiting photosynthetic CO$_2$ assimilation rate, but it is unclear whether this role is only due to their influence on $g_m$. As for the thermodynamics of their involvement in $g_m$, simulations based on molecular models have further indicated high activation energy for CO$_2$ passage through aquaporin monomers, suggesting that CO$_2$ movement through aquaporins is energetically unfavorable compared with passive diffusion through membranes, except for highly polar membranes with low CO$_2$ solubility [46-48]. However, the aquaporin family contains a large number of homologs [49, 50], and molecular simulation studies indicate that the energetic barrier against CO$_2$ movement can be lowered by only a few point mutations [47, 48], suggesting that in principle, aquaporins most probably exist with widely varying permeability for dissolved gases. Arabidopsis aquaporin AtPIP1;2-1 has a much higher permeability for CO$_2$ than AtPIP2;3-1 [32], supporting this concept. It is possible that having different aquaporins with different activation energies allows plants to regulate CO$_2$ diffusivity and H$_2$O diffusivity more or less independently, although water and CO2 conductances are often co-regulated (see Section 7). In addition, aquaporin tetramers rather than monomers may
be functionally active in CO$_2$ conductance in tobacco [51]. This suggestion is also corroborated by molecular simulations suggesting that the central cavity of the tetramer requires almost 50% less activation energy for facilitating CO$_2$ diffusion than the aquaporin monomer [48]. However, this barrier is still relatively large compared with direct diffusion through some highly hydrophobic membranes [48], although the theoretical physical parameters of a lipid bilayer are not necessarily equal to those of a biological membrane [52]. It is also important to consider that all studies on aquaporins have been conducted in herbaceous species with mesophytic leaves that have intrinsically high $g_m$ due to thin cell walls and high surfaces of chloroplasts exposed to intercellular air species [14, 15]. In such species, the relative contribution of lipid phase conductance to total diffusion conductance is expected to be larger than in species with thick cell walls and low surfaces of chloroplasts exposed such as trees, in particular evergreen trees (Fig. 2). The bulk of diffusion conductance in evergreens is expected to be in the liquid phase, for which the diffusion distance can be largely variable.

### 3.3. Changing the diffusing distance: anatomical properties of leaves and cells

The diffusion distance is given by the size of the different anatomical components of the diffusion path. In early studies, leaf anatomical characteristics were considered to constitute the chief limitation of $g_m$ [1, 53-57], and anatomical traits were used to infer $g_m$ [54, 56]. At the time of these early studies, the methods to estimate $g_m$ had not yet been established, and a separation of physical diffusion conductance (known as $g_m$ today) and biochemical conductance, a variable quantifying the photosynthetic capacity (chiefly the carboxylase activity of Rubisco) under a conductance formalism, could not be reliably achieved. On the basis of anatomical measurements, it was concluded that the physical diffusion conductance inside the leaves was large compared with the stomatal and biochemical conductances [56, 58]. However, these early studies did not precisely characterize the thickness of cell walls and cytosol. In addition, they assumed that the
diffusion flux in aqueous and lipid phases varies with the ratio of exposed mesophyll area ($S_{mes}$) to total leaf area ($S$) ratio ($S_{mes}/S$) that characterizes the number of parallel diffusion pathways [54, 55]. This assumes that the exposed surface of mesophyll cells is covered by chloroplasts. Yet, recent studies have shown that the surface of exposed chloroplasts ($S_c$) to leaf area ratio ($S_c/S$) is generally smaller than $S_{mes}/S$ [15, 59-61], implying that the use of $S_{mes}/S$ as a substitute of $S_c/S$ underestimates the anatomical constraints on diffusion.

So far, strong negative correlations between $g_m$ and cell wall thickness have been demonstrated, especially when differences in $S_c/S$ are considered by calculating $g_m$ per unit exposed chloroplast surface area [16]. However, there have been surprisingly few quantitative analyses linking $g_m$ to leaf anatomy. The few studies available demonstrate that $g_m$ can be quantitatively estimated from leaf anatomical measurements, although certain assumptions on the tortuosity of diffusion path length in gas phase, porosity of cell wall, effective diffusion coefficient in cytosol and chloroplast and permeability of plasmalemma and chloroplast envelope are needed [15, 62]. These quantitative analyses have corroborated the correlative findings of the role of cell wall thickness and $S_c/S$ as the main determinants of $g_m$ across species with widely varying anatomy [16]. Linking $g_m$ to detailed anatomical measurements is promising, but tedious. Conversely, $g_m$ has also been correlated with integrated leaf traits such as leaf dry mass per unit area, thickness and density [12, 14, 63]. Although strong relationships have been observed when pooling data from multiple studies, especially for non-stressed plants [14], significant outliers such as Australian sclerophylls have been denoted in other studies, reflecting the circumstance that high dry mass per unit leaf area, thickness and density are not always associated with thick cell walls [63, 64]. The correlations between dry mass per unit leaf area and $g_m$ can also vary across environmental gradients in water availability, that can alter cell wall thickness [15], and in light availability that can alter $S_c/S$ [59, 65] at given values of leaf traits such as dry mass per unit area, thickness and density.
4. Which environmental conditions does $g_m$ respond to?

Mesophyll conductance to CO$_2$ responds to environmental factors either in the long term or rapidly, i.e. in minutes-hours [10]. Recent reviews have already highlighted the incidence of varying environmental conditions such as soil water availability, salinity, growth irradiance and temperature on $g_m$ [12, 14]. In the recent years, the important contribution of $g_m$ in limiting photosynthesis during drought and salinity has been emphasized, knowledge has improved as for nutrient stress effects on $g_m$, and many more data have been obtained regarding the controversial effects of rapid changes in CO$_2$ concentration on $g_m$.

4.1. Acclimation to and recovery after drought and salinity

Under drought and salinity, the degree of photosynthesis limitation by $g_s$ and $g_m$ is similar (see Section 5), but $g_m$ can respond to water deficit and subsequent re-watering at different velocities than $g_s$ [66-69]. Most remarkably, $g_m$ acclimates and recovers during prolonged drought in tobacco and grapevine plants [66, 67], while in stressed soybeans it was hardly affected [70] despite a significant reduction of $g_s$ in all cases. In a study on tobacco [67], $g_m$ and $g_s$ first decreased during the onset of drought, whereas during sustained drought, $g_m$ recovered to control values despite maintenance of low $g_s$. Moreover, drought interacted with environmental conditions, since $g_m$ did not decrease when the experiment was repeated at lower light intensities and milder temperatures [59]. Thus, $g_m$ does not necessarily respond in the same manner as $g_s$ when stress is prolonged and factors other than hydraulics or water status impact on $g_m$.

