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Review Article

Mesophyll conductance: the leaf corridors for photosynthesis

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Besides stomata, the photosynthetic CO_2 pathway also involves the transport of CO_2 from the sub-stomatal air spaces inside to the carboxylation sites in the chloroplast stroma, where Rubisco is located. This pathway is far to be a simple and direct way, formed by series of consecutive barriers that the CO_2 should cross to be finally assimilated in photosynthesis, known as the mesophyll conductance (g_m). Therefore, the g_m reflects the pathway through different air, water and biophysical barriers within the leaf tissues and cell structures. Currently, it is known that g_m can impose the same level of limitation (or even higher depending of the conditions) to photosynthesis than the wider known stomata or biochemistry. In this mini-review, we are focused on each of the g_m determinants to summarize the current knowledge on the mechanisms driving g_m from anatomical to metabolic and biochemical perspectives. Special attention deserve the latest studies demonstrating the importance of the molecular mechanisms driving anatomical traits as cell wall and the chloroplast surface exposed to the mesophyll airspaces (S_c/S) that significantly constrain g_m. However, even considering these recent discoveries, still is poorly understood the mechanisms about signaling pathways linking the environment a/biotic stressors with g_m responses. Thus, considering the main role of g_m as a major driver of the CO_2 availability at the carboxylation sites, future studies into these aspects will help us to understand photosynthesis responses in a global change framework.

Photosynthesis: a three-team 'match'

In C3 plants, three major physiological and biochemical processes drive photosynthesis: the stomatal conductance (g_s), the mesophyll conductance (g_m) and the biochemistry lead by the Rubisco enzyme. Besides stomata, the photosynthetic CO_2 pathway also involves the diffusion of CO_2 from the sub-stomatal air spaces inside to the carboxylation sites in the chloroplast stroma (where Rubisco is located). This pathway is far from being a simple and direct way, consisting of a complex of consecutive barriers up to the stroma carboxylation sites, when considered jointly is referred as the internal or mesophyll conductance [1]. Therefore, the g_m reflects the pathway through different air, water and biophysical barriers, which we discuss in more detail here.

Mesophyll conductance is usually obtained by three different methodologies, combining gas exchange measurements with either online carbon and oxygen isotope discrimination [2–5] or chlorophyll fluorescence [6,7]. But a combination of both techniques is the most used [8–10]) and also by the curve-fitting method employing A_N-C_i curves [11–13]. In earlier works, g_m was considered infinite and thus not a limiting factor of photosynthesis, so g_s and photo-biochemistry were considered the main players driving the photosynthesis 'game' (Figure 1A). In this simplified vision, only one factor would be the most limiting for photosynthesis under a certain condition and improving such a single
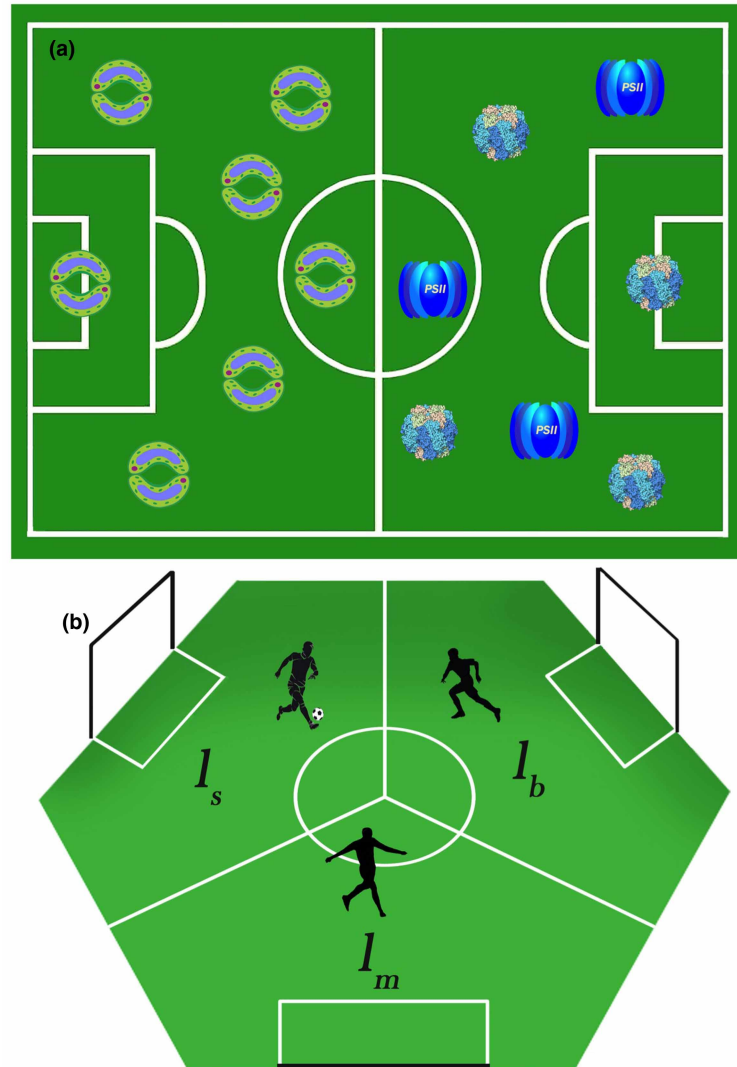
Figure 1. Photosynthesis: a three-team 'match'.

