Title: Mesophyll diffusion conductance to CO_2: an unappreciated central player in photosynthesis

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Keywords

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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 ecophysiological 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 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 (*Triticum*, *Oryza* and *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 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 not 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., CO_2 or 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_i-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]:

$$\begin{aligned} l_s &= \frac{g_{tot} k}{g_{s,CO2}(g_{tot} + k)} \\ l_m &= \frac{g_{tot} k}{g_m (g_{tot} + k)} \\ l_b &= \frac{g_{tot}}{g_{tot} + k} \end{aligned} \quad (1)$$

where $g_{s,CO2}$ is the stomatal conductance to CO_2, g_{tot} is the total diffusion conductance for CO_2 determined as:

$$g_{tot} = \frac{1}{\frac{1}{g_{s,CO2}} + \frac{1}{g_m}}, \quad (2)$$

and k is the first derivative of net assimilation rate A_n with respect to chloroplastic CO_2 and is given as [44]:

$$k = \frac{V_{cmax}(\Gamma^* + K_m)}{(C_c + K_m)^2}, \quad (3)$$

where V_{cmax} is the maximum carboxylase activity of Rubisco, Γ*is the hypothetical CO_2 compensation point in the absence of dark respiration, and K_m is the effective Michaelis-Menten constant for CO_2 that considers the competitive inhibition by 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_2O 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 μmol 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 μmol mol^{-1} in Fig. 5, which reflects a range of ambient CO_2 concentrations of about 500 to 1000 μmol 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_i-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 CO_2 diffusion share partly common diffusion pathways in the mesophyll [13, 16]. The involvement of aquaporins in the diffusion of water and possibly CO_2 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 CO_2 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_{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_{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_{leaf} values below 5 mmol H_2O 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_{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_{leaf} as a surrogate for the hydraulic conductance of the mesophyll is that K_{leaf} involves both mesophyll and xylem resistances, and although the former plays a significant role in whole K_{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_{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_{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.

[149] showed a tight link between L_{eff} and both K_{leaf} and g_m in response to experimental treatments (Fig. 8), although the relationship between L_{eff} and g_m reached an asymptote at higher values of g_m (g_m >200 mmol CO_2 m^{-2} s^{-1}; Fig. 8b). These findings provided empirical evidence in support of the theoretical link between L_{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_{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 NtAQP1lines (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 CO_2 conducting aquaporins (PIP1) are required to reach maximum 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 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 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 sub-stomatal 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_{liq} 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 imitations sum up to 1. Maximum carboxylase activity of Rubisco, V_{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_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 $HgCl_2$ treated (0.3-1.2 mM) and non-treated control plants were from [39] (*Vicia faba*) and [37, 38] (*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_2 concentration reduces mesophyll conductance. g_m correlates with C_i over a broad range of ambient CO_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_{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_{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_{lamina}), scaled effective pathlength (L_{eff}) and mesophyll conductance for CO_2 (g_m) in Grenache grape. Treatment averages for g_m were calculated excluding values above 200 mmol CO_2 m^{-2} s^{-1}, which were beyond the linear range for the relationship between g_m and L_{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 *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.

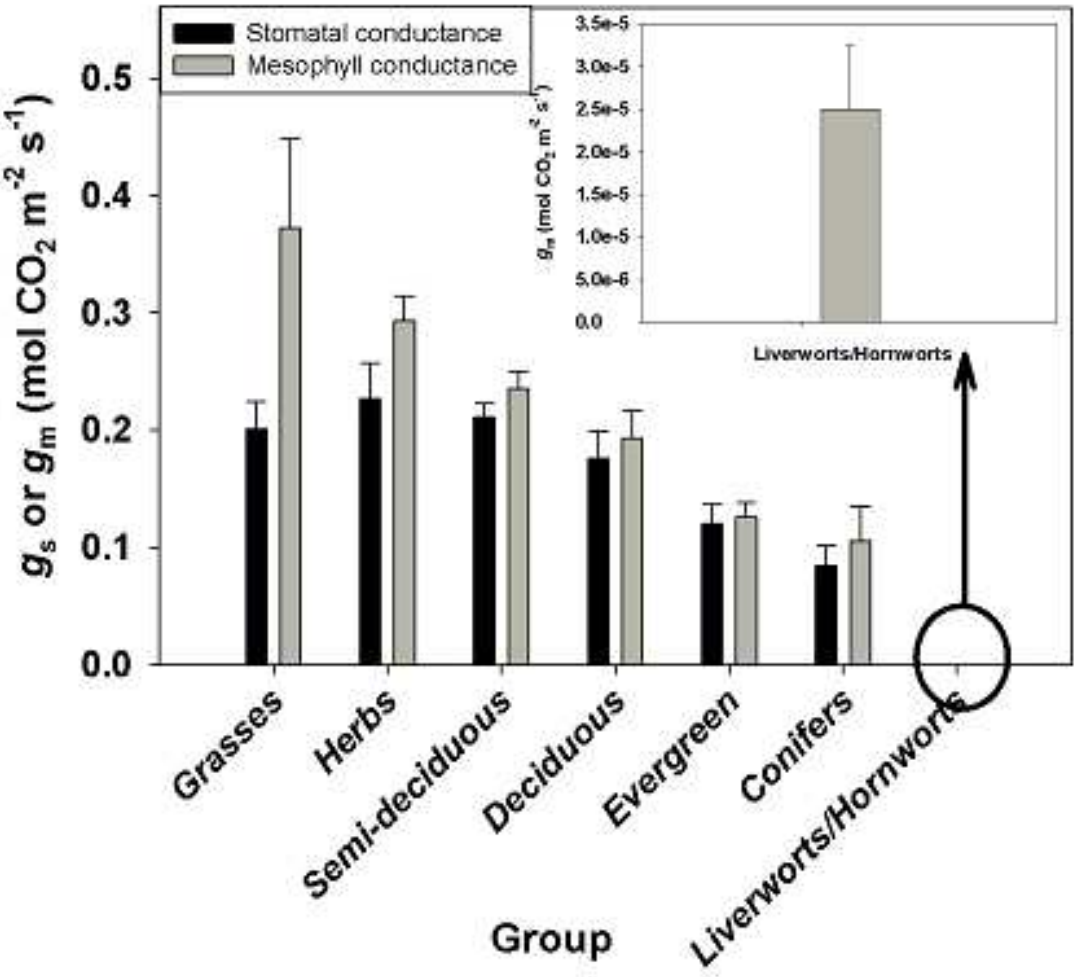
Stomatal conductance
Mesophyll conductance
g_s or g_m (mol CO_2 m^{-2} s^{-1})
0.0
0.1
0.2
0.3
0.4
0.5
Grasses
Herbs
Semi-deciduous
Deciduous
Evergreen
Conifers
Liverworts/Hornworts
Group
3.5e-5
3.0e-5
2.5e-5
2.0e-5
1.5e-5
1.0e-5
5.0e-6
0.0
Liverworts/Hornworts