Furthermore, $g_m$ exceeded control levels after re-watering in the Mediterranean semi-deciduous shrub *Cistus albidus* [71]. In *C. albidus*, consecutive drought and re-watering cycles resulted in a drought cycle-dependent adjustment of leaf gas exchange towards reduced limitation by $g_m$ and enhanced water use efficiency with each consecutive cycle, while the evergreen oak *Quercus ilex*
had a very stable response during successive cycles, lowering $g_m$ and $g_s$ in parallel [71]. Acclimation of $g_m$ during prolonged drought and facilitated recovery after re-watering represent an effective way of optimizing CO$_2$ diffusion transiently under limited CO$_2$ supply, thereby boosting photosynthetic activity and water use efficiency after relief of stress. On the other hand, $g_m$ and photosynthetic activity can vary among ecotypes of the same species under similar water availability due to differences in e.g. photosynthetic capacity, osmotic adjustment and leaf structure [69, 72]. Thus, plant growth form alone cannot explain the differences in $g_m$ response to limited water availability, but may allow for contrasting general trends in photosynthesis among evergreens and deciduous species [73]. Whole plant structure also affects leaf diffusion components and hence the capacity to recover from and survive drought, as observed in pre-conditioned nursery plants of different age and size [74] and in the woody legume *Prosopis velutina* [75]. Low root to shoot ratios [74] as well as lasting effects of hydraulic failure [75] resulted in a delayed or only partial recovery of $g_m$ and leaf gas exchange after re-watering.

### 4.2. Nutrient stress

Compared with responses to drought, little is known about the influence of plant nutrition on $g_m$, and only recently have the effects of nutrition been addressed. In line with some earlier reports, leaf nitrogen content correlates positively with photosynthetic activity and $g_m$ across several woody and herbaceous species [76-78], whereas a negative relationship of leaf nitrogen and $g_m$ was observed when related to tree height in *Pinus densiflora* [79, 80]. Such a decline of $g_m$ with tree height can be related to decreasing water potential affecting leaf expansion and structure [80, 81].

Photosynthetic activity and $g_m$ correlated well with the supply of K in hickory seedlings [82], whereas biochemical modifications and/or structural changes seemed to primarily limit photosynthesis. More research is needed to gain further insight into the K - $g_m$ relationship.
Changes in P nutrition revealed no direct effect on $g_m$ [69, 83]. Excess of Zn in *Beta vulgaris* and excess nickel Ni in *Populus nigra* strongly decreased leaf conductance ($g_m$ and $g_s$) [84, 85]. This was presumably caused by changes in mesophyll structure, which affected leaves developing during stress more than mature ones [85]. Therefore, photosynthetic activity under excess Zn and Ni was primarily limited through impaired leaf conductance.

4.3. Changing CO$_2$ concentration around leaves

The effects of varying CO$_2$ concentrations on $g_m$ display different trends for long-term (acclimation) and for short term responses. No general trend has been observed for plants grown under elevated CO$_2$ (i.e. 500-600 ppm) with no change, decreased or increased $g_m$ being reported, possibly depending on the species and time [86, 87]. In contrast, changes in $g_m$ under short-term exposure to different CO$_2$ concentrations seem to follow a general trend across many species [88-94], with a negative correlation between $g_m$ and sub-stomatal and/or ambient CO$_2$ concentration (Fig. 5). However, some exceptions have been published [95], leading to a controversy as to whether the observed responses are real or a simple measurement artefact. The data recorded under low CO$_2$ (< 100 ppm) have to be taken with caution due to errors with the available techniques [10, 89] and it seems very likely that $g_m$ declines with rising CO$_2$ levels. The same trend was observed with very different techniques, each relying on very different assumptions [see e.g. 9] [11] [86] [96] [97]. As a general pattern, $g_m$ largely differs at the ends of a $C_i$ gradient (0 to 2000 $\mu$mol mol$^{-1}$) (Fig. 5). Still, variation of $g_m$ within a smaller range of CO$_2$ concentrations might be small. This is particularly true when referring to CO$_2$ changes projected for the next few decades (e.g., the range from 300 to 800 $\mu$mol mol$^{-1}$ in Fig. 5, which reflects a range of ambient CO$_2$ concentrations of about 500 to 1000 $\mu$mol mol$^{-1}$).

Several explanations for the decline of $g_m$ with increasing CO$_2$ have been proposed, including fine adjustments for balancing $C_i$ and $C_c$, and avoiding large decreases of cell pH [12].
and increased cell leakiness under high pCO$_2$ [98]. However, these adjustments might be restricted by structural conditions at the mesophyll/chloroplast level, allowing $g_m$ to change within a relatively narrow range (see Section 3 on the possible limit of the contribution of aquaporins to $g_m$). Moreover, other internal factors such as respiration may also affect the determination of $g_m$ under varying CO$_2$ concentrations, e.g. if a constant value is assumed. The true rate varies with CO$_2$, but activities of respiratory pathways can change the resistance of the diffusion pathway, e.g. by altering the mean path length [98]. In fact several reports, either theoretical [98] or empirical [92, 99], but not others (Flexas), have described an effect of O$_2$ concentration on $g_m$ and its dependency on CO$_2$ concentration. The reasons for this effect remain unclear, although the influence of photorespiration and of changes in the spatial distribution of CO$_2$ emission (in mitochondria) and carboxylation (in the chloroplasts) have been claimed. Clearly, more detailed studies are required to fully understand the effect of O$_2$ concentration on $g_m$.