(**A**) Photosynthesis was traditionally considered as a balance between the stomatal conductance (g_s) regulating the CO_2 diffusion (i.e. the 'supply') and the pool of photo-biochemistry reactions, here represented as a classical soccer match game with two players: 'g_s team' *versus* the 'photo-biochemistry team'. Like in a soccer game, only three results are possible: (1) one 'wins' (i.e. limits photosynthesis the most), (2) the other does, or (3) the two 'tie' (i.e. co-limited photosynthesis). (**B**) However, mesophyll conductance (g_m) can impose significant limitations to photosynthesis (l_m), of the same order of magnitude than the limitations imposed by the stomata (l_s) and the photo-biochemistry (l_b), which is illustrated as a much complex football match being played by three teams simultaneously, i.e. largely increasing the number of potential 'results' (i.e. combinations of main limiting factors).

factor should lead to increased photosynthesis. Additionally, under this assumption C_i should be equal to C_c, but it is now well accepted that g_m can impose significant limitations to photosynthesis (in the same order of magnitude or even more than g_s) depending on the plants and the prevailing environmental conditions, implying that C_c will be considerable minor than C_i [1,14–16]. It is important to note that the achieved C_c is not just depending of the CO_2 flow within the leaf, but also to the consumption velocity of the CO_2 by the Rubisco integrated to the whole photosynthetic metabolism (i.e. the electron transport rate (ETR) in the thylakoids, the maximum carboxylation rate by Rubisco ($V_{c,max}$), and the RuBP regeneration in the Calvin cycle) [17]. Thus, the photosynthesis game is not just a matter of two, but three players interacting (Figure 1B). In this context,

maximal potential photosynthesis can be limited by one, two or even three of them (if they are co-limiting in a balanced manner). Indeed, it was recently reported that angiosperms showed the largest photosynthetic rates as compared with other phylogenetic plant groups with a co-balanced limitation between these three factors [16].

In this mini-review, we are focused on the latest advances about the mechanisms driving g_m from anatomical to metabolic and biochemical perspectives. Considering the main role of g_m as a major driver of the CO_2 availability at the carboxylation sites, future studies into these aspects will help us to understand photosynthesis responses in a changing environment. If the reader is also interested into the mechanisms driving the role of g_s and Rubisco carboxylation we can suggest several recent works and reviews with the latest insights [18–22].