FIGURE 1

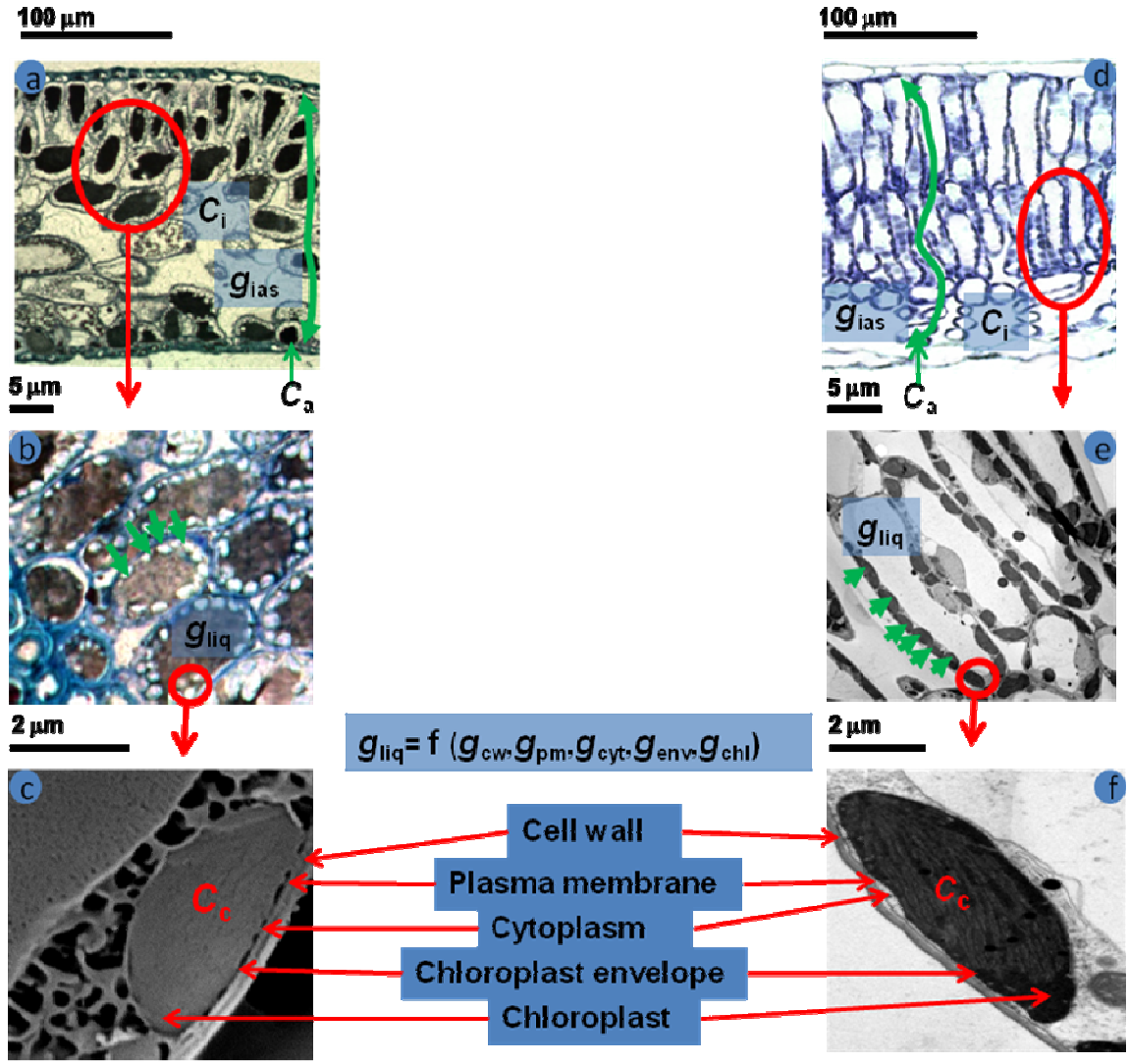
100 µm
a
c_i
g_{ias}
5 µm
c_a
100 µm
d
g_{ias}
c_i
5 µm
c_a
b
g_{liq}
2 µm
e
g_{liq}
2 µm
$g_{liq} = f\ (g_{cw}, g_{pm}, g_{cyt}, g_{env}, g_{chl})$
c
c_c
Cell wall
Plasma membrane
Cytoplasm
Chloroplast envelope
Chloroplast
f
c_c

