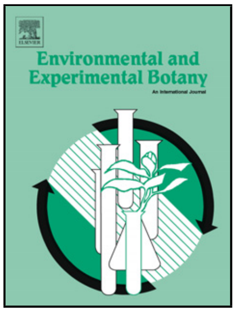

The photosynthesis game is in the ïnter-plaÿ: mechanisms underlying CO_2 diffusion in leaves

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The photosynthesis game is in the "inter-play": mechanisms underlying CO_2 diffusion in leaves

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Highlights

- At optimum conditions diffusional limitations (driven by stomatal conductance (g_s) and mesophyll conductance (g_m)) impose around two-thirds of the total photosynthetic limitation in plants
- -Under stress photosynthetic limitations due to diffusional restrictions can reach 85% of the total
- Knowledge about each of the conductances differs into their focus, meanwhile signalling pathways regulating g_s are highly known, the biochemical and signalling pathways regulating g_m remains almost unexplored.
- -Knowledge about each of the conductances is unbalanced, molecular and anatomical determinants of g_s are highly advanced meanwhile
- -The possible molecular mechanisms that drive the known coupled behaviour between both conductances remains almost unexplored

Abstract

Ensuring global food security is a worldwide major concern considering the predicted climate change scenarios for the main agricultural regions of the world. Stomatal conductance (g_s) and mesophyll conductance (g_m) are major drivers limiting photosynthesis (A). Both conductances frequently impose about two-thirds of the total photosynthetic limitation under optimum conditions. However, under abiotic stress, like drought or salinity, the diffusional limitations can reach more than 85% of the total. Thus, knowledge about both conductances is essential to improve water use efficiency (WUE) through targeted crop breeding programs and to promote sustainable, resource-efficient, and environmental-friendly agriculture strategies.

Intriguingly, knowledge obtained from both conductances from decades of research differs importantly by their focus. Whilst the role of both mechanics and metabolism of guard cells on the regulation of stomatal movements remains much less understood, the signaling pathways that regulates stomatal movements are well-documented. The opposite is true for g_m, in which the biochemical regulation and signaling pathways remain mostly still unexplored. Even more surprising is the lack of information about the putative molecular mechanisms that should drive the known coupled behavior of both conductances in response to the environment. Here, we discussed the main mechanisms driving the responses of each of the conductances, but highlighting a special focus into the possible common determinants that could link their coupled behavior. Further integrative multidisciplinary studies joining molecular biology and ecophysiology are required in order to improve our understanding of both conductances, the major actors limiting photosynthesis and WUE in a changing environment.

Keywords: photosynthesis; water use efficiency; stomatal conductance; mesophyll conductance; diffusional limitations;

1. Out of the atmosphere and into the leaf: the complex CO_2 pathway to photosynthesis

The origin and early diversification of land plants began in the mid-Paleozoic era (ca. 470 Mya), where earlier plants faced new conditions in an aerial environment, requiring adaptations to obtain a positive carbon balance without being dehydrated out of the aquatic environment (Kenrick and Crane, 1997; Quirk et al., 2014). About 20 million years later, flat-bladed megaphylls, highly specialized photosynthetic structures resembling the so-called ‘leaves’ (*sensu lato*), suddenly spread throughout land floras (Osborne et al., 2004). Another unprecedented evolutionary innovation was the development of impermeable cuticles, closely followed by the appearance of the stomata ca. 400 million years ago, consisting of the stomatal pore surrounded by two guard cells mainly at the leaf epidermis. While it appears that, originally, the main function of stomata was to regulate water loss allowing, for instance, sporophytes to desiccate

(Ducket and Pressel, 2018; Brodribb et al., 2020), this exclusive evolutionary innovation was soon also used by leaves to regulate the CO_2 and water exchange between the leaf and the surrounding atmosphere (Edwards et al., 1998). It is still a matter of debate how leaf evolution, stomata, and the changing climatic conditions interacted and influenced each other leading to the current leaf structure types and functions (Osborne et al., 2004; Franks and Beerling, 2009; Leakey and Lau, 2012). Nevertheless, compelling evidence suggests that stomata have appeared before bryophytes, although the true identity of the stomata ancestor remains unknown (Harris et al., 2020).

Stomata most likely represent the very first step for the photosynthetic CO_2 pathway in vascular plants, and their highly regulated opening / closing leads to a largely variable and dynamic stomatal conductance (g_s). Subsequently, the CO_2 reaches the substomatal cavities at a certain concentration (C_i). Openness of the stomata pore has additional consequences including transpiration of the water vapour present in sub-stomatal cavities (W_i). Thus, the balance among leaf carbon gain and water loss constitutes an essential relationship for plants living in environments with limited water, such as those with important abiotic stresses like drought, salinity and/or high atmospheric vapor pressure deficit. This relationship is known as the water use efficiency (WUE), an essential parameter for agriculture that can be considered in different spatial and temporal scales, and becoming even more important now considering the climatic change predicted scenarios for the agricultural regions (Gago et al., 2014; WWAP, 2018; Leakey et al., 2019).

From the substomatal cavities, the CO_2 diffuses into the mesophyll tissues to the carboxylation sites in the chloroplast, the so-called mesophyll conductance (g_m). This pathway is highly complex because CO_2 should cross different media within the leaves such as airspaces, water and biophysical barriers (such as cell walls -CWs- and membranes), and, moreover, it can be actively regulated by e.g. transmembrane channels like aquaporins and enzymes like carbonic anhydrases to finally reach the Rubisco in the stroma (Flexas et al., 2012). The gas-phase within the mesophyll is known as the intercellular air spaces diffusion conductance (g_{ias}), that is driven by the effective mesophyll thickness and porosity to the outer surface of the CWs. Subsequently, the liquid phase consists of a series of conductances (i.e. the inverse of resistances) including the cell wall (g_{cw}), the plasma membrane (g_{pm}), the cytoplasm (g_{cyt}), the chloroplast envelope (g_{env}) and the chloroplast stroma (g_{st}) itself (Terashima et al., 2011), where finally CO_2 reaches the carboxylation sites in the stroma at a certain concentration (C_c).

While g_s has been measured repeatedly since the onset of gas exchange systems (Gaastra, 1959; Björkman and Holmgren, 1963), it is only more recently that estimates of g_m have been obtained, combining gas exchange measurements with either on-line carbon isotope discrimination (Evans et al., 1986) or chlorophyll fluorescence (Harley et al., 1992) (see Pons et al. 2009 for a review on currently used techniques).