Mesophyll diffusion conductance
General features

If stomatal conductance can be viewed as the degree of opening of a single door from the atmosphere to inside the leaves, mesophyll conductance can be viewed as an integrative degree of opening of the multiple corridors allowing CO_2 to move from the sub-stomatal cavity to the site of carboxylation inside chloroplasts' stroma. This complex pathway (Figure 2) includes a gas phase component (i.e. the so-called intercellular air spaces conductance, g_{ias}), several aqueous components (cell wall conductance, g_{cw}; cytosol conductance, g_{cyt}; and stroma conductance, g_{st}) and two lipid–protein components (plasma membrane conductance, g_{pl}, and chloroplast membrane conductance, g_{cm}). While these components can potentially vary independently each other, most current methods to estimate internal diffusion only permit and integrative estimate of the diffusion conductance of the whole pathway, i.e. the so-called mesophyll conductance (g_m). For this reason, in the next sections we will mostly refer to g_m only, yet indicating which of the partial conductance is mostly involved whenever this information is available. However, it is worth noting that novel advances are questioning this approach to CO_2 diffusion due to the potential artifacts in g_m when considering the re-assimilation of the CO_2 produced during photorespiration [23]. New reaction-diffusion and two-resistance models that consider all processes affecting C_c may provide more accurate estimations of g_m and insight of the additional structural features that affect it, such as mitochondria positioning and the 3-D structure of the mesophyll [24–27].

The anatomical determinants of mesophyll diffusion conductance

Both maximum values of g_m and g_s can be achieved under the same physiological and environmental non-stress conditions [1]. However, g_m is a much more complex photosynthetic trait, since it results from the total CO_2 diffusion efficiency of each of the different gas- and liquid-phase components comprised between the intercellular airspaces and the carboxylation sites [28,29]. In turn, the conductance to CO_2 diffusion of each component is determined by several properties: (1) the CO_2 diffusivity of each phase (e.g. diffusion in the liquid phase is by four orders of magnitude slower than in the gas phase), (2) the diffusion path length, being the shortest pathway the most effective one, and (3) the effective porosity. This last one, is also determined by (i) the structure and composition of the component, which sets the tortuosity and the porosity (effective porosity = tortuosity/porosity), and (ii) the presence of mediators like aquaporins and carbonic anhydrases (CAs) [28,30–32], discussed in more detail in next section. Moreover, the liquid-phase conductance is escalated by both mesophyll and chloroplast surface areas exposed to intercellular airspaces per unit of leaf area (S_m/S and S_c/S, respectively), which allow to increase the area for CO_2 dissolution and to decrease the effective pathway for CO_2 diffusion [29]. The cytosol comprised between the plasma and chloroplast membranes only plays a minor role in limiting g_m, as chloroplasts tend to be closely lined up with cell walls (CWs) under high light conditions to reduce the CO_2 effective pathway [28,29,32]. However, cases have been reported in which chloroplasts detach from the plasma membrane, which leads to a decrease in g_m [33].