FIGURE 2

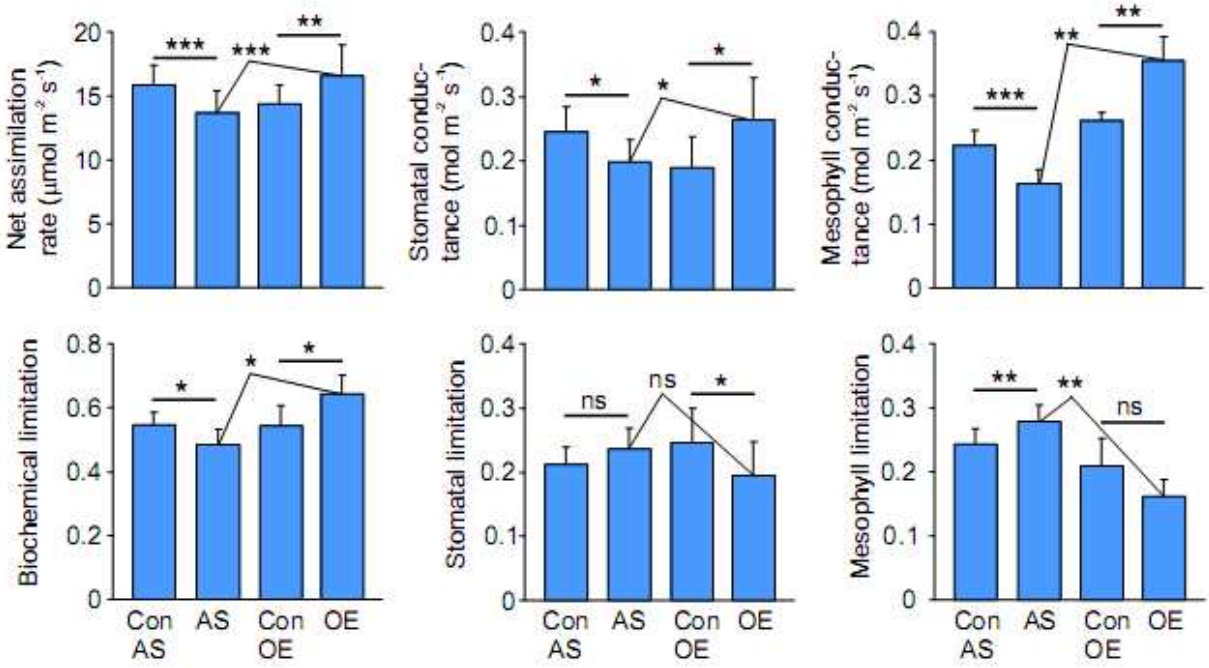
Net assimilation rate (μmol m-2 s-1)
Stomatal conduc-tance (mol m-2 s-1)
Mesophyll conduc-tance (mol m-2 s-1)
Biochemical limitation
Stomatal limitation
Mesophyll limitation
Con AS
AS
Con OE
OE
ns