Leaf photosynthesis (A) is totally dependent on the CO_2 availability in the sites of carboxylation and it is thus strongly influenced by both g_s and g_m. Hence, high photosynthetic capacity can be expected associated to high g_s and g_m (Flexas et al., 2013; Onoda et al., 2017; Nadal and Flexas, 2019), and a positive relationship between g_s and g_m can be found across species under optimum conditions (Flexas et al., 2013; Nadal and Flexas, 2018). Nonetheless, there is still a significant variation in the g_m/g_s ratio across species and even genotypes (Flexas et al., 2013), and this ratio changes in responses to abiotic factors like water availability (Flexas et al., 2013) or plant´s nutritional status (Franco-Navarro et al., 2019). This variation has to be considered an important feature for e.g. selection of genotypes based on WUE, especially under drought conditions (Galmés et al., 2011; Théroux-Rancourt et al., 2015). Furthermore, the dynamic behavior of both conductances plays a major role in driving terrestrial primary productivity, but it is still a great challenge to implement the leaf-scale knowledge concerning both conductances into ecosystems and global models. The challenge for modelling studies will be thus to integrate the dynamic of g_s and g_m responses with different remote sensing technologies in order to improve both carbon and water flux predictions at a global scale (Guanter et al., 2014; Sun and Gu, 2014; Knauer et al., 2019, 2020).

In this review, we will focus in the latest advances about the mechanisms driving both conductances (g_s and g_m) as the major drivers of the CO_2 availability at the carboxylation site and leaf-level WUE. In addition, we discuss the essential metabolism interplay between them in an attempt to improve the current understanding of the complex physiological responses of both conductances and photosynthesis.

2. The importance of g_s and g_m and current knowledge on their regulation

As outlined in the above section, g_s and g_m similarly limit photosynthesis in higher plants. However, as recently shown by Gago et al. (2019), their importance as photosynthesis-limiting factors is variable across the land plant's phylogeny as determined in the absence of stress conditions and at saturating light. Combined, g_s and g_m account for more than 75% of the total limitation in bryophytes, lycophytes, and pteridophytes, and they account for about 70% in gymnosperms. In angiosperms, on average, each of the three limiting factors account for about 1/3 of the total limitation, that is, combined diffusional limitations account for 2/3 while photo-biochemical limitations account for only 1/3 of the total limitation. Under abiotic stress e.g. water or salt stress, the diffusional limitations typically increase far more than photo-biochemical limitations, hence accounting for an even larger fraction of total limitations (Flexas et al., 2006; Chaves et al., 2009).

The well-proven importance of the two diffusional photosynthetic limitations contrasts with the scarce knowledge available on some aspects of their respective regulation (Fig. 1). Although the importance of stomata morpho-anatomical features as size and distribution on the regulation of stomatal conductance have been widely investigated (Woolfenden et al., 2018; Zoulias et al., 2019), knowledge concerning the signaling pathways that regulate stomatal movements are also significantly understood. However, meanwhile the major structural determinants of g_m are well-studied, the molecular and biochemical mechanisms behind g_m regulation remain rather unexplored. In the following sections, we will summarize the current knowledge of the anatomical and molecular determinants of g_s (section 3) and g_m (section 4) including their responses to the environment, and finally the coupled behavior and interplay between both conductances driving photosynthesis (section 5).

3. Stomatal conductance: the leaf gates for photosynthesis

Stomata is a key structure for plant environmental signal perception. Environmental cues such as light, CO_2 concentration and water availability are known to impact both stomatal movement and density (Casson and Hetherington, 2010). These signals are directly perceived by guard cells and indirectly through signalling molecules that tightly connect mesophyll photosynthetic activity with guard cells function. At geological time-scale, it is believed that the CO_2 atmospheric concentration was a major force driven stomata morphology and physiology (Franks and Beerling, 2009; Brodribb and McAdam, 2017). For instance, the decrease in environmental CO_2 concentration leads to decreased stomatal size and increased stomatal density, which is believed to be a response to optimize the flux of CO_2 from the atmosphere to the internal leaf space (Franks and Beerling, 2009). Additionally, it has been shown that stomatal movements have acquired an active control over plant phylogenetic groups, resulting in angiosperms with faster stomatal responses, in which the main stress-related phytohormone abscisic acid (ABA) play a central role in coordinating stomatal responses to closure stimulus such as drought, darkness or high CO_2 concentration (Assmann and Jegla, 2016). Deciphering stomata evolution and functionality promoted a great interest from molecular to ecophysiological perspectives in the last decades (Doi et al., 2006; Doi and Shimazaki, 2008; McAdam and Brodribb, 2012; Franks and Britton-Harper, 2016; Deans et al., 2019). Whilst some authors have suggested a gradualistic evolution of stomatal control (Brodribb and McAdam, 2011), others suggest that ancient stomata from basal land plant groups already possess all mechanisms regarding sensitivity and response to the environment as the modern angiosperms (Ruszala et al., 2011; Franks and Britton-Harper, 2016; Chater et al., 2017). In any case, despite some common mechanisms of stomatal regulation, tremendous differences in both stomatal behaviour and morphology are found among the different plant groups. It is clear that basal lineage of plants (e.g. ferns) have much lower g_s, which is, at least to some extent, associated with a lower stomatal density (Lima et al., 2019). However, it is noteworthy that significant differences in stomata morphology are also found even within angiosperm species (McAusland et al., 2016; Lawson and Vialet-Chabrand, 2018). For instance, whilst eudicots typically present kidney-shaped stomata, monocots can have either kidney-shaped or dumbbell-shaped stomata with adjacent subsidiary cells (Bertolino et al., 2019). Therefore, it seems that the combination of patterning of stomata formation and regulation follows specific environmental requirements toward plant WUE improvement.

Several recent evidence indicate that stomata from ferns are able to respond to changes in light/dark and different CO_2 concentrations, although at a much lower velocity (Franks and Britton-Harper, 2016; Lima et al., 2019). Thus, the main achievement of angiosperms stomata is the ability to rapidly respond to environmental cues (McAusland et al., 2016; Lawson and Vialet-Chabrand, 2018). Faster stomata in angiosperms could be associated to genetic modifications such as the appearance of new genes or simply the duplication of a function of old ones coupled to anatomical and physiological differences among these and basal plants (Lima et al., 2019;

Sussmilch et al., 2019a; Sussmilch et al., 2019b). Given that the velocity of stomatal responses are pivotal for plant responses to abiotic stress and improving WUE (Lawson and Blatt, 2014; Lawson and Vialet-Chabrand, 2018), future studies aiming to unveil the evolution and the regulatory mechanisms of stomatal speediness are of crucial importance to improve plant WUE, particularly in crops. Whilst slower stomatal opening may lead to unnecessary water losses and improved WUE in a scenario in which g_s is partially uncoupled to A (McAusland et al., 2016), plants with faster stomatal closure would avoid water loss during stress conditions. Compelling evidence has recently revealed that guard cell starch degradation is a key regulator of light stomatal speediness (Flutsch et al., 2020). It remains unclear, however, how and to which extent this mechanism affects plant performance under fluctuating environmental conditions.