Consequently, in order to maximize g_m efficiency, leaves need to reduce the diffusion path length (anatomically determined) and increase both S_c/S and the effective porosity of each cellular component of g_m [28,29]. S_c/S and g_m are tightly correlated across species, genotypes and treatments [9,34–38]. Recent efforts have attempted to manipulate the mesophyll properties to maximize S_c/S and thus consequently increasing the photosynthetic capacity [39]. Besides S_c/S, other important traits appear determining g_m, but, how can we know which are these other traits? For that, analytical 1-D and 3-D models of g_m allow dissecting the mesophyll CO_2 diffusion pathway by modeling the partial limitation imposed by each pathway component on g_m [25,31,35,37,40–42]. Interestingly, these models have revealed that usually the main constraints on g_m reside in the CW and in the chloroplast stroma.
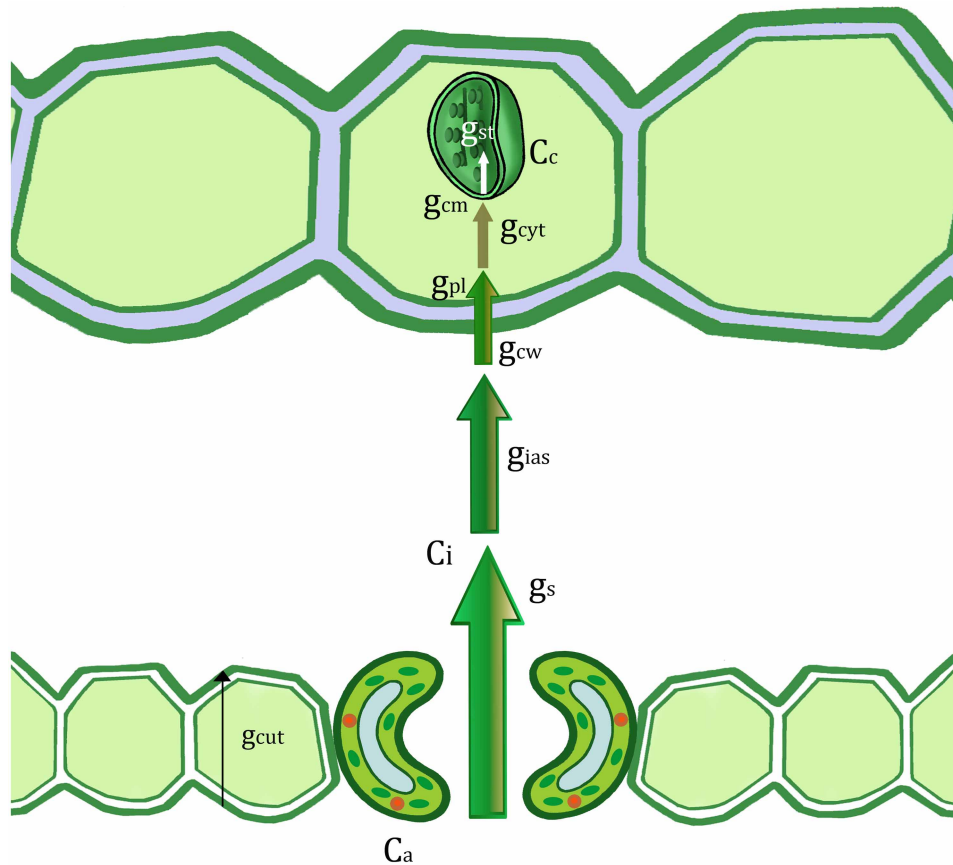
Figure 2. The gates and leaf corridors for photosynthesis.

Both stomatal and mesophyll conductance (g_s and g_m, respectively) are the CO_2 diffusion pathways from the stomata guard cells into the mesophyll tissues where photosynthesis take place inside chloroplasts. From the atmosphere the CO_2 (C_a) diffuses through the guard cells of stomata (g_s) into the sub-stomatal cavities at a certain concentration (C_i). From the sub-stomatal cavities the CO_2 crosses a series of biophysical barriers composed by air, water and lipid elements that are reflected by g_m. The internal gas-phase diffusion (g_{ias}) is the CO_2 pathway from the sub-stomatal cavities to the outer surface of the mesophyll cell walls and is determined by the effective mesophyll thickness, porosity and tortuosity. From this point the CO_2 diffuses through the liquid-phase diffusion (g_{liq}) that basically comprises the cell structure barriers up to the carboxylation sites in the chloroplast stroma. This phase consists of the conductance through the cell wall (g_{cw}), the plasma membrane (g_{pl}), the cytoplasm (g_{cyt}), chloroplast membrane (g_{cm}) and the chloroplast stroma (g_{st}) up to the carboxylation sites (C_c) where Rubisco is located to perform the photosynthetical process.