FIGURE 3

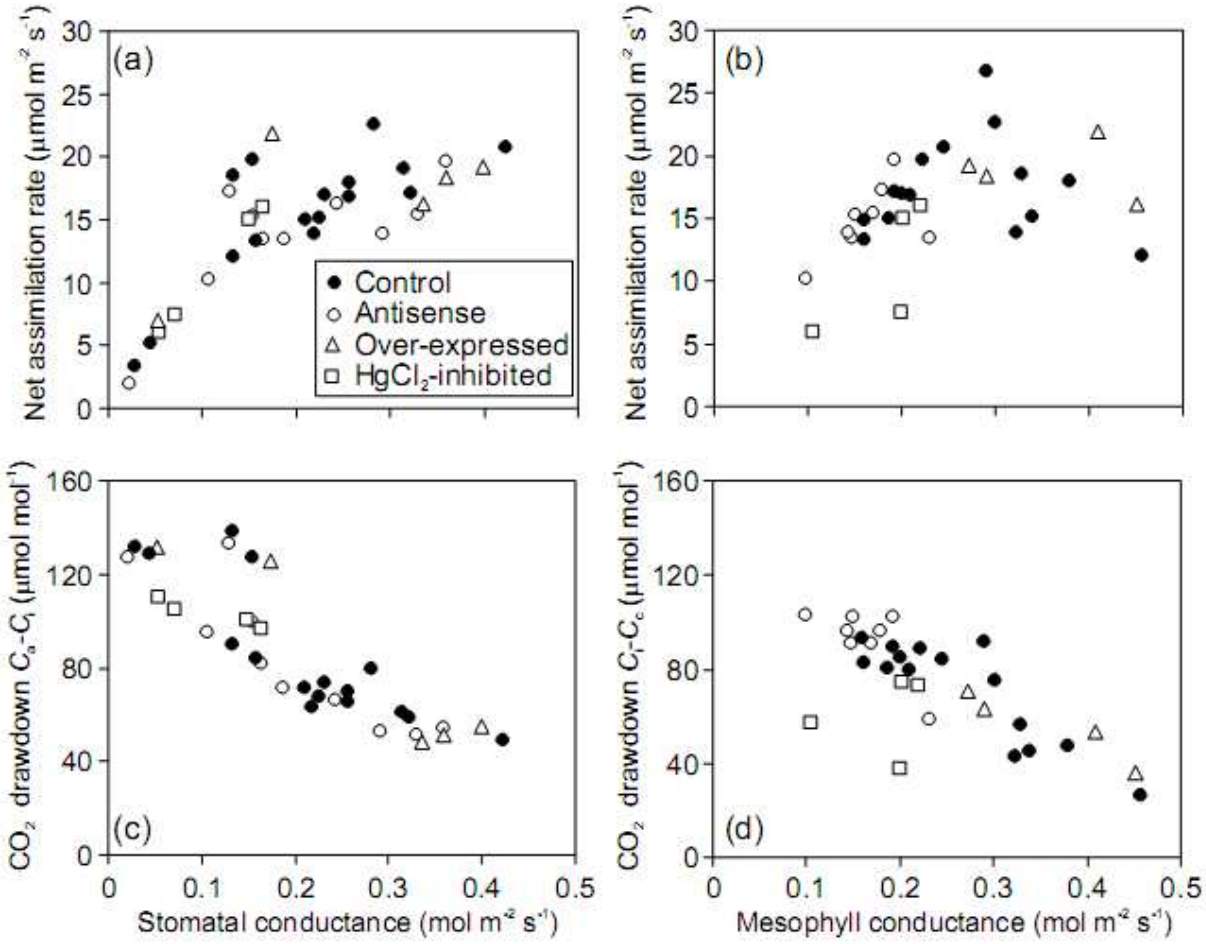
(a)
(b)
(c)
(d)
Net assimilation rate (µmol m-2 s-1)
CO2 drawdown Ca-Ci (µmol mol-1)
CO2 drawdown Cc-Cc (µmol mol-1)
Stomatal conductance (mol m-2 s-1)
Mesophyll conductance (mol m-2 s-1)
Control
Antisense
Over-expressed
HgCl2-inhibited

FIGURE 4

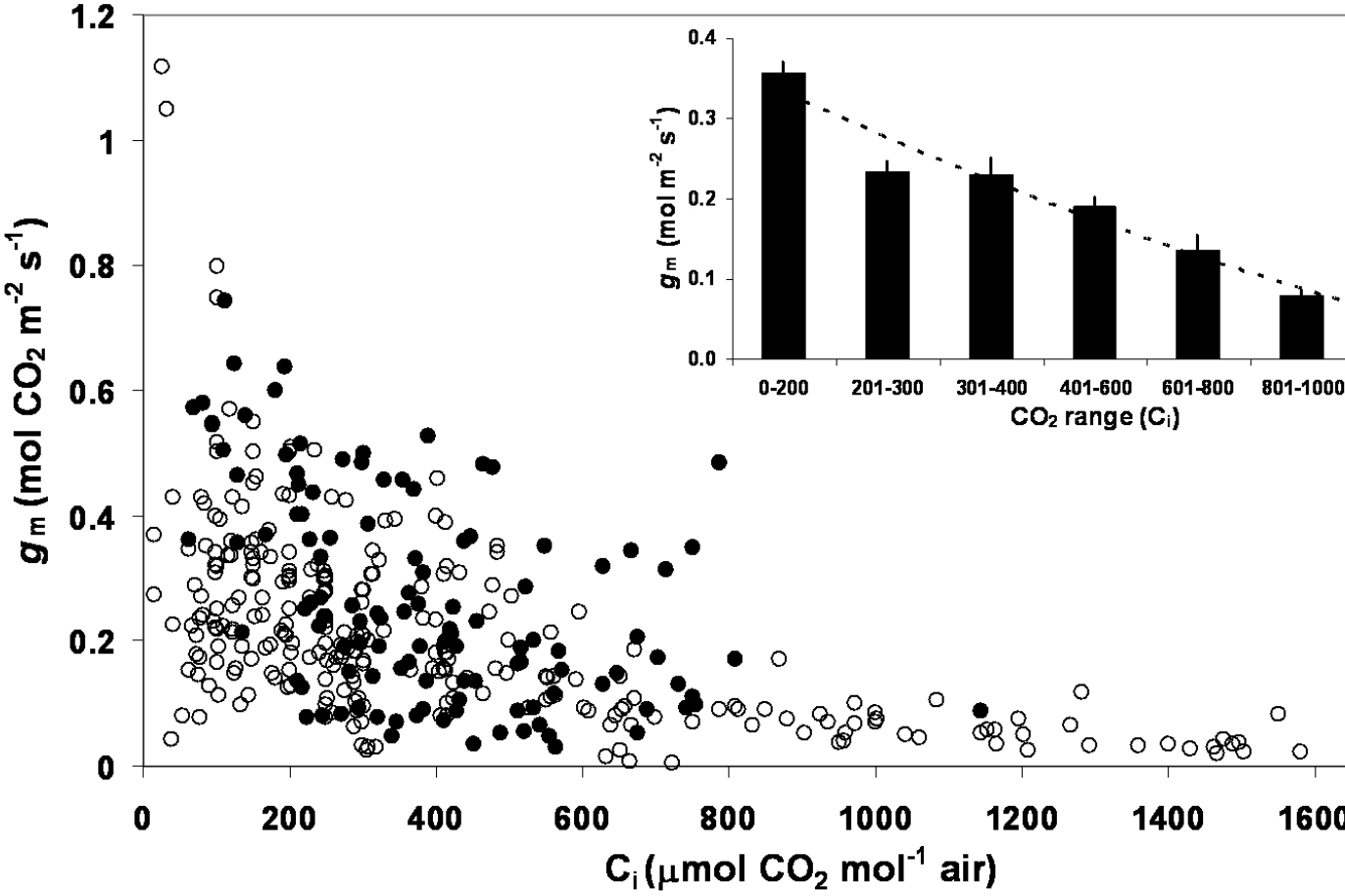
1.2
1
0.8
0.6
0.4
0.2
0
g_m (mol CO_2 m^{-2} s^{-1})
0
200
400
600
800
1000
1200
1400
1600
C_i (µmol CO_2 mol^{-1} air)
0.4
0.3
0.2
0.1
0.0
g_m (mol m^{-2} s^{-1})
0-200
201-300
301-400
401-600
601-800
801-1000
CO_2 range (C_i)