5. How important is $g_m$ in limiting photosynthesis?

5.1. Photosynthesis limitations in response to environmental variables

Once it was demonstrated and accepted by most of the scientific community that $g_m$ is finite, and possibly dynamically regulated, it became important to quantify how much mesophyll diffusion limits photosynthesis. In the 90’s and beginning of this century, photosynthesis limitation by $g_m$ was ignored - for simplicity and because of the difficulty to estimate $g_m$ with methods available - despite the early warnings that $g_m$ was finite, variable and limiting photosynthesis ([3] [100]). Recently, a comprehensive analysis was described where total photosynthesis limitations were estimated and disentangled into its three components: stomatal, mesophyll and biochemical limitation [43]. This is a relative analysis because the percentage of reduction of the net assimilation rate for each limiting component is estimated on the basis of a control value without any of these limitations. As the severity of the stress progresses total limitation increases and so
potentially does each of the three components. Up to now, such analysis has been applied mainly to quantify water stress-induced limitations in herbaceous [90, 101] and woody species [102-105], as well as during recovery from water stress [67, 71, 101]. The limitation of photosynthesis by $g_m$ was followed as well during seasonal changes [106, 107], leaf ontogeny [81, 90, 108], temperature acclimation [109], Zn contamination [84] and nitrogen and phosphorus fertilization [83]. Data for limitation analysis from all these studies are pooled in Figure 6, in which the Total limitation was considered 0 for the ‘control’ plants (i.e., non stress conditions), and increasingly higher for stressed plants up to a maximum of 1. It can be observed that, as total limitation increases with increasing stress, stomatal mesophyll conductance and, to a lesser extend, biochemical limitations increase (Fig. 6). The scattered results are a consequence of including a number of species with different growth forms with a range of structural and anatomical characteristics, different types of stress, and varying severities of the same type of stress from mild to severe stress in the same analysis. Yet, it can be observed that up to a total limitation of 25%, limitations are mainly represented by stomata and mesophyll. From this point to larger total limitation, all three limitations increase, but the relative contribution of stomata becomes generally the larger, followed by that of mesophyll and, finally biochemical limitations. Cases in which mesophyll limitations account for more than 75% of TL are under mild water stress [101], first stages of re-watering [101], where atmospheric demand was impaired from soil water availability [105], or in the response of photosynthesis to temperature [109]. All the data available point out that mesophyll conductance limitations to photosynthesis are of similar magnitude as stomatal limitations, and generally greater than biochemical limitations. Besides limiting photosynthesis in response to environmental clues, the spatial variability of $g_m$ within canopies, especially in relation to height in tall trees, has also an impact in limiting photosynthesis.

**5.2. Photosynthesis limitations within complex canopies**
As mentioned in Section 3, $g_m$ is highly determined by leaf structure, as suggested by the negative relationship between $g_m$ and leaf mass per unit area. Additionally, $g_m$ scales positively with leaf photosynthetic capacity. However, both variables, leaf mass per unit area and photosynthetic capacity, are positively correlated with local irradiance in canopies, so that opposite forces operate when increasing height, controlling the $g_m$ of leaves. The situation is further complicated by the fact that in evergreens leaves of different age are found at different tree heights. Hydraulic limitations in very tall trees exert a limitation on $g_s$ especially in the upper leaves which, in turn, may exert a negative feedback on $g_m$ but at the same time older leaves with expectedly reduced $g_m$ might be found at the lower parts of the canopy. As expected, different results have been obtained when analyzing tree height / leaf canopy position on $g_m$.

For instance, sun leaves at the top of a 34 m tall conifer *Pseudotsuga menziesii* were compared with leaves collected at the bottom (10% incident PPFD) [110]. Despite a significant difference in leaf mass per unit area there was a strong correlation between $A_n$ and $g_m$. The variability of $g_m$ was high within each irradiance level or leaf type, and the differences in $g_m$ between the locations were not significant. Mesophyll conductance was also studied across the canopy profile in different-aged leaves of the oak *Quercus ilex*. Here, in contrast to *Pseudotsuga*, strong positive curvilinear relationships between $g_m$ and mean irradiance were found for all leaf age classes except in oldest leaves [111]. However, the degree of limitation of photosynthesis by $g_m$ was actually slightly larger at higher irradiance, suggesting increasing photosynthetic limitation by $g_m$ at the top of the canopy [111]. The spatial distribution of structural (leaf N and chlorophyll content and leaf mass per unit area), and functional leaf traits (maximum velocity of carboxylation, maximum capacity for electron transport and $g_m$) were studied along the canopy of the deciduous *Fagus sylvatica* in relation to irradiance and leaf age [112]. A multivariate approach was used based on path analysis to disentangle the relationship among these variables. The primary role of structural adjustment was confirmed i.e., the plastic response of leaf mass per unit area which was
in this case negatively related to $g_m$, for the acclimation of leaves to the local irradiance in a canopy. In much taller trees, however, hydraulic limitations could exert a larger role than local irradiance in setting photosynthetic characteristics of the top leaves. For instance the response of leaf mass per unit area to local irradiance was altered in *Sequoia sempervirens* (113 m) as height increased [113]. There was a transition region in the canopy where the primary determinants of leaf morphology and structure switched from local irradiance to hydraulics. It was concluded that structural changes due to hydraulics indirectly reduced net CO$_2$ assimilation rates via increased respiration rates and decreased $g_s$ and $g_m$.

The other question is whether total tree height itself (i.e., not the height of a given leaf inside a tree, but the total height of the tree itself) can alter $g_m$ and its role in constraining photosynthesis. Specifically, leaf mass per unit area increases as trees increase in height and this is associated with reductions of net CO$_2$ assimilation [114-116], $g_s$ [117] and, perhaps, $g_m$ [114, 118, 119]. Lower $g_m$ in taller trees has recently been confirmed experimentally. There was a decrease of $g_m$ with total tree height in the conifer *Pseudotsuga menziesii* [120], suggesting that gravity and the water path length were likely the main determinants of trends in foliar characteristics via their effects on leaf water potential during leaf expansion. Also, there was a tight relationship between leaf mass per unit area and $g_m$ in *Pinus densiflora* that was the inevitable consequence of the morphological acclimation to height [79]. Similarly, the age effect on $g_m$ in leaves of the deciduous *Nothofagus solandri* was only evident in tall trees (15 m tall), while in shorter trees (2 m tall) no differences were found [81]. It seems that changes in $g_m$ with tree height occur in proportion to changes in $g_s$ and photosynthetic capacity, such that photosynthesis is limited to a similar degree by $g_m$ in different-size trees [14, 116].

To sum up, most studies conclude that the main determinant of $g_m$ modifications with tree height is leaf structure represented by leaf mass per unit area, which can be modulated by local
irradiance, leaf age and hydraulic gradients. The complexity of their interactions does not allow yet a clear prediction of $g_m$ changes with tree height.