Cell wall, the first component of the liquid phase, can impose up to 90% of the g_m limitations [38] and a recent data compilation of CW thickness (T_{cw}) has been measured in hundreds of species [16]. It is tightly correlated with g_m, describing a linear negative function when accounting for angiosperms and ferns [29,36,37,39], which turns into an exponential decay function when including the thick-walled gymnosperms and bryophytes [38,43]. The other main physical limitation is the chloroplast stroma, where the carbon fixation by Rubisco occurs [28,44]. Due to the low affinity of Rubisco for CO_2, it is suggested that the resistance for CO_2 diffusion in the stroma decreases with the Rubisco content per unit S_c/S (i.e. the thinner and elongated the chloroplast (thus, exposing a higher surface of the chloroplast to the mesophyll airspaces) the better photosynthesis increase) [29]. Thus, chloroplast stroma would be the major limitation to g_m in species with high photosynthetic capacity and very thin CWs [35,45], or in species that present very thick chloroplasts as is the case of some lycophytes [37,38]. Nevertheless, as it happens with CWs, the effective diffusivity (or porosity) of the chloroplast stroma is still unknown. Keeping the focus inside the cell, the role of intercellular space on setting g_m is presumably smaller. Due to its high diffusivity is normally considered to be the easiest component to go

through, reason by which some studies neglect it [26,28,30]. However, mesophyll porosity can vary greatly between species from 3 to 73% [26,46] and, in species with thick leaves and especially dense mesophyll tissues, the tortuosity, the connectivity and the lateral path lengthening of this component can be especially affected, causing important intercellular space limitations to g_m [26,47].

Last but not least, in the last few decades some studies have reported that cytosol receives, apart from the CO_2 flux from the plasma membrane, a flux of CO_2 photorespired by mitochondria [30] and released by chloroplasts [48]. Although the presumably contribution of this photorespired CO_2 in the general CO_2 flux would depend on the arrangement of mitochondria and chloroplasts, traditional photosynthesis models assume a tight arrangement of chloroplasts closely lined up to the plasma membrane, being mitochondria located behind chloroplasts. So, CO_2 released by mitochondria into the cytosol could diffuse to other sinks than chloroplasts and, consequently, it would convert artifactual g_m estimations since these wouldn't be represented in the sum of physical resistances [25,27,41].

Mesophyll conductance responses to the environment

Mesophyll conductance is nowadays widely recognized as a determinant of photosynthesis changes in response to abiotic environmental variations from the short- to the long-term [1,32]. At the short-term studies (i.e. seconds to minutes), g_m has been established to respond, although neither in all species nor under all conditions, to CO_2 [49–53], light [49,50,52–54], temperature [14,40,55–59], drought [60–62] and salinity [13,63–65]. However, these short-term responses have to be taken with caution, as in most cases there is no clear evidence of the mechanistic basis regulating those g_m changes [28,38,59,66] and several potential artifacts or problems in the calculations of the models have been detected (e.g. type-II errors, effects of photorespired CO_2, intra-leaf light gradients) [25,27,30,67,68]. On the other hand, at long-term studies more evidences of g_m regulations have been obtained in response to CO_2, light, temperature, ozone, water stress and/or nutrients, including anatomical variations [31,57,69–73], changes in aquaporin (AQPs) as well as CAs expression and activity [74]. The mechanisms by which environmental conditions are sensed and signaled to induce modifications of g_m are still poorly understood and an important matter of debate [13,14,59,75]. As with g_s, hydraulic signaling has been hypothesized — but not firmly demonstrated — because water and CO_2 share a significant fraction of their respective pathways inside leaves [32,58,76,77].

To cope with the environmental stressors, abscisic acid (ABA) is the main abiotic stress-related phytohormone in seed plants [78], which also has been shown to induce modifications of g_m [51,79]. However, in these studies it could not be discerned whether the effect of ABA on g_m was direct or indirect through modulating g_s nor both conductance co-regulation through an independent mechanism. Recently, uncoupled responses between g_s and g_m to ABA have been described in *Arabidopsis* mutant lines lacking OST1 and SAC1, suggesting a direct effect of ABA on g_m through a pathway independent of that for g_s [75]. Nevertheless, more studies are needed to understand the signaling pathways linking the environment a/biotic stressors with g_m change responses.