3.1. The determinants of maximum stomatal conductance

Under optimal conditions (i.e. well-hydrated plants, saturating light and low vapour pressure deficits), leaves are able to attain a maximum stomatal conductance (g_{smax}). Potential g_{smax} is achieved by anatomically-driven traits which are established during leaf development: the stomatal density (D_s, i.e. the number of stomata per unit leaf surface) and the stomatal pore depth and maximum area, which are related to size (S_s) and the shape of stomata (Franks and Beerling, 2009; Sack and Buckley, 2016; Harrison et al., 2019). Higher g_{smax} values are achieved by increases in D_s, which are often correlated with decreases in S_s, coordination that has been widely observed as a result of both acclimation and adaptation responses to variations in environmental factors (Franks and Beerling, 2009; Doheny-Adams et al., 2012; Harrison et al., 2019). Coordination between D_s and S_s is apparently under tight selection in angiosperms, determining the optimal use of the epidermal area for g_{smax} (de Boer et al., 2016; Haworth et al., 2018). Only a few species, like some species within *Begonia* genus, have been able to overcome the minimum one-epidermal cell spacing between stomata (Gan et al., 2010; Harrison et al., 2019). Regarding stomatal shapes, which are species-specific but diverse across land plants, some allow opening comparatively wider than others hence being able to achieve higher g_{smax} values. For instance, in angiosperms, dumbbell-shaped stomata allow for wider opening than kidney-like-shaped stomata; and kidney-shaped stomata with thinner guard cells allow for wider opening than the same with wider guard cells (Franks, 2006; Franks and Farquhar, 2007).

Leaves do not usually operate at g_{smax} and thus g_s typically remains at around 20-30 % of g_{smax}, reaching for some angiosperm species up to 39-43%, corresponding to a turgor pressure at which guard cells more efficiently control the aperture of the pore (Dow et al., 2014; McElwain et al., 2016). Even so, it sets a maximum evaporative demand that needs to be matched by a hydraulic supply of the same magnitude. Otherwise, this would result in damage to the water transport tissues, potentially even causing the embolism of the vascular tissue (Brodribb and Feild, 2000; Hubbard et al., 2001; Brodribb and Holbrook, 2004; Brodribb and Buckley, 2018). Thereby, as the leaf hydraulic capacity should coordinate with g_{smax}, the optimization to achieve higher CO_2 diffusion rates of the stomatal properties and density came necessarily associated with a concomitant increase of this hydraulic supply over the more of 400 My of land plant evolution (Brodribb et al., 2020). This coordination occurs, and there is a conservative investment in the water conductive tissues to fair enough supply g_{smax} (Medlyn et al., 2011; Martins et al., 2014; Carins Murphy et al., 2016; Brodribb et al., 2017). Thus, since leaves impose a disproportionately large resistance to the hydraulic system (Sack and Holbrook, 2006), leaf hydraulic conductance (K_{leaf}) is usually tightly correlated to g_s and photosynthesis (Brodribb et al., 2007; Scoffoni et al., 2016). From a mechanistic perspective, the leaf hydraulics link with g_{smax} and D_s is phylogenetically mostly driven by a positive correlation with vein density (D_v) (Boyce et al., 2009; McElwain et al., 2016), although they do not always correlate at the short phylogenetic scale (Gleason et al., 2016; McElwain et al., 2016; Rockwell and Holbrook, 2017).

In summary, g_{smax} is mainly anatomically determined by a link between D_s and D_v and through changes in the leaf cell size (i.e. miniaturizing the size of stomata and veins, but also of other leaf cell types) (Brodribb et al., 2013; Feild and Brodribb, 2013; Carins Murphy et al., 2016; de Boer et al., 2016; Carins Murphy et al., 2017). Interestingly, this, in turn, appears to be scaled by the genome size (Simonin and Roddy, 2018; Roddy et al., 2019). However, despite the great advance in our understanding concerning stomatal development and evolution, how the different stomata morphologies associated with their intrinsic hydraulic and biochemical regulation contribute to stress tolerance remains far from clear (Medeiros et al., 2019). This is particularly due to the fact that the regulation of stomatal movements from highly stress-tolerant groups such as bryophytes, ferns, resurrection and CAM plants remains poorly and fragmentarily studied (Males and Griffiths, 2017), which clearly hampers our understanding on how stomatal physiology could be used in plant breeding programs toward plant stress tolerance.

3.2. Stomatal responses to the environment: perception and signalling

Changes in guard cell metabolism are known to regulate the magnitude of stomatal opening according to the prevailing environmental conditions (Lawson et al., 2014a). Guard cells are thus able to directly recognize light, CO_2 and other signals and then modulate stomatal opening or closure. In general, stomata open in the light and close during dark conditions, with the exception of CAM plants. However, a significant transpiratory stream is detected in the night for many species independent of their origin, and the precise function of this nocturnal conductance (g_n) is still a matter of debate: why plants sustain g_n under dark conditions losing water without carbon gains? Recently, through a multi-species meta-analysis, it was demonstrated that g_n was related to the relative growth rate (RGR) (Resco et al., 2019). These results do not relate with the previous hypothesis explaining the g_n role as the removal of CO_2 excess produced by respiration, O_2 delivery to the vascular system, nutrient supply or stomata leakiness. Notably, it has been postulated that higher g_n would optimize predawn g_s and subsequently earlier carbon gains (Resco et al., 2019 and references therein). Curiously, it appears that g_n responds roughly to the very same environmental factors as day-time g_s (Caird et al., 2007). Nevertheless, and besides the still not fully understood role of g_n, most of our current knowledge on stomatal responses to environmental cues refer to day-time dynamics.

The dynamics of stomatal movement during the day follow a circadian rhythm with a further complex network of interaction with endogenous signals, including the synthesis and transport of phytohormones, ions, metabolites and possibly electrical signals and still highly unknown from mesophyll to guard cells (Gallé et al., 2015; Daloso et al., 2017). Guard cells are thus the hub that integrates environmental and endogenous signals to regulate stomatal movements (Mott et al., 2009). During the diel course, signals from mesophyll cells are key to close the stomata in periods of high *A*, as a mechanism to optimize plant WUE. Some authors indicated that these molecules could be a vapour-phase ions, possibly hydronium ions able to change the pH of the guard cells (Mott et al., 2014). Although the real identity of these signals still remains to be identified, additional evidence suggests that the compounds linking mesophyll and with guard cells could range in the size of 100 to 500 Da. This idea is based in the fact that stomatal responses are disrupted when a membrane excluding molecules in this size range are placed between mesophyll and guard cells (Fujita et al., 2019), which may include sucrose (342 Da), malate (134 Da) and ABA (264 Da). In fact, genetic manipulation of sucrose and malate metabolism leads to altered stomatal movements (Laporte et al., 2002; Nunes-Nesi et al., 2007; Araújo et al., 2011; Antunes et al., 2012; Antunes et al., 2017a; Kelly et al., 2013; Lugassi et al. , 2015; Daloso et al., 2016b; Wang et al., 2019).