6. Modeling and including $g_m$ in photosynthesis models

Mesophyll diffusion of CO$_2$ must be taken into account in leaf gas exchange models, since considering an infinite $g_m$ is not correct. The difficulty rises when deciding a value of $g_m$ to be applied in each specific scenario, and as a function of how it varies in space and time. Currently, we are not able to incorporate $g_m$ in models with a mechanistic basis due to the lack of sufficient knowledge on the mechanisms involved in the regulation of $g_m$. This being said, there have been several attempts to empirically include $g_m$ in models. One of the first examples was proposed modeling the soil-plant-atmosphere continuum in a Quercus-Acer forest [121]. These authors, as [122], concluded that $V_{cmax}$ and $J_{max}$ were underestimated in most studies due to neglecting $g_m$. They included a different constant value of $g_m$ for each species. Later on, two approaches were used in rice to include a variable $g_m$ as a function of N or as a function of $g_s$ [123]. The models were based on previous work where $g_m$ usually scaled with $g_s$. The model based on $g_s$ explained more variation in measured $A_n$ than the model based on N. It was argued that $g_m$ changes during different developmental stages of the crop, and the apparent coordination between $g_m$ and $g_s$ allowed the use of $g_s$ as a scaling factor. The same approach was used in modeling the response of C$_3$ and C$_4$ plants to water stress [124]. The inclusion of $g_m$ in a photosynthesis model was further justified by the response of $g_m$ to temperature [11]. None of these approaches allows for a flexible dependency between $g_m$, $g_s$ and photosynthetic capacity like the one that occurs, for instance, under water stress.

One of the remaining challenges in leaf gas exchange models is to take into account the effects of seasonal water stress. Water stress, as mentioned above, affects the relative importance of each component in limiting photosynthesis, depending on the degree of stress. In this sense,
some have used a limitation analysis [43] to infer what must be included in the models that mimic the observed behavior of $A_n$. Three values of $g_m$ were used to simulate $A_n$ assuming that stomatal limitation is equal, smaller or larger than mesophyll limitation [125]. It was concluded that diffusive limitations can explain water flux responses to seasonal changes in soil water availability only if $g_m$ was included in the models. The use of either stomatal or biochemical limitation alone did not mimic the observed data. The same conclusion was reached about the importance of combining several components in the limitation during acclimation to stress [108]. They concluded that to reproduce the general pattern of $C_3$ photosynthesis during water stress, the highest limitation strength must be imposed by $g_m$, then by $g_s$, and finally by the biochemical capacity.

The impact of $g_m$ on model predictions of carbon isotope discrimination has been also assessed by testing whether a fixed or a variable $g_m$ depending on $g_s$ or time of day improved model predictions in mature juniper trees [126]. The incorporation of $g_m$ in the model did not consistently improve carbon isotope discrimination. These results contrast with those were the inclusion of a variable $g_m$ (as a function of $g_s$), improved the model predictions of isotope composition of respired carbon from a coastal Douglas-fir forest in comparison with a model with a fixed $g_m$ [124]. Recently, [127] have warned about the need of including the ternary effect of transpiration rate in the equations for carbon isotope discrimination. The effect is greatest when the leaf-to-air vapor mole fraction difference is greatest, which could explain some of the contradictory results commented above.

Although it is obvious that a realistic model for predicting $A_n$ should incorporate $g_m$, Oliver et al. [128] concluded that the use of $g_m$ did not improved the performance of the $A_n$ model, as long as $V_{cmax}$ was seasonally tuned. Effectively, a similar prediction of $A_n$ can be obtained either reducing $g_m$ or overreducing $V_{cmax}$, i.e., $C_c$ or $C_t$-basis. However, if we are interested in using a mechanistic model, the actual regulation of $g_m$ and $V_{cmax}$ as a function of the degree of stress should be taken into account. In this sense, Niinemets et al. [14] showed how the inclusion of $g_m$ in
models results in a description of leaf acclimation to changing environmental conditions, and in a more realistic description of daily photosynthesis, especially in leaves under stress. While \( A_n-C_i \) parameterization predicted a negative carbon balance at midday in plants under water stress, actual measurements and simulations with the \( A_n-C_c \) approach yielded a carbon gain. Similarly, a biochemical photosynthesis model on a \( C_c \)-basis was used to explain the potentially favorable response of evergreens plants to climate change due to their robust leaves and low \( g_m \) [15]. Currently, the determination of the seasonal evolution of \( g_m \) or its dynamic in cycles of stress and recovery, and even distribution within canopies, is seen as a huge drawback in the process of incorporation in models of process-based land-surface schemes [128]. Definitely, more information is needed to make possible the inclusion of \( g_m \) in leaf-gas exchange models. This information is hard to be obtained at a large scale of space and time due to the limitations in the use of most used techniques of \( g_m \) determination [10]. However, more efficient and straightforward methods for determining average canopy \( g_m \), like that proposed by Ubierna and Marshall [129] based on \( \delta^{13}C \) of phloem content, can be useful in the future for ecophysiological and ecosystem model applications.

7. Can mesophyll conductance be uncoupled from the water path regulation?

7.1. Co-regulation of \( g_m \) and stomatal conductance

The previous sections have demonstrated that \( g_m \) and \( g_s \) are very often co-regulated, although not under all instances. Some degree of co-regulation has been suggested between \( g_m \) and plant hydraulics. Water vapor and \( CO_2 \) share at least a part of their pathways in leaves. Both gases are exchanged with the atmosphere through stomata. In addition, both water vapor and \( CO_2 \) must cross the aerial sub-stomatal cavity. Additionally, after leaving the leaf xylem, liquid water not only moves along apoplastic pathways but also (partly mediated by aquaporins) crosses cell membranes and flows through the plasmalemma (symplastic pathway) and cell vacuoles.
(transcellular pathway) to the sites of evaporation [130, 131]. Accordingly, liquid water and CO₂ diffusion share partly common diffusion pathways in the mesophyll [13, 16]. The involvement of aquaporins in the diffusion of water and possibly CO₂ also suggests at least partly common pathways for both molecules, although it is also possible that different aquaporins could be involved in each case.

Because of overlapping transport pathways, some degree of co-regulation is expected between \( g_m \) and water transport in leaves. Indeed, variations of \( g_m \) are generally closely related to those in \( g_s \), e.g., variations among species (see Section 2), induced by water stress (see Section 4), etc, but not necessarily in transgenic plants with different levels of aquaporins (see Section 3) or under combined water stress and low irradiance (see Section 4). From a purely photosynthetic perspective, this co-regulation is expected and may optimize photosynthesis. For instance, energy and water-consuming stomatal opening does not translate into effective photosynthesis if \( g_m \) equals zero, but requires higher \( g_m \).