FIGURE 5

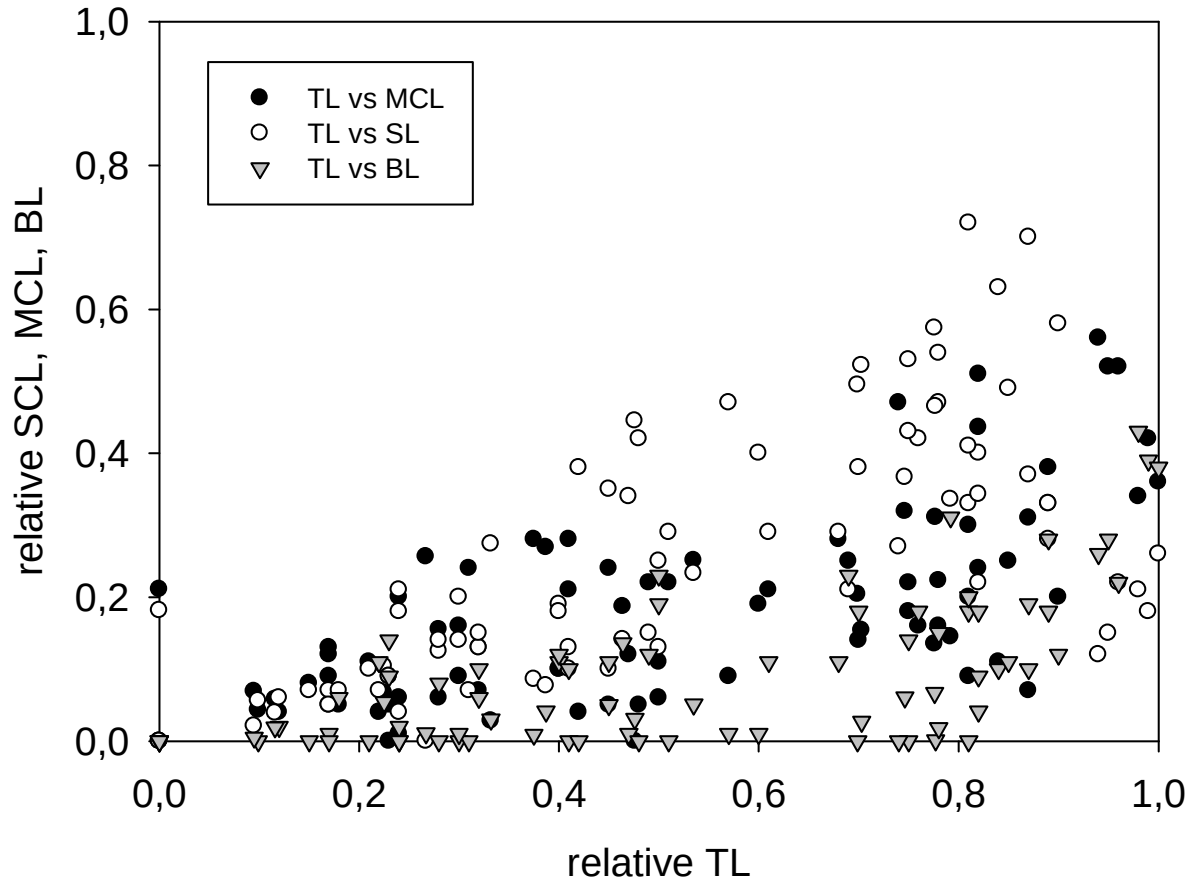
TL vs MCL
TL vs SL
TL vs BL
relative SCL, MCL, BL
relative TL
0,0
0,2
0,4
0,6
0,8
1,0