Beyond endogenous signals, it is interesting to highlight that environmental cues such as water availability as well as the light quantity and quality strongly influence g_s. Water deficit is a well-recognized signal that induces stomatal closure via ABA and perhaps hydraulic and electrical signals (see next sections 3.2.1 and 3.2.2, respectively), being an important mechanism that restricts CO_2 assimilation in periods of moderate stress (Galmés et al., 2007; Flexas et al., 2009). By contrast, light is the main opening signal where red light was for long believed to influence stomatal behaviour via mesophyll photosynthetic activity (Baroli et al., 2007). However, recent evidence indicates that red light can be directly perceived by guard cells and induces plasma membrane H^+-ATPases phosphorylation (Ando and Kinoshita, 2018), similarly to the well-described blue-light signalling pathway (Inoue and Kinoshita, 2017). Taken all the endogenous and environmental cues that modulate g_s together, it is clear that g_s is very dynamic and highly dependent on the prevailing environmental conditions, in which guard cells act as sensors to integrate all hydraulic, metabolic and light signals to modulate g_s under both non-stress and stress conditions.

3.2.1. Hydraulic signalling

Early works assumed a hydro-passive mechanism for stomatal closure under e.g. water stress or increased vapour pressure deficit (VPD), which intrinsically implies interplay between plant hydraulics and transpiration (see Jones 1998 for a review). In essence, whenever transpiration exceeds the hydraulic capacity of the plant to supply water to leaves, a water deficit occurs in these resulting in decreased leaf water potential and turgor pressure in the guard cells, thus provoking stomatal closure. It has been proposed that such hydro-passive mechanism operates mostly in plants belonging to phylogenetic groups such as lycophytes and pteridophytes (Cardoso et al., 2019), while the ABA-based hydro-active signalling described in the above section is the main system used by seed plants (Brodribb and McAdam, 2011). However, a water potential-based mechanism for the regulation of g_s in response to irradiance and other factors was also proposed (Pieruschka et al., 2010). Additionally, recent evidence indicates that

VPD-stomatal responses appears to involve a passive hydraulic mechanism as well as a signalling pathway that entails OST1 (Open Stomata 1) and SLAC1 (Slow anion channel 1), which would be controlled by either ABA or by a still unknown ABA-independent mechanism (Merilo et al ., 2018).

Besides the hydro-passive mechanism, direct evidence for xylem cavitation driving stomatal closure has been provided. For instance, Salleo et al. (2000) showed that dehydrating air-detached leaves increased stomatal aperture (i.e. the so-called ‘wrong-way response’ or ‘Iwanoff effect’) only after the threshold water potential for the onset of petiole cavitation was reached, after what stomata progressively closed thereafter. Moreover, during a transition from darkness to light, attached leaves initially showed progressively increased stomatal opening and decreased water potential, with no cavitation detected by ultra-acoustic methods. When the water potential for the onset of petiole cavitation was reached g_s was at its maximum and cavitation events started being recorded ultra-acoustically. Thereafter, g_s slightly decreased to reach a steady-state value corresponding to a constant water potential just at the onset of petiole cavitation and the cease of cavitation events (Salleo et al., 2000). Hubbard et al. (2001) provided direct evidence for the involvement of cavitation in stomatal closure. After carefully removing bark and phloem from pine stems, they were able to directly inject discrete amounts of air into the xylem to provoke different degrees of cavitation. After each step injection, an immediate decrease of g_s was recorded, resulting in a perfect linear correlation between g_s and the hydraulic conductance when pooling data for all the injection steps (Hubbard et al., 2001).

Stomata water relation is a challenging topic where several authors proposed recent models based on mechanisms related to ABA, hydraulic and osmotic potential, among others, but it is still needed much more knowledge to develop reliable mechanistic models about stomatal responses to water status (reviewed in Buckley, 2019). Regardless of the mechanism, there is compelling evidence for the existence of a hydraulically-based signalling pathway inducing stomatal closure. The predominance of hormonal *versus* hydraulic stomatal regulation in higher plants is currently the subject of ample debate (Wang et al., 2018; Flexas et al., 2018). While the dominance of either mechanism could be species-specific (Flexas et al., 2018), it is more likely that there is an interplay between the two. In grapevines, for instance, Pou et al. (2008) showed that ABA-based g_s regulation was dominant during the imposition of drought while hydraulic-based regulation was the most important during the subsequent recovery. Additionally, it was shown that grapevines stomatal closure is induced by hydraulic signals but maintained by ABA during drought (Tombesi et al., 2015). Another example of the interplay between the two mechanisms is the indirect involvement of ABA in embolism refilling (Secchi et al., 2013).

3.2.2. Electrical signalling

The operation of electrical signaling controlling stomatal aperture has been much less studied than the hormonal and hydraulic ones. Two different types of electrical signals have been described in plants: action potentials and variation potentials, both involved in the regulation of multiple metabolic and developmental effects (see Fromm and Lautner, 2007 and Galle et al., 2013 for reviews). As it was known for long that watering a dry root is an efficient way of producing action potentials (Pickard, 1973), Fromm and Fei (1998) followed the concomitant variation of g_s and photosynthesis with action potentials during water stress and re-watering of maize plants. They found a perfect co-variation among these parameters, action potential decreasing in parallel to g_s during the imposition of water stress and peaking strong – and preceding stomatal opening by about 15 min – immediately upon re-watering. The latter result led them to hypothesize that action potentials induced by re-watering roots were directly responsible for stomata re-opening. To test this hypothesis, Grams et al. (2007) used a design consisting of potted maize plants with the pots inside a root pressure chamber – to compensate / eliminate hydraulic signals – and a part of the leaf cooled by two aluminum blocks that were cooled by flushing with a mixture of glysanthin/water – to eliminate the propagation of action potentials through the leaf. Using this design, and measuring variations of leaf turgor, action potential and g_s, they observed that upon re-watering stomata first started closing (another ‘wrong-way response’) in a mechanism regulated by hydraulic signals (turgor changes), to then start opening after several minutes in a mechanism regulated by action potentials.

In addition to these examples related to water stress and recovery, Gallé et al. (2013) showed in soybean that wound-induced action potentials in a leaflet provoked a transient stomatal opening in the adjacent leaflets of the leaf and a direct and large decrease of mesophyll conductance and photosynthesis, i.e. a transitory uncoupling of g_s and g_m. It seems reasonable to assume that more studies are clearly required to fully disentangle the role of electrical signalling in controlling g_s (and g_m).