However, from the perspective of leaf water use efficiency, theoretical considerations suggest that some uncoupling between the two conductances may be advantageous. Under steady-state, net photosynthesis is:

\[
A_n = g_s (C_a - C_i) = g_m (C_i - C_c),
\]

where \( C_a \), \( C_i \) and \( C_c \) are the atmospheric, sub-stomatal and chloroplastic CO₂ concentrations, respectively. \( A_n / g_s \) is of the intrinsic water use efficiency at the leaf level, as \( g_s \) controls transpiration in a constant environment. From the equation, at constant photosynthetic activity (i.e., ‘demand’ for \( C_c \)), increased \( A_n / g_s \) can be achieved by increasing the ratio of \( g_m \) to \( g_s \). Some degree of uncoupling between the two conductances, i.e. variation in \( g_m / g_s \), has been observed. There is a progressive increase in \( g_m \) along the leaf of the monocot Triticale, but little variation in \( g_s \) [132]. In this case, \( g_m \) is the main driver for changes in observed carbon isotope discrimination (\( \Delta^{13}C \)), an indicator of \( C_c / C_a \). Dry climate populations of the deciduous trees Picea
[133] and Populus [134] have larger $g_{m}/g_{s}$ than their relatives from milder climates. Genetic- and drought-induced variability in $g_{m}/g_{s}$ in grapes [135] and tomato [136] is significantly and positively correlated with water use efficiency. In all these examples, increased $g_{m}/g_{s}$ and water use efficiency was accompanied by decreased $A_{n}$, suggesting that manipulating water use efficiency by means of $g_{m}$ regulation may always result in decreased production capacity. However, a simultaneously higher $A_{n}$, $g_{m}/g_{s}$ and water use efficiency was observed in the evergreen conifer Abies pinsapo than in its close relative A. alba (Peguero-Pina, unpublished). Similarly, a close relationship was found between $A_{n}$ and $g_{m}$ along a range of Pseudotsuga menziesii trees varying in total height, but virtually no change in $g_{s}$ i.e., a higher $g_{m}/g_{s}$ as total height declined and $A_{n}$ and water use efficiency increased [80]. Understanding the mechanisms regulating $g_{m}$ in such a way that it can uncouple from $g_{s}$ to some extent may be an essential step for future genetic manipulation of plants aiming simultaneous increases in photosynthesis and water use efficiency.

7.2. Co-regulation of $g_{m}$ and hydraulic conductance in the mesophyll

At least in part, tight co-regulation of $g_{m}$ and $g_{s}$ may arise from the close relationship often found between $g_{s}$ and the conductance of water within the mesophyll. Up to now, it has not been possible to determine hydraulic conductance of the mesophyll directly, but several methods to measure whole leaf hydraulic conductance ($K_{\text{leaf}}$) are available, mainly based on the measurement of water flow relative to a water potential gradient[137]. A general positive relationship occurs across species between $g_{m}$ and $K_{\text{leaf}}$, with fast growing species showing the highest values for both variables and conifers falling in a group with the lowest values (Fig. 7a). The underlying reason for such relationship might be the existence of anatomical limitations to CO$_2$ and water conductances, which are likely to be higher in species with thicker mesophyll layers and greater surface of mesophyll cells [80, 138-142]. Nevertheless, this does not seem to be a universal
relationship, and herbaceous monocots with high $g_m$, around 0.5 mol m$^{-2}$ s$^{-1}$ [36, 132, 143], may have $K_{\text{leaf}}$ values below 5 mmol H$_2$O s$^{-1}$ MPa$^{-1}$ m$^{-2}$ [144, 145]. Whether this is due to anatomical or biochemical particularities of these species is still a matter of debate. In this sense, at least for some species the pattern of change in response to drought and tree height for $g_m$ and $K_{\text{leaf}}$ is comparable to the general pattern observed across species (Fig. 7b), suggesting that not only anatomical but also differences in biochemical regulation are involved in interspecific differences for both variables.

The main limitation of $K_{\text{leaf}}$ as a surrogate for the hydraulic conductance of the mesophyll is that $K_{\text{leaf}}$ involves both mesophyll and xylem resistances, and although the former plays a significant role in whole $K_{\text{leaf}}$, its relative contribution may vary with species and experimental conditions [130, 137, 146, 147]. Studies on the environmental response of leaf water isotopic enrichment offer a new way to assess short-term changes in mesophyll hydraulic resistance [148-151]. During transpiration, leaf water becomes enriched in the heavier isotopes, $^{18}$O and $^2$H. The enrichment at the sites of evaporation can be modeled from environmental variables [152, 153]. (see Appendix I for details). However, the observed enrichment in the leaf lamina does not generally agree with modeled values at the site of evaporation, since back diffusion of enriched water from the sites of evaporation to the rest of the leaf is counteracted by a mass flow of non-enriched water driven by transpiration (Péclet effect [154]). The magnitude of this effect is proportional to the transpiration rate, the distance from the xylem to the evaporative surface, and a scaling factor, which accounts for the higher velocity of water through a porous media than if it were moving through the leaf as a slab (i.e. as derived from transpiration). From these models, a “scaled effective path length” ($L_{\text{eff}}$, the product of the actual distance and the scaling factor) can be determined by comparing modeled enrichment at the site of evaporation with observed values [154-156]. Since $L_{\text{eff}}$ accounts both for the length of the water pathway and its tortuosity, it is theoretically related to mesophyll hydraulic resistance to water flow. In a recent work, Ferrio et al.
showed a tight link between $L_{\text{eff}}$ and both $K_{\text{leaf}}$ and $g_m$ in response to experimental treatments (Fig. 8), although the relationship between $L_{\text{eff}}$ and $g_m$ reached an asymptote at higher values of $g_m$ ($g_m > 200 \text{ mmol CO}_2 \text{ m}^{-2} \text{s}^{-1}$; Fig. 8b). These findings provided empirical evidence in support of the theoretical link between $L_{\text{eff}}$ and hydraulic conductance of the mesophyll. Most interestingly, they also showed that at least in response to certain environmental variables, changes in $g_m$ and hydraulic conductance of the mesophyll are closely and positively related, implying that the $g_m / g_s$ ratio can vary but over a limited range. Nevertheless, the lack of relationship between $g_m$ and $L_{\text{eff}}$ at higher values of $g_m$ suggests that diffusion of CO$_2$ can be enhanced beyond the common limitations for water and CO$_2$. It is likely, for example, that well-watered plants may have reached their maximum values of hydraulic conductance of the mesophyll, determined by anatomical limitations, while $g_m$ may still respond to other regulations that maximize photosynthesis. One possible explanation for the uncoupling between CO$_2$ and water conductances has been proposed [51]: the aquaporin NtAQP1, from the PIP1 family, did not increase water transfer, but enhanced CO$_2$ diffusion. Conversely, NtPIP2;1 from the PIP2 family, favored water transport but did not affect CO$_2$ diffusion. The changing of the proportion of the two different aquaporins in a tetramer progressively varied the water and CO$_2$-related functions. As a consequence, even though both leaf hydraulics and CO$_2$ diffusion respond to changes in aquaporin conductivity, different combinations of aquaporin subunits in aquaporin tetramers may promote either water or CO$_2$ transfer, or both, depending on the proportion of PIP1 or PIP2. Thus, regulation of the function of aquaporins would take place as a result of a competition among subunits for the formation of tetramers, in a way that would allow enhancing CO$_2$ fixation and at the same time reducing water use. Indirect evidence in vivo in support to this hypothesis was obtained by comparing the values of $g_m$ and effective path length in a wild type and two tobacco mutants and antisense and overexpressing NtAQP1 lines (Fig. 9) (Kodama, unpublished, plants courtesy of Dr. R. Kaldenhoff). Plants with increased $g_m$ had increased effective path length (i.e., decreased hydraulic
conductance of the mesophyll). The overexpressing line (with a greater expression of PIP1s) had the highest \( g_m \) and effective path length, and the antisense line had the lowest values. We speculate that this is the result of different aquaporin forms proportion in tetramers following altered expression of one of the two forms (PIP1 and PIP2). According to [51], one water conducting aquaporin (PIP2) is enough to facilitate water transport to a level close to maximum, while 3 or 4 \( \text{CO}_2 \) conducting aquaporins (PIP1) are required to reach maximum \( \text{CO}_2 \) diffusion. We suggest that, in antisense lines, the proportion of PIP1 aquaporin in most aquaporin tetramers falls well below 3, while in overexpressing lines the proportion is between 3 and 4. In contrast, the effective path length and \( g_m \) were negatively correlated when comparing individual plants within each mutant type, suggesting a common trend for \( \text{CO}_2 \) and water conductance. In this case, we suggest that variations in the total expression of aquaporins among individuals, while keeping identical proportions among subunits, results in a concomitant increase in conductance of \( \text{CO}_2 \) and of water vapor.