Biochemical regulation of the mesophyll diffusion conductance

The biochemical mechanisms driving g_m still remain mostly unknown, however in the last years this is becoming an emergent and exciting topic within the scientific community. Several of the different traits previously mentioned are driven and/or modified by known metabolic routes, but almost never explored in relation to g_m.

We stated previously that CW thickness (T_{cw}) is one of the most important anatomical parameters related to g_m. However, there are no direct measurements of the effective porosity of CWs to CO_2 diffusion for terrestrial plants, in which only some approximations or assumptions about its relationship with T_{cw} are available [29,31,35,80]. It is known that CW pores are an order of magnitude larger than CO_2 molecules [28], for which how T_{cw} affects CO_2 diffusion still is unclear. Otherwise, it could be not only the thickness of the CW what matters but other non-anatomical factors could determine g_m. In particular, how both the way CW structure and its biochemical composition could affect structural features (such as porosity and tortuosity) and/or provoke different physico-chemical interactions in the CO_2 diffusion pathway deserves further exploration. CWs are not stationary structures within the tissues; in which continuously take place-remodeling processes in response to environmental and physiological *stimuli* by abiotic/biotic factors [81,82]. Indirect evidences from multi-species meta-analysis modeling showed exclusive associations between g_m with metabolites mostly related with CW metabolism, such as xylose, arabinose, hydroxybenzoate and gluconate [83]. More recently, different authors have reported how changes in CW composition (specifically hemicelluloses and pectins) can affect g_m. For example, rice mutants with defective production of mixed-linkage glucan showed reductions in g_m [84]. Under salinity and drought stress, leaves tobacco plants

displayed modifications in the CW composition (mainly changes in pectins and the ratio pectins/hemicelluloses) associated with gm functionality [85]. In turn, it was related with the apoplast redox state and its antioxidant enzymatic activity, such as peroxidases, so altogether driving CW composition changes [85]. These novel studies offer additional information that may enable us to understand the biochemical and molecular mechanisms driving g_m and its responses to abiotic stressors.

Besides CWs within the liquid phase, an additional 'barrier' of lipid nature consists of both cell and chloroplast membranes. Still is a matter of debate their permeability to the CO_2 because the proposed current values differ in orders of magnitude [1]. Even more uncertainties can be expected considering that lipid and protein membrane composition can be strongly remodeled in response to the environment [86,87]. Under stress conditions, membrane antioxidant lipophilic composition (carotenoids and tocopherols) can be altered [88–90], as well as the integral transmembrane proteins activity [91,92]. Unfortunately, how membrane composition affects the direct permeation coefficient still remains unexplored in the field.

On the other hand, AQPs are channel proteins that can facilitate CO_2 diffusion into the cells [93,94] and its activity were tested *in vivo* showing higher g_m in AQPs overexpressing plants [95–98]. Indeed, those increases were lately related with higher productivity in rice plants overexpressing the *Oryza sativa* Plasma membrane Intrinsic Protein 1;2 (abbreviated as PIP1;2) with increased g_m by 150% compared with the wild type [99]. Interestingly, in general g_m increases concomitantly co-ordinates with increases in g_s, thus increasing A_N but not the WUEi [17]. In addition, employing tobacco NtAQP1 RNA interference (RNA$_i$) plants it was shown that CO_2 permeability was reduced by 90% in chloroplast envelopes, however just 10% in the cell membrane. Interestingly, these reductions just have a slight effect in g_m (*ca.* 20%) [97]. Although there is an important uncertainty regarding the permeability of the lipid membranes, AQPs presumably constitute a compensatory mechanism to ensure CO_2 supply into the stroma.