3.2.3. Hormonal signalling

As several physiological processes, the stomatal movement is also highly controlled by phytohormones, in which especially ABA, have been closely associated with the formation of stomata (Chater et al., 2017). Remarkably, an evident cross-talk among stomata formation and stomatal movement regulation is usually observed, in which the role of ABA, auxin, brassinosteroids, gibberellins, ethylene, cytokinins, and jasmonic acid, as well as the genes associated with stomatal development, have been revealed (Bergmann, 2006; Qi and Torii, 2018). Both the generation and the regulation of stomatal movements are extremely sensitive to changes in the environment around the leaf and, broadly characterized and especially rich in complexity, involving gene expression, post-translational modifications, protein-protein interaction, synthesis of signalling compounds such as nitric oxide and reactive oxygen species (ROS) as well as ion transporter activity (Kim et al., 2010). In this vein, the role of ABA is well-documented and will be thus the focus of this section.

ABA is the main phytohormone that connects plant water potential with stomatal movement (Buckley, 2019). Guard cells have been used as a model for the establishment of the ABA signalling pathway, in which the complete signalling pathway including PYR/PYL/RCAR receptors and the upstream components have been identified and confirmed to act in tandem *in vitro* (Fujii et al., 2009; Park et al., 2009; Santiago et al., 2009; González-Guzmán et al., 2012). Given that plants subjected to water deficit conditions have stomatal closure as one of the main physiological responses, which is usually associated with substantial increases in ABA levels, ABA has been associated to be an important regulator especially under stress conditions (Jarzyniak and Jasinski, 2014). Notwithstanding, ABA is seemingly important also under non-stress conditions (Yoshida et al., 2019b), possibly as an important regulator of transpiration, stomatal density (Hetherington and Woodward 2003; Lake and Woodward 2008) and the primary metabolism, as recently has been demonstrated (Yoshida et al., 2019c, a).

ABA has also been pointed as an important contributor to the increased velocity of stomatal responses found in angiosperms when compared to ferns. This idea comes from several studies that show a lack or, at maximum, a slower stomatal CO_2 and ABA responses observed in ferns (Brodribb and McAdam, 2011; McAdam and Brodribb, 2012; Franks and Britton-Harper, 2016; McAdam et al., 2016; Hõrak et al., 2017; Lima et al., 2019). Although key genes of the ABA signaling pathway have also been identified in ferns, it seems likely that they have acquired their role in the control of stomatal movements only in recent evolutionary groups (McAdam et al., 2016; Hedrich and Geiger, 2017). Thus, it seems that ABA acts as an important modulator of the changes in stomatal density and stomatal responsiveness to environmental cues, especially the changes in CO_2 concentration observed at a geological scale. Notably, it suggests that ABA determines not only the plant CO_2 responses but also strongly influencing the earth's CO_2 biogeochemistry cycle (Hetherington and Woodward, 2003; Lima et al., 2018).

3.3. Biochemical regulation of stomata movements

Guard cells have been widely used to understand the molecular and signalling mechanisms of hormonal regulation, especially those associated with ABA metabolism (Assmann and Jegla, 2016). This hormone acts as a hub in the molecular network associated with stomatal closure induced by drought, low humidity, high CO_2 and dark (Kim et al., 2010; Bauer et al., 2013a,b; Engineer et al., 2016b). After its perception by PYR/PYL/RCAR receptors, ABA response is triggered by inhibition of PP2C phosphatases, activation of different kinases such as SnRK2.6/Srke/OST1, synthesis of important second messengers such as ROS, nitric oxide and Ca^{+2} and nitric oxide and, ultimately, activation of ion channels such as QUAC1, SLAH3 and SLAC1 (Yoshida et al., 2019b). It is noteworthy, however, that each stimulus activates particular signaling pathways culminating in stomatal closure.

Beyond that, evidences suggest that stomatal closure is regulated by an intricate regulatory network that involves not only ABA but also ethylene, brassinosteroid, jasmonic acid among other phytohormones (Desikan et al., 2006; Acharya and Assmann, 2009; Zhu et al., 2014). Thus, although the ABA-induced mechanisms regulate both stomata formation and stomatal closure have been widely investigated in *Arabidopsis*, our understanding of how these mechanisms act together with other phytohormones and how they control stomatal movement under stress conditions, where closing and opening stimulus are found simultaneously, remains largely unknown. Given the complexity of these mechanisms, we argue that the only perspective to fully understand stomatal movement is by using systems biology approaches (Li et al., 2006; Wang et al., 2014b; Medeiros et al., 2015; Medeiros et al., 2018).

Beyond the crosstalk between phytohormones on g_s regulation, recent evidence indicates a complex regulation of guard cell metabolism and several particularities that differ substantially from mesophyll cells (Santelia and Lawson, 2016). For instance, guard cell CO_2 metabolism is unique in terms of functioning and regulation. The molecular mechanisms by which guard cells recognize CO_2 involves a complex network of carbonic anhydrases, kinases such as OST1, HT1, GHR1, and MPK12; as well as anion channels such as SLAC1 and QUAC1 (Engineer et al., 2016a; Hedrich and Geiger, 2017; Negi et al., 2018). Alterations in any of these molecular mechanisms may have significant effects in the stomata response to the environment or under stress. For instance, it was described that mutations in the plasma membrane protein *SLAC1* (SLOW ANION CHANNEL ASSOCIATED 1) that mediate guard cell anion efflux by slow anion (S-type) channels altered most of the typical responses to light, CO_2, ozone, ABA, humidity, Ca^{2+}, H_2O_2 and NO (Vahisalu et al., 2008). Furthermore, higher CO_2 fixation in the dark and higher PEPc-mediated CO_2 assimilation have been demonstrated in guard cells of C3 plants, highlighting that the photosynthetic metabolism of these cells does not resemble of those of mesophyll C3 cells (Gotow et al., 1988; Robaina-Estévez et al., 2017). Given the low level of chlorophyll and the few chloroplast numbers found in guard cells (Willmer and Fricker, 1996), RubisCO-mediated CO_2 assimilation was for long-neglected (Outlaw et al., 1979). However, recent evidence highlights the importance of guard cell chloroplasts for stomatal opening (Wang et al., 2014a; Azoulay-Shemer et al., 2015, 2016). By contrast, guard cells possess a higher mitochondria number and higher respiration rate, when compared to mesophyll cells (Willmer and Friker, 1996; Araújo et al., 2011). These features lead to a unique mode of metabolism which resembles a combination of sink and C4/CAM cells (Cockburn, 1983; Daloso et al., 2016a; Daloso et al., 2017).