8. Concluding remarks and future prospects

We have stressed that the share of overall photosynthetic limitation by mesophyll conductance is large and can be the most significant factor limiting photosynthesis under certain conditions and certain plant functional types. This statement is backed up by ample evidence, and we argue that \( g_m \) should be included in any study analyzing limitations to photosynthesis, as well as in models for predicting rates of photosynthesis.

Significant progress has recently been made in quantitatively linking \( g_m \) to foliage anatomical and structural traits. Cell wall thickness and chloroplast distribution seem to play a dominant role in determining the upper limit of \( g_m \). However, rapid variations in response to environmental cues might not be regulated by anatomical traits. Aquaporins seem to be only partly responsible, although their mechanistic bases remain unclear.
We conclude that further developments in the field require more advanced understanding of the currently most obscure points, which include:

1. The role of aquaporins in diffusion conductance, especially in species growing in stressful environments and having particularly low values of $g_m$.

2. To what extent water and CO$_2$ transport processes are coordinated and how does this affect photosynthesis? It seems that the coordination is not necessarily maintained across environmental gradients and gradients of tree height.

3. What is the genetic basis of $g_m$ and its genetic variability? Data on genetic variability within species, the degree of heritability of $g_m$, as well as for entire phylogenetic groups, notably ferns and mosses are far too scarce.

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**Figure legends**

Figure 1. Mesophyll conductance is greatest the more a species is phylogenetically evolved. Average ± S.E. values for $g_s$ and $g_m$ in different pooled groups of plants. Data from liverworts and hornworts from [17], data for all other groups from [41].

Figure 2. Mesophyll conductance reflects the CO$_2$ diffusion pathway, which is composed of air, water and lipid barriers. Comparison of CO$_2$ diffusion pathway within the fully expanded needle of evergreen conifer *Abies alba* (a-c) and within the leaf of broad-leaved deciduous *Populus tremula* (d-f) according to light (a, b, d), scanning (c) and transmission electron (e, f) micrographs. Leaf cross-sections (a, d) illustrate the CO$_2$ gas phase diffusion pathway from ambient air ($C_a$) to substomatal cavities ($C_i$) and from sub-stomatal cavities to outer surface of cell walls, $C_{i,w}$. The CO$_2$ concentration drawdown, $C_a-C_i$, depends on stomatal conductance, while the drawdown $C_i-C_{i,w}$ is characterized by internal gas-phase diffusion conductance ($g_{ias}$) that is determined by effective mesophyll thickness and porosity. The micrographs of palisade tissue (b, e) demonstrate parallel diffusion pathways within cells (indicated with arrows for representative cells) that is determined by the exposure of chloroplasts to intercellular airspace. The representative micrographs of palisade cells (c, f) illustrate CO$_2$ diffusion pathways in liquid phase from mesophyll cell outer surface to chloroplasts. The CO$_2$ drawdown from outer surface of cell walls to chloroplasts ($C_c$, $C_{i,w}-C_c$) is determined by liquid-phase diffusion conductance $g_{aq}$ that consists of cell wall (cw), plasma membrane (pm, not visible in the micrographs), cytoplasm (cyt), chloroplast envelope (env, not visible in the micrographs), and chloroplast stroma (chl). Micrographs from Peguero-Pina *et al.* [62] (a-c) and Tosens *et al.* [15] (d-f), with permission.

Figure 3. Altered expression of aquaporins in relation to mesophyll conductance limitations of photosynthesis. Average (error bars show +SE) net assimilation rate, stomatal conductance to CO$_2$ and mesophyll diffusion conductance (upper panels) and relative photosynthetic limitation due to limited biochemical capacity, stomatal conductance and mesophyll diffusion conductance (lower panels) in transformed plants with reduced (AS, RNA-interference) and over-expressed (OE) aquaporins and corresponding controls (either wild type or plants transformed with the same construct used for AS and OE, but lacking the modified aquaporin expression phenotype). As the data represent one-to-one correspondence between data pairs (control vs. AS and control vs. OE and AS vs. OE within the given study), the averages between the treatments and corresponding controls and between AS and OE were compared by paired $t$-tests that is a more powerful statistical test than standard ANOVA [141]. Statistical significance as: ns – not significant, * - $P<0.05$, ** - $P < 0.01$, *** - $P < 0.001$. The data are for *Arabidopsis thaliana* aquaporins atpip1;2-1 and atpip2;3-1 [32], *Nicotiana tabacum* aquaporin NtAQP1 [33-35] and * Hordeum vulgare* aquaporin HvPIP2;1 [36]. The data correspond to saturating light, leaf temperature of 25 °C and chamber CO$_2$ concentration of 280-400 µmol mol$^{-1}$. The relative limitations of photosynthesis were calculated according to [43] and the three limitations sum up to 1. Maximum carboxylase activity of Rubisco, $V_{\text{cmax}}$, needed for these calculations was determined by inverse modeling as in [44]. Means between treatment and corresponding controls and between AS and OE were compared by paired $t$-tests (ns – not significant, * - $P<0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