FIGURE 6

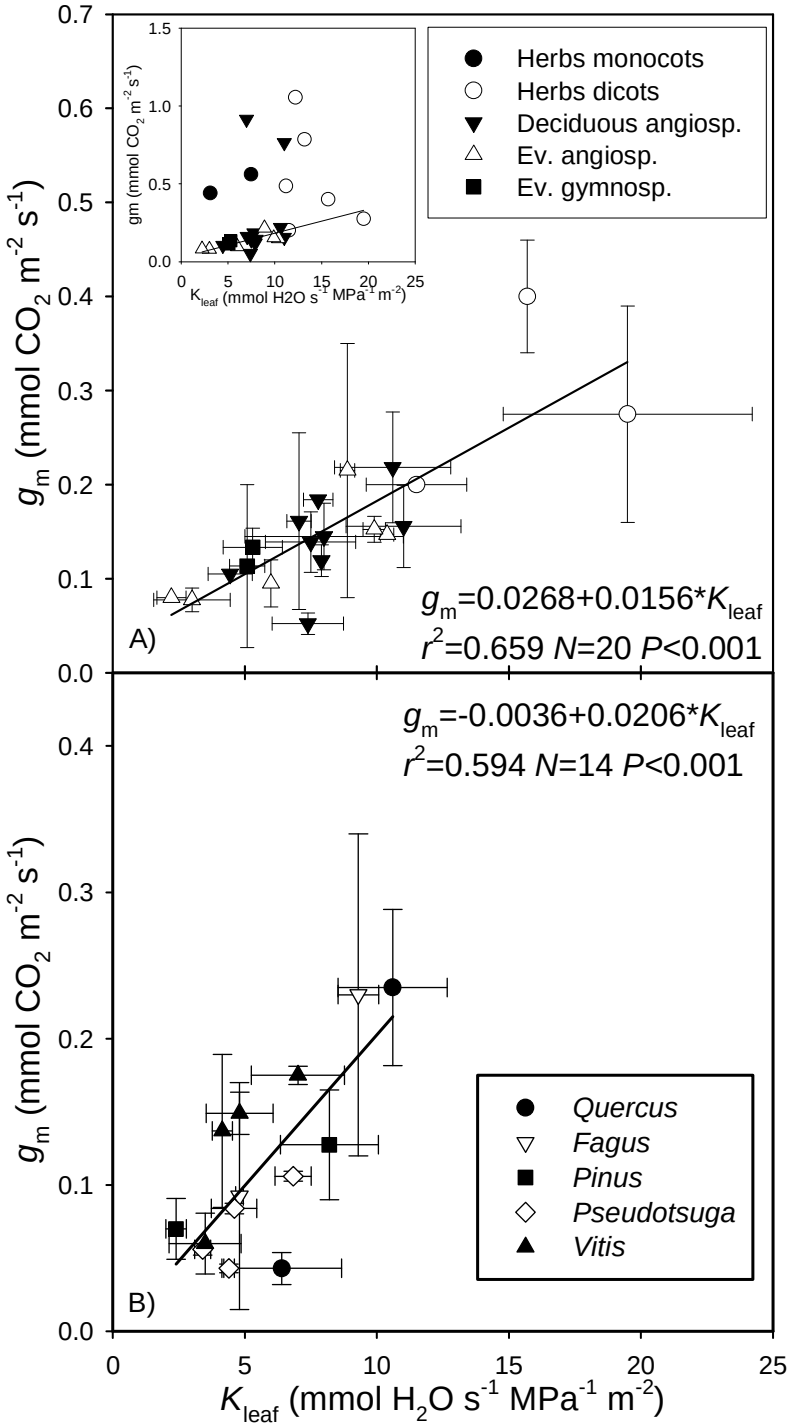
Herbs monocots
Herbs dicots
Deciduous angiosp.
Ev. angiosp.
Ev. gymnosp.
gm (mmol CO2 m-2 s-1)
Kleaf (mmol H2O s-1 MPa-1 m-2)
gm=0.0268+0.0156*Kleaf
r2=0.659 N=20 P<0.001
A)
gm=-0.0036+0.0206*Kleaf
r2=0.594 N=14 P<0.001
Quercus
Fagus
Pinus
Pseudotsuga
Vitis
B)
gm (mmol CO2 m-2 s-1)
Kleaf (mmol H2O s-1 MPa-1 m-2)