As a sink cell, guard cells depend on sucrose and other metabolites from source tissues. Evidence supporting this assumption comes also from transcriptomic studies that have shown high expression of sucrose/hexoses transporters found in guard cells and by the higher activity of sink marker enzymes in guard cells (Hite et al., 1993; Ritte et al.,1999; Bates et al., 2012; Bauer et al., 2013a; Daloso et al. 2015). Furthermore, photosynthesis-derived sucrose has been pointed as an important mesophyll to guard cell connectors. Whilst the over accumulation of sucrose and other sugars induce stomatal closure (Kelly et al., 2013; Gago et al., 2016; Li et al., 2016; Medeiros et al., 2018), antisense inhibition of a sucrose importer to guard cells leads to lower guard cell sucrose content and lower g_s (Antunes et al., 2017b). This indicates that sugars are important metabolites for the A-g_s trade-off regulation (Daloso et al., 2016a; Granot and Kelly, 2019). Indeed, alteration of a subsidiary cell-localized glucose transporter, a SWEET transporter that is regulated by the amount of photoassimilates, substantially alter g_s (Wang et al., 2019). Thus, it seems likely that sugars, especially sucrose, mediate stomatal closure in periods of high A, as a mechanism to improve WUE (Kang et al., 2007; Daloso et al., 2016a).

Not only sucrose but also mesophyll-derived malate has also been closely associated to the regulation of stomatal movements (Hedrich and Marten, 1993; Talbott and Zeiger 1993; Laporte et al., 2002; Nunes-Nesi et al., 2007; Araújo et al., 2011; Penfield et al., 2012; Medeiros et al., 2016, 2017). Furthermore, compelling evidence indicates that guard cell malate metabolism does not only affect g_s but also g_m (Medeiros et al., 2016), suggesting that both g_s and g_m may have common regulation points. Indeed, both g_s and g_m have been shown to be couple in a wide range of conditions (Flexas et al., 2002, 2013). Thus, it is clear that both g_s and g_m respond directly to changes in the environment, which affects A. In turn, the CO_2 assimilated by mesophyll cells can be converted into molecules that will be transported to guard cells where it induces stomatal closure (Lu et al., 1995; Lu et al., 1997; Mott et al., 2008; Mott, 2009). This complex interplay among A, g_s and g_m is extremely important in a fluctuating environment, in which opening and closing environmental signals are combined with (a)biotic interactions in the continuum soil-plant-atmosphere to regulate the CO_2 flux through the leaf and, consequently, overall plant WUE.

4. Molecular and physiological mechanisms driving mesophyll conductance

Mesophyll conductance to CO_2 (g_m) refers to the facility offered by the leaf for CO_2 diffusion from the sub-stomatal cavity to the chloroplast stroma where the CO_2 fixation occurs. This is a complex pathway, comprising aerial, aqueous and lipid phases and composed of several steps each one representing a partial component of g_m: intercellular air spaces (g_{ias}), cell walls (g_{cw}), plasma membranes (g_{pm}), cytosol (g_{cyt}), chloroplast membranes (g_{chl}) and stroma (g_{st}). Contrary to stomatal conductance, the regulatory pathways of g_m are poorly understood at the biochemical and molecular levels (Fig. 1).

g_m can be estimated by combining its partial conductance components, which in turn can be approximated dividing values for the diffusivity of CO_2 at a given temperature in each medium (air, water or lipid) by the distance represented by each step pathway (Evans et al., 2009). For instance, g_{cw} is calculated considering CO_2 diffusivity in water and cell wall (CW) thickness (Evans et al., 2009). This is the basis of the widely-used anatomical method to estimate g_m (Niinemets and Reichstein, 2003; Tosens et al., 2012; Tomas et al., 2013; Carriquí et al., 2019). However, this is a simplistic view as it assumes that each partial step is composed of a single medium. Hence, this might be unrealistic as, for instance, cell membranes are formed by lipids but also by abundant proteins (Kjellbom and Larrson, 1984; Engelman, 2005); cytosol and stroma are far from being 'pure water' and, although CW pores might be filled mostly of water, chemical interactions between water, apoplast and CW components (including proteins and phenols) (Mercado et al., 2004; Schiraldi et al., 2012) and diffusing CO_2 should be expected and thus affect the CO_2 passage through the CW. In addition, the presence of active facilitating mechanisms in any of the step pathways would affect the 'apparent' diffusivity of such pathways. Indeed, while the anatomical method for estimating g_m often correlates well with *in vivo* estimates of g_m (e.g. using combined gas exchange and chlorophyll fluorescence or isotope discrimination; see e.g. Pons et al., 2009 and Flexas et al., 2012, 2018 for an ample description of the most commonly used methods) in non-stressed plants, the correlation frequently deviates from the 1:1 relationship (Tomás et al., 2013; Fini et al., 2016; Tosens et al., 2016; Peguero-Pina et al., 2017a,b; Veromann-Jürgenson et al., 2017; Carriquí et al., 2019). Moreover, g_m as determined from anatomy often differs strongly from g_m estimated *in vivo* in plants subject to abiotic stresses, as the former often remains unaffected while abiotic stresses generally induce significant decreases of g_m (Tomás et al., 2014, but see also Tosen et al. 2012). This fact – if not an artifact (e.g. Carriquí et al., 2019a) – most likely reflects stress-induced changes in the partial pathways composition and / or in the activity of potential facilitating mechanisms (Flexas and Díaz-Espejo, 2015). However, as said, the regulatory effects of partial pathway compositions and facilitating mechanisms on g_m are poorly known. Among the former, effects of CW composition on g_m have been proposed (Niinemets et al., 2009; Gago et al., 2016; Ellsworth et al., 2018; Clemente-Moreno et al., 2019; Roig-Oliver et al., 2020) among potential facilitating mechanisms, chloroplast movements, aquaporins and carbonic anhydrases are the most frequently-invoked candidates (Bernacchi et al., 2002; Terashima et al., 2011; Tomás et al., 2013). Nevertheless, it is likely that the composition of other steps in the g_m pathway – such as the specific lipid composition of membranes and/or the abundance and typology of membrane-bound or membrane-integral proteins, or the solutes of cytosol and stroma – also have relevant effects on CO_2 diffusion and hence g_m, yet none of these have been investigated yet.

4.1. Cell wall dynamics

The structure and interaction of cellulose, hemicelluloses, pectins, lignin, phenols and structural proteins will determine the size of the free spaces within the CW (Carpita et al., 1979; Baron-Epel et al., 1988; Fleischer et al., 1999; Lamport and Kieliszewski, 2005) that the CO_2 should cross in its way to the stroma carboxylation sites (Gago et al., 2019). CWs are dynamic structures that can quickly respond to their environment changing both chemical composition and physical structure (Houston et al., 2016). For instance, under salt or drought stress, the apoplastic media that is usually acidic, increase their pH which may difficulting the CO_2 solubilization and alter proteins functionality. Moreover, in these stress conditions, the oxidative response includes both ROS increase and the activation of different apoplastic enzymes that are also key players in CW remodelling (Pitann et al., 2009; O'Brien et al., 2012; Tenhaken, 2015). Moreover, it is known that apoplastic ROS levels are major signalling components (as it is also known for guard cell movements as described above) for proteins in charge of the communication between the extracellular environment to the intracellular compartment (Pignocchi and Foyer, 2003), that should drive subsequently highly regulated molecular mechanisms and metabolic fluxes responses through the CW (Verbancic et al., 2018).