Figure 4. Co-regulation of stomatal conductance, mesophyll conductance and photosynthesis in wild type and mutants with altered expression of aquaporins. Net assimilation rate ($A_n$) in relation to (a) stomatal conductance to CO$_2$ ($g_s$) and (b) mesophyll diffusion conductance ($g_m$), and correlations between (c) the CO$_2$ drawdown due to limited $g_s$ (difference of CO$_2$ concentrations in ambient air and sub-stomatal cavities, $C_a-C_i=A_n/g_s$) and (d) the CO$_2$ drawdown due to limited $g_m$
(difference in CO\textsubscript{2} concentrations in sub-stomatal cavities and chloroplasts, $C_i-C_c = A_n/g_m$). Every data point corresponds to an average value of either control, aquaporin overexpressed, aquaporin antisense or aquaporin-inhibited plants reported in original studies. Data sources for transformed plants as in Fig. 3. Data for HgCl\textsubscript{2} treated (0.3-1.2 mM) and non-treated control plants were from [39] (\textit{Vicia faba}) and [37, 38] (\textit{Nicotiana tabacum}). Environmental conditions during measurements as in Fig. 3. The number of data points in different panels differs because not all studies reported the whole suite of characteristics $A_n$, $g_s$, $g_m$, $C_i$ and $C_c$.

Figure 5. Increasing CO\textsubscript{2} concentration reduces mesophyll conductance. $g_m$ correlates with $C_i$ over a broad range of ambient CO\textsubscript{2} concentrations and across a number of different species and growth forms. The isotopic method ($\delta^{13}$C) and the combined chl fluorescence and infrared gas exchange analysis method are shown in closed and open circles, respectively. Data are taken or calculated from [86], [88], [157], [89], [99], [96], [90], [91], [97], [158], [87], [92], [93], [94], [11].

Figure 6. Mesophyll conductance limitations increase as photosynthesis declines (total limitation increase) in response to stress. Empty circles represent stomatal limitation (SCL), filled circles mesophyll conductance limitation (MCL) and triangles biochemical limitation (BL). Data have been compiled from the following references: [83], [124], [102], [108], [66, 90], [67, 71], [101], [159], [160], [104], [107], [105], [84], [81] and [109].

Figure 7. Mesophyll conductance co-regulated with hydraulic conductance. A) Strong relationship between average leaf hydraulic conductance ($K_{\text{leaf}}$) and mesophyll diffusion conductance ($g_m$) for different plant species, based on literature data (see Supplemental Information for the list of references). B) Comparative response of different species to changes in growing conditions affecting both $g_m$ and $K_{\text{leaf}}$: high/low water stress for Quercus, Fagus and Pinus; canopy height for Pseudotsuga; a combination of drought and vein severing for Vitis.

Figure 8. Mesophyll conductance and hydraulic conductance co-regulate with the effective pathlength of mesophyll water movement. Strong relationship between treatment averages for leaf lamina hydraulic conductance ($K_{\text{lamina}}$), scaled effective pathlength ($L_{\text{eff}}$) and mesophyll conductance for CO\textsubscript{2} ($g_m$) in Grenache grape. Treatment averages for $g_m$ were calculated excluding values above 200 mmol CO\textsubscript{2} m\textsuperscript{-2}s\textsuperscript{-1}, which were beyond the linear range for the relationship between $g_m$ and $L_{\text{eff}}$ (see inset with individual leaf values). Circles: control plants, triangles: drought plants. Open symbols: intact leaves. Closed symbols: vein-severed leaves. Data from [149].

Figure 9. Altered expression of PIP 1 aquaporins results in co-variations of the mesophyll conductance and the effective pathlength of mesophyll water transfer. Upper panel: the relationship between $g_m$ and the path length of mesophyll water transfer (L) in wild-type (circles), anti-sense (downwards triangles) and over-expressing (up-wards triangles) tobacco plants with altered levels of NtAQP1. Empty dots are individual plants within each genotype, and filled dots average values per genotype. Lower panel: potential explanation for the opposite relationships found between $g_m$ and L within and between genotypes, based on the findings by Otto \textit{et al.} [51]: negative correlations between $g_m$ and the path length of mesophyll water transfer occur between genotypes, likely reflecting that altered expression of one aquaporin class results in different proportions of aquaporin classes within tetramers; positive correlations between $g_m$ and the path length of mesophyll water transfer occur between individuals within each genotype, likely reflecting different concentrations of aquaporins rather than different proportions of aquaporin classes within tetramers.
FIGURE 1
\[ g_{\text{liq}} = f \left( g_{\text{cw}}, g_{\text{pm}}, g_{\text{cyt}}, g_{\text{env}}, g_{\text{chl}} \right) \]

**FIGURE 2**
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6
Herbs monocots
Herbs dicots
Deciduous angiosp.
Ev. angiosp.
Ev. gymnosp.

\[ g_m = 0.0268 + 0.0156 \times K_{\text{leaf}} \]
\[ r^2 = 0.659 \quad N = 20 \quad P < 0.001 \]

\[ g_m = 0.0036 + 0.0206 \times K_{\text{leaf}} \]
\[ r^2 = 0.594 \quad N = 14 \quad P < 0.001 \]

FIGURE 7
FIGURE 8
FIGURE 9
Table S1. Average and standard deviation of leaf hydraulic conductance \((K_{\text{leaf}})\) and mesophyll diffusion conductance \((g_m)\) across the different genera included in Fig. 8. The “OUT” column indicates the values not included in the linear regression plotted in Fig. 8a.