FIGURE 7

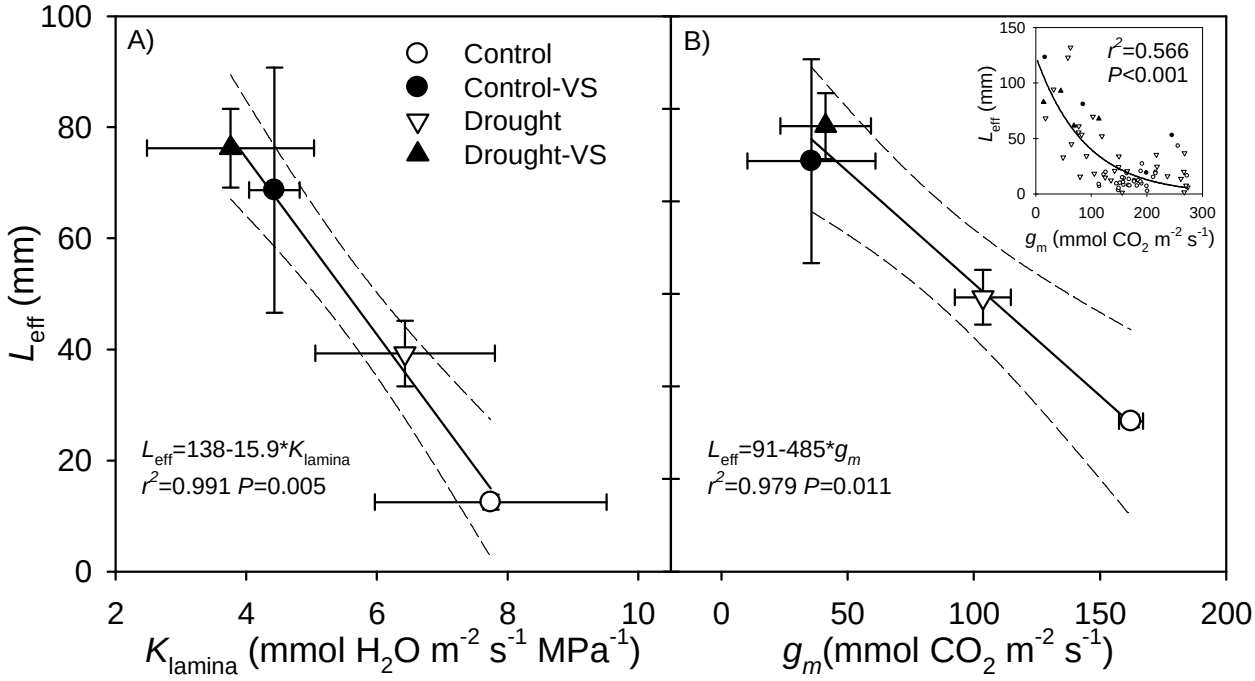
A)
B)
Control
Control-VS
Drought
Drought-VS
L_{eff}=138-15.9*K_{lamina}
r^2=0.991 P=0.005
L_{eff}=91-485*g_m
r^2=0.979 P=0.011
r^2=0.566
P<0.001
L_{eff} (mm)
g_m (mmol CO_2 m^{-2} s^{-1})
K_{lamina} (mmol H_2O m^{-2} s^{-1} MPa^{-1})
g_m(mmol CO_2 m^{-2} s^{-1})

FIGURE 8

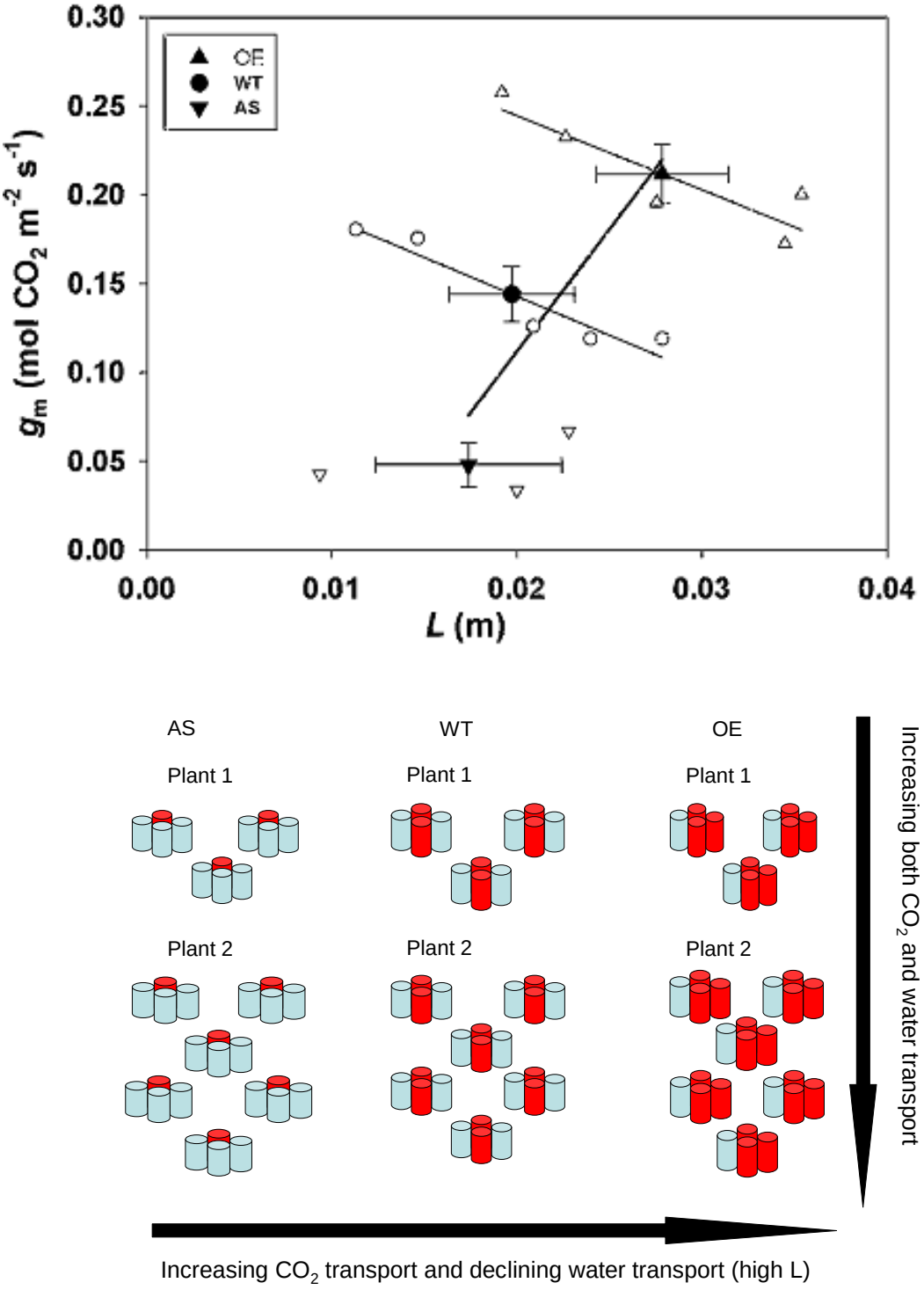
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00
0.01
0.02
0.03
0.04
OE
WT
AS
g_m (mol CO_2 m^{-2} s^{-1})
L (m)
AS
WT
OE
Plant 1
Plant 1
Plant 1
Plant 2
Plant 2
Plant 2
Increasing both CO_2 and water transport
Increasing CO_2 transport and declining water transport (high L)