Antioxidant apoplastic enzymes as peroxidases (POXs) or superoxide dismutases (SODs) can act as stiffening agents catalysing the cross-linking between different CW matrix compounds including phenolic groups and lignification (Wu and Cosgrove, 2000). For instance, even short-term drought frequently increases lignification of CWs (Escudero et al., 1992; Sobrado, 1992; Henry et al., 2000) and has been suggested to decrease g_{cw} due to the occlusion of pores (Monties, 1989; Niinemets et al., 2009). As well, POXs can also drive the CW loosening via oxidative cleavage of other polymers that show important hydrocolloid properties to retain water as xyloglucan and pectins, thus affecting CW composition and porosity (Cathala et al., 2001; Panchev et al., 2010; Tenhaken, 2015; Schmidt et al., 2016). Interestingly, ascorbate, the most abundant non-enzymatic antioxidant in the apoplast, and the symplastic ascorbate oxidase activity might regulate POXs activity controlling hydrogen peroxide (H_2O_2) levels (Ros-Barceló et al., 2006) and could have a regulatory effect on POX-CW remodelling, indicating the complex network regulation between apoplast and symplast. Additional line of evidence can be observed in the CW profile of angiosperm resurrection species that show higher levels of hemicelluloses, pectines and structural proteins (pectin-arabinans, arabinogalactan proteins and arabinoxylans) than other non-tolerant desiccation angiosperm species. This exclusive profile is related to highly flexible CW that can support significant mechanical pressures without collapse, plus subsequently, these compounds facilitate rehydration after a desiccation event (Moore et al., 2013).

Interestingly, significant associations of CW compounds were described including, for example, galactose, xylose and arabinose (precursors of pectins and hemicellulose) and the shikimate pathway through hydroxybenzoate (precursor of several phenolic compounds) relate specifically with g_m but were mostly not observed for g_s or A_n through a multi-species modelling analysis. Additional information was obtained because g_m associations showed a higher number of amino acids han g_s and A (Gago et al., 2016), being speculated if these associations can refer to the activity and levels of aquaporins, carbonic anhydrases and structural CW proteins. These data indirectly signalled that the intrinsic CW composition could directly affect the CO_2 pathway beyond the known inverse anatomical relationship with its thickness (Gago et al., 2019). Lately, Ellsworth et al. (2018) also observed a reduction of g_m in mutants with disrupted synthesis and accumulation of the hemicelluloses (1.3)- and (1,4)-linked β-glucosyl polysaccharides. Further evidence was found recently by Clemente-Moreno and collaborators (2019), showing that g_m was related to the peroxidase activity at the apoplast level but not at the symplast level in response to salt and drought stress (and its recovery). In parallel, g_m reductions were also related significantly with pectins increase and positively with the ratio hemicelluloses/pectins, and again with specific CW primary metabolites as galactose (pectins precursor), GABA, glucosamine, and *p*-coumarate (phenolic compound); altogether, indicating a complex response affecting g_m by oxidative stress and redox status driving CW remodeling.

Further research is needed to disentangle if the polymer hydrocolloids accumulation (hemicelluloses, pectins and phenols) can enlarge CW lattice (thus, increase the CO_2 pathway) or these increases in the hydrophilic lattice would facilitate CO_2 solubilization and movement across CW under stress. In this sense, the unknown role of the structural proteins remains as much challenging as exciting. These studies provided the first evidence that CW composition affects g_m, yet the complete mechanisms remain mostly unknown.

4.2. Chloroplast movements

Chloroplasts are generally as close to CWs as possible in order to short the liquid pathway of CO_2 through cytosol – maximize g_{cyt} (Evans et al., 2009; Flexas et al., 2018). In fact, the chloroplast surface area facing intercellular air spaces per unit leaf area (S_c/S) is one of the anatomical parameters that explain more variance of g_m in studies of non-stressed diverse species (Syvertsen et al., 1992; Hanba et al., 2002; Carriquí et al., 2015; Tosens et al., 2016; Veromann-Jürgenson et al., 2017; Peguero-Pina et al., 2017a,b; Carriquí et al., 2019b). This parameter also changes under drought, sunlight and potassium treatments (Tosens et al., 2012a, b; Fini et al., 2016; Lu et al., 2016; Han et al., 2019), with changes in leaf development (Miyazawa and Terashima, 2001), as well as in some mutants and transgenic plants with altered chloroplast shapes, numbers or distribution (Sharkey et al., 1991; Evans et al., 1994; Weraduwage et al., 2018). Thus, some authors have suggested that changes in S_c/S could explain the short-term responses of g_m e.g. to light (Ho et al., 2016; Théroux-Rancourt and Gilbert, 2017) and high temperatures (Flexas and Díaz-Espejo, 2015). However, several studies have observed scarce changes of g_m associated with chloroplast movement (Gorton et al., 2003; Loreto et al., 2009; Carriquí et al., 2019a) and only Tholen et al. (2008) observed a correlation between short-term variations of g_m in response to light quality with changes in chloroplast placement and S_c/S. The involvement of chloroplasts' movements on g_m was further corroborated using phytochrome-mutants lacking chloroplast movements (Tholen et al., 2008). Therefore, more pieces of evidence are needed to associate the short-term decrease of g_m with fast removal of chloroplast from the outer cell surface.

4.3. Aquaporins

During the last decades, the importance of aquaporins as facilitators of the diffusion of CO_2 across membranes has been identified (reviewed in Uehlein et al., 2017 and Flexas et al., 2016). First, Terashima and Ono (2002) demonstrated that g_m decreased after the application of $HgCl_2$, a general inhibitor of aquaporins. Subsequently, the implication of tobacco NtAQP1 aquaporin in the permeabilization for CO_2 of plasma membranes (Uehlein et al., 2003; Otto et al., 2010) and chloroplast membranes (Uehlein et al., 2008) was evidenced. Genetically-modified plants had been also used to verify *in planta* the involvement of NtAQP1 (Flexas et al., 2006; Uehlein et al., 2008; Sade et al., 2014) and some other aquaporins (Hanba et al., 2004; Heckwolf et al., 2011; Kawase et al., 2013) on g_m. While all those studies were performed in non-stressed plants, Miyazawa et al. (2008) suggested by treating irrigated and water-stressed plants with $HgCl_2$ a specific involvement of aquaporins in drought-induced decreases of g_m. A similar suggestion was made by Perez-Martin et al. (2014) after monitoring aquaporin transcription in water-stressed plants. Despite of all these proofs, recently Kromdjik et al. (2019) have shown a null effect of aquaporins knock-out on g_m measured with multiple methods. One of the explanations suggested by these authors is that redundancy of different aquaporins isoforms would mask the effects of a single gene knock-out. The number of aquaporins belonging to 10 different aquaporin families and multiple isoforms present in each species is generally unknown (Kjellbom et al., 1999; Maurel et al., 2008) and the CO_2 transport activity may differ among aquaporin types (Otto et al., 2010). Furthermore, the role that aquaporins play in the short-term response of g_m to temperature, CO_2 and light (Bernacchi et al., 2002; Flexas et al., 2007; Xiong et al., 2015) is not fully understood yet.