<table>
<thead>
<tr>
<th>Genus</th>
<th>(K_{\text{leaf}}) (\text{mmol} \text{ H}_2\text{O s}^{-1} \text{MPa}^{-1} \text{m}^{-2})</th>
<th>(g_m) (\text{mmol} \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1})</th>
<th>OUT</th>
<th>References (K_{\text{leaf}})</th>
<th>References (g_m)</th>
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<tbody>
<tr>
<td><strong>Herbaceous monocots</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Triticum</td>
<td>3.1 ±0.00</td>
<td>0.44 ±0.072</td>
<td>X</td>
<td>[1],[2],[3]</td>
<td>[4],[7],[5],[6],[8]</td>
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<td>Oryza</td>
<td>7.5 ±0.39</td>
<td>0.56 ±0.410</td>
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<td>[9]</td>
<td>[10],[11],[8]</td>
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<tr>
<td>Phaseolus</td>
<td>19.5 ±4.72</td>
<td>0.28 ±0.115</td>
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<td>[12],[13]</td>
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</tr>
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<td>0.79 ±0.545</td>
<td>X</td>
<td>[12],[20],[21]</td>
<td>[14],[15],[16],[17],[18],[8],[19]</td>
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<td>Vicia</td>
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<td>[26]</td>
<td>[6],[27]</td>
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<tr>
<td>Glycine</td>
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<td>Arabidopsis</td>
<td>11.5 ±1.90</td>
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<td>[33],[34]</td>
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<td>0.30 ±0.205</td>
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<tr>
<td>Gossypium</td>
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<td>1.06 ±0.745</td>
<td>X</td>
<td>[28],[43]</td>
<td>[22],[44]</td>
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<tr>
<td>Quercus</td>
<td>7.5 ±1.71</td>
<td>0.14 ±0.032</td>
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<td>[45],[46],[48],[20],[47]</td>
<td>[31],[49],[6],[50],[51],[52],[53],[54]</td>
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<td>Fagus</td>
<td>7.1 ±0.46</td>
<td>0.16 ±0.094</td>
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<td>Acer</td>
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<td>0.05 ±0.011</td>
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<td>[58],[17],[57],[50]</td>
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<td>alnus</td>
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<td>0.12 ±0.017</td>
<td></td>
<td>[59]</td>
<td>[17],[57],[50]</td>
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<td>populus</td>
<td>10.6 ±2.20</td>
<td>0.22 ±0.059</td>
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<td>betula</td>
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<td>0.77 ±0.335</td>
<td>X</td>
<td>[47],[62]</td>
<td>[63],[64],[65],[66]</td>
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<td>Tilia</td>
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<td>0.92 ±0.415</td>
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<td>[65],[24]</td>
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<td>Juglans</td>
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<td>0.15 ±0.035</td>
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<td>Corylus</td>
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<td>[50]</td>
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<td>0.11</td>
<td></td>
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<td>[22],[55],[68]</td>
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<td>0.16 ±0.044</td>
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<td>[69],[1],[70],[71],[15],[33],[72]</td>
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<tr>
<td>Quercus</td>
<td>3.0 ±1.44</td>
<td>0.08 ±0.013</td>
<td></td>
<td>[20]</td>
<td>[73],[17],[74],[6],[75],[76],[52]</td>
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<tr>
<td>Olea</td>
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<td>0.22 ±0.135</td>
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<td>[20]</td>
<td>[77],[78],[79],[33],[80],[75]</td>
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<td>Camellia</td>
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<td>0.10 ±0.025</td>
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<tr>
<td>Hedera</td>
<td>10.4 ±0.26</td>
<td>0.15</td>
<td></td>
<td>[47]</td>
<td>[6]</td>
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<tr>
<td>eucalyptus</td>
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<td>0.15 ±0.014</td>
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<td>Laurus</td>
<td>2.2 ±0.56</td>
<td>0.08</td>
<td></td>
<td>[20]</td>
<td>[75]</td>
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<tr>
<td>Pinus</td>
<td>5.3 ±1.12</td>
<td>0.13 ±0.020</td>
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<td>[87],[48],[88]</td>
<td>[14],[50],[89]</td>
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<td>Pseudotsuga</td>
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<td>0.11 ±0.087</td>
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<td>[14],[91],[50],[92],[90]</td>
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</table>
References Appendix A


Appendix B

Leaf water enrichment models and scaled effective path length

Steady-state isotopic enrichment over source water at the site of evaporation ($\Delta_e$) has been described by the Craig & Gordon model [1-2]:

$$\Delta_e = \varepsilon^* + \varepsilon_k + \left( \Delta_v - \varepsilon_k \right) \frac{e_a}{e_i}$$

(Eq. S1)

where $\varepsilon^*$ is the equilibrium fractionation between liquid water and vapour [3]; $\varepsilon_k$ is the kinetic fractionation as vapour diffuses from leaf intercellular spaces to the atmosphere [4-5], $\Delta_v$ is the isotopic enrichment of atmospheric water vapour relative to plant source water, and $e_a/e_i$ is the ratio of ambient to intercellular vapour pressures.

The steady-state isotopic enrichment of mean lamina mesophyll water ($\Delta_{LsP}$) can be described by the above steady-state Craig & Gordon model corrected for the gradient from xylem source water to enriched water at the evaporating sites, the so-called Péclet effect [6]:

$$\Delta_{LsP} = \Delta_e \frac{1 - e^{-\varphi}}{\varphi} \text{ with } \varphi = \frac{v \cdot l}{D}$$

(Eq. S2)

where $\varphi$ (Péclet number) is the ratio of convection to diffusion, $v$ is the linear velocity of water movement (m s$^{-1}$), $l$ is the length of the water pathway along which diffusion occurs (i.e., the distance between the xylem and the evaporation surface), and $D$ the tracer-diffusivity (m$^2$ s$^{-1}$) of heavy water isotopologues (either H$_2^{18}$O or $^2$H$^1$HO) in ‘normal’ water. Linear velocity through the water pathway ($v$) can be related to transpiration rate ($E$, mol H$_2$O m$^{-2}$ s$^{-1}$) as follows [7]:

$$v = k \frac{E}{C}$$

(Eq. S3)

where $C$ is the molar concentration of water (55.56 10$^3$ mol m$^{-3}$) and $k$ is a scaling factor to convert the velocity of water moving through the leaf as a slab ($E/C$) to the actual velocity in a porous medium, and usually ranges from 10$^2$ to 10$^3$. Accordingly, $\varphi$ can be also expressed as:

$$\varphi = \frac{E \cdot L_{eff}}{C \cdot D}$$

(Eq. S4)

where $L_{eff}$ stands for the scaled effective pathlength, i.e. the product of $l$ and $k$. Thus, $L_{eff}$ is expected to be much larger than the actual distance between the xylem and the evaporation sites, and would vary not only with changes in $l$ but also with changes in the tortuosity of the water pathway (e.g. varying the proportion of apoplastic and cell-to-cell water pathways; [8]).
References Appendix B