Another family of proteins related to g_m, the carbonic anhydrases (CAs, located mostly in the stroma but also in mitochondria, cytosol and plasma membrane) are zinc metalloenzymes that catalyze the interconversion of CO_2 into HCO_3^- with higher efficiency [100]. Despite earlier experiments overexpressing CAs showed little improvements in g_m and A_N [101,102], recent studies have shown evidence of their potential role in g_m [74,103]. Further studies in a latitudinal genotype transect in *Populus trichocarpa* reported that northern genotypes showed higher A_N relating positively g_m with their elevated CAs activity [104]. Altogether, the role of CAs in g_m in C3 species is still poorly understood, most probably because of the redundant functions of CAs, their multiple cell locations and roles in any reaction that implies CO_2 or HCO_3^- [17,105].

Conclusion

If the stomata are considered the gates of photosynthesis, there is no doubt that mesophyll conductance can be considered the final corridors. However, its complex nature still avoids to fully understanding the main mechanisms driving its responses. Here, we reviewed the most important g_m determinants to summarize the current knowledge of the mechanisms driving it from anatomical to metabolic and biochemical perspectives. In accordance, gas, liquid and lipid barriers determine g_m, in turn all of them can be affected by responses to environmental factors (mainly, light, CO_2 concentration and water availability). For this reason, more studies unraveling and integrating the knowledge from anatomical to metabolomic and biochemical determinants of g_m is needed. This information will be essential to address crop improvement on maximal photosynthesis capacity and WUE in the global change scenario.

Perspectives

- **Importance of the field:** Mesophyll conductance (g_m) is a major actor driving photosynthesis and water use efficiency (WUE). It describes the CO_2 pathway from the sub-stomatal cavities to the Rubisco carboxylation sites in the chloroplast stroma of the mesophyll cells. Its importance relies on the fact that g_m can limit photosynthesis as much as stomatal conductance and the photobiochemistry.

- **Summary of the current thinking:** Besides its well-recognized importance, the large complexity of g_m has limited the knowledge acquisition about its mechanistic basis. While the CO_2 pathway through stomatal cavities is simple and straight; across the mesophyll CO_2 should

cross a series of consecutive biophysical barriers through leaf tissues and cell structures diffusing in different media (air, lipids, and aqueous phases). Therefore, understanding the main molecular mechanisms driving changes in the relevant anatomical traits affecting g_m is currently a major research priority.

- **Future directions:** More research efforts are needed to understand the mechanisms driving g_m (and thus photosynthesis) responses to both abiotic and biotic factors. Any of the biophysical barriers that constraints g_m can be affected by these factors (light, CO_2 concentration, water availability…) in a complex manner and at different time scales. Thus, studies integrating different scales to attempt deciphering the molecular mechanisms from metabolism to physiology are needed. Moreover, this knowledge will help designing new crop breeding strategies to maximize photosynthesis and WUE in the global change scenario.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Author Contribution
J.G. and J.F. conceived and designed the idea of this mini-review. J.G., D.M.D. and J.F. wrote the first draft of the paper with subsequent inputs from all co-authors. M.N., M.M. and J.F. developed the figures and dataset compilation.

Abbreviations
ABA, abscisic acid; A_N, net photosynthesis rate; AQPs, aquaporins; CAs, carbonic anhydrases; C_c, chloroplast CO_2 concentration; C_i, intercellular CO_2 concentration; CO_2, carbon dioxide; CW, cell wall; ETR, electron transport rate; g_{cm}, chloroplast membrane conductance; g_{cw}, cell wall conductance; g_{cyt}, cytoplasm conductance; g_{ias}, gas-phase diffusion conductance; g_m, mesophyll conductance; g_{pm}, plasma membrane conductance; g_s, stomatal conductance; g_{st}, chloroplast stroma conductance; Rubisco, Ribulose-1,5-bisphosphate carboxylase/oxigenase; S_c/S, chloroplast surface areas exposed to intercellular airspaces per unit of leaf area; S_m/S, mesophyll surface areas exposed to intercellular airspaces per unit of leaf area; $V_{c,max}$, the maximum carboxylation rate by Rubisco; WUE, water use efficiency.

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