4.4. Carbonic anhydrases

Carbonic anhydrases (CAs) are cytosol, mitochondrial and stromal enzymes that catalyse the reversible reaction $CO_2 + H_2O \rightarrow HCO_3^- + H^+$ to allow a suitable diffuse through the liquid phase and a sustained CO_2 supply at the carboxylation site of Rubisco. It is under debate if CAs have an influence on the changes of g_m or whether CO_2 interconversion is not a limiting factor of g_m (Tholen and Zhu, 2011; Flexas et al., 2012; Veromann-Jürgenson et al., 2017). In C_4 plants, photosynthetic activity and g_m seemed reliant on the activity of CAs by assisting the high CO_2 concentration of chloroplast of sheath cells (Badger and Pfanz, 1995; Ludwig, 2012; Studer et al., 2014; Osborn et al., 2017; Kolbe et al., 2018; but see also Cousins et al., 2006). In C_3 plants, pieces of evidence are not as clear. Variation of CAs expression or activity by the use of inhibitors, mutants or water stress has produced inconclusive results, as studies have reported either an effect on g_m (Badger and Pfanz, 1995; Gillon and Yakir, 2000; Perez-Martin et al., 2014; Momayyezi and Guy, 2017a, b; Momayyezi and Guy, 2018) or a null effect (Price et al., 1994; Williams et al., 1996; Sasaki et al., 1998). Thus, the role of CAs in determining g_m remains a matter of debate (Momayyezi et al., 2020), as well as, the relative importance of the biochemical limitations to anatomical determinants of g_m.

5. The molecular and physiological inter-play between both conductances and photosynthesis

It is widely described that both g_s and g_m show a notable coupled behaviour largely influencing photosynthesis and WUE (Flexas et al., 2013; Gago et al., 2014). There is a significant variation in the g_m / g_s ratio across species and even genotypes (Flexas et al., 2013), which can be an important feature for WUE selection, especially under drought conditions (Galmés et al., 2011; Théroux-Rancourt *et al.*, 2015). An increased g_m / g_s ratio (i.e. a decreased mesophyll conductance limitation to photosynthesis) seems to be also at the basis of the observed progressive increase in WUE along land plant's phylogeny, from lowest values in bryophytes to the largest and more variable in angiosperms (Flexas and Carriquí, 2020). In fact, stomatal density and regulation have been often genetically manipulated and resulted in plants with improved WUE (Antunes et al., 2012; Kelly et al., 2013; Wang et al., 2014c; Medeiros et al., 2016; Daloso et al., 2016b; Antunes et al., 2017b; Hughes et al., 2017; Vráblová et al., 2017). Recently, it was demonstrated in tobacco plants the effect of one macronutrient, the chloride (Cl^-), prompted higher WUE driven by lower g_s and higher g_m, increasing g_m / g_s ratio without penalizing *A*. These trends were driven by anatomical traits at stomata level as reducing stomata density but increasing their size, in combination with an elevated S_c / S (higher number of smaller chloroplast facing the mesophyll airspaces) leading to higher g_m (Franco-Navarro et al., 2019).

Hence, both conductances, although coordinated when considering large interspecific datasets, show a certain degree of uncoupling under short-term conditions, which may arise from their different underlying mechanisms (Gallé et al., 2011; Flexas et al., 2012; Clemente-Moreno et al., 2019). The response of g_m and g_s to water and salt stresses has already been extensively reviewed elsewhere (Flexas et al., 2013, 2018; Nadal and Flexas, 2019), but the response of g_m and its potential drivers to well-known stimuli affecting stomatal response are still poorly understood.

We compiled a multi-species dataset from 13 papers published in the literature covering in 23 species the responses of g_s and g_m to short-term changes in atmospheric CO_2, light irradiances as well exogenous ABA application (Table 1). Notably, in most cases g_s and g_m show a similar trend, indicating a rather conserved response to stimuli and directly affecting photosynthesis (light intensity, CO_2). This positive coordination may arise from the need to reduce water losses and maximize CO_2 availability for photosynthesis during limited optimum conditions, as for example in the case of sunflecks in understorey vegetation (Campany et al., 2016).

The short-term response of g_m to both high light and CO_2 was first reported by Flexas et al. (2007), where g_m showed a rapid increase under low CO_2 (< 400 ppm) and a decrease under high CO_2 (> 400 ppm) (Table 1), in a similar manner as the reported response of g_s in angiosperms (Brodribb et al., 2009). However, this pattern may partially arise from mathematical circularity due to the inclusion of C_i in the methods used for g_m estimation (Gu and Sun, 2014). The lack of an anatomical basis for the apparent variation of g_m with CO_2 adds to the potential artifactual nature of this response (Carriquí et al., 2019a) and the lack of good understanding of the molecular factors that could drive quick responses of g_m (Shatil-Cohen et al., 2011; Grondin et al., 2015; Gago et al., 2016; Clemente-Moreno et al., 2019; Mizokami et al., 2019) avoids further concluding on this aspect of g_m regulation, a topic that clearly deserves further investigation. Similarly, g_m has been reported to vary with light intensity (Douthe et al., 2011, 2012) (Table 1), which again may partially arise from model limitations (Gu and Sun, 2014). Nonetheless, the fact that the response of g_m to light and CO_2 is not universal across all species (Xiong et al., 2018) seems to indicate that part of the observed variation is not entirely artifactual but responds to some species-specific features. In this case, leaf anatomy may play an important role as the apparent response of g_m to light intensity would emerge from the differential light penetration in the mesophyll layers, instead of any biochemistry-driven process (Theróux-Rancourt and Gilbert, 2017). On the other hand, the response of g_m to blue light is well supported by changes in S_c due to chloroplast re-arrangements (Tholen et al., 2008). Interestingly, blue light is one of the few cases where g_m and g_s show an opposite response (Table 1).

During drought, the decline of g_m and g_s could be partially attributed to a hydraulic (K_{leaf}) decline (Rodríguez-Domínguez et al., 2016; Wang et al., 2018), although the interplay between these parameters is still under debate (Flexas et al., 2018). Interestingly, at the leaves, ABA is mostly produced in the mesophyll cells (McAdam and Brodribb, 2018) and its