FIGURE 9

Appendix A: Supplementary data

Table S1. Average and standard deviation of leaf hydraulic conductance (K_{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.

Genus	K_{leaf} (mmol H_2O s^{-1} MPa^{-1} m^{-2})	g_m (mmol CO_2 m^{-2} s^{-1})	OUT	References K_{leaf}	References g_m
Herbaceous monocots					
Triticum	3.1	0.44 ±0.072	X	[1],[2],[3]	[4],[7],[5],[6],[8]
Oryza	7.5 ±3.89	0.56 ±0.410	X	[9]	[10],[11],[8]
Herbaceous dicots					
Phaseolus	19.5 ±4.72	0.28 ±0.115		[12],[13]	[14],[15],[16],[17],[18],[8],[19]
Helianthus	13.2 ±3.31	0.79 ±0.545	X	[12],[20],[21]	[22],[23],[17],[24],[25]
Vicia	15.7	0.40 ±0.060		[26]	[6],[27]
Glycine	11.2	0.49 ±0.165	X	[28]	[29],[30],[31]
Arabidopsis	11.5 ±1.90	0.20		[32]	[33],[34]
Nicotiana	26.0 ±4.00	0.30 ±0.205	X	[35]	[36],[37],[33],[30],[38],[39],[31],[40],[41],[42],[8]
Gossypium	12.2 ±1.10	1.06 ±0.745	X	[28],[43]	[22],[44]
Woody deciduous angiosperms					
Quercus	7.5 ±1.71	0.14 ±0.032		[45],[46],[48],[20],[47]	[31],[49],[6],[50],[51],[52],[53],[54]
Fagus	7.1 ±0.46	0.16 ±0.094		[46]	[55],[19]
_Acer	7.4 ±1.35	0.05 ±0.011		[56],[20]	[58],[17],[57],[50]
alnus	7.9	0.12 ±0.017		[59]	[17],[57],[50]
populus	10.6 ±2.20	0.22 ±0.059		[45]	[60],[17],[57],[50],[51],[61],[52],[18]
betula	11.0 ±3.00	0.77 ±0.335	X	[47],[62]	[63],[64],[65],[66]
Tilia	7.0 ±0.10	0.92 ±0.415	X	[45]	[65],[24]
Juglans	8.0 ±3.00	0.15 ±0.035			[67]
Corylus	7.8 ±0.56	0.18		[20]	[50]
Castanea	4.4 ±0.83	0.11		[20]	[22],[55],[68]
Vitis	11.0 ±2.18	0.16 ±0.044		[1],[47]	[69],[1],[70],[71],[15],[33],[72]
Woody evergreen angiosperms					
Quercus	3.0 ±1.44	0.08 ±0.013		[20]	[73],[17],[74],[6],[75],[76],[52]
Olea	8.9 ±0.28	0.22 ±0.135		[20]	[77],[78],[79],[33],[80],[75]
Camellia	6.0	0.10 ±0.025		[21]	[17],[74]
Hedera	10.4 ±0.26	0.15		[47]	[6]
eucalyptus	9.9 ±0.42	0.15 ±0.014		[81] [82],[83]	[49],[6],[8],[84],[85],[86]
Laurus	2.2 ±0.56	0.08		[20]	[75]
Woody evergreen gymnosperms					
Pinus	5.3 ±1.12	0.13 ±0.020		[87],[48],[88]	[14],[50],[89]
Pseudotsuga	5.1 ±0.67	0.11 ±0.087		[48],[90]	[14],[91],[50],[92],[90]

References Appendix A

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Appendix B

Leaf water enrichment models and scaled effective path length

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

$$\Delta_e = \varepsilon^+ + \varepsilon_k + \left(\Delta_v - \varepsilon_k\right)\frac{e_a}{e_i} \qquad \text{(Eq. S1)}$$

where ε^+ is the equilibrium fractionation between liquid water and vapour [3]; ε_k is the kinetic fractionation as vapour diffuses from leaf intercellular spaces to the atmosphere [4-5], Δ_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 (Δ_{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]:

$$\Delta_{LsP} = \Delta_e \frac{1 - e^{-\wp}}{\wp} \quad \text{with } \wp = \frac{v \cdot l}{D} \qquad \text{(Eq. S2)}$$

where $\wp$ (*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 $^2H^1HO$) in 'normal' water. Linear velocity through the water pathway (v) can be related to transpiration rate (E, mol H_2O m^{-2} s^{-1}) as follows [7]:

$$v = k\frac{E}{C} \qquad \text{(Eq. S3)}$$

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, $\wp$ can be also expressed as:

$$\wp = \frac{E \cdot L_{\text{eff}}}{C \cdot D} \qquad \text{(Eq. S4)}$$

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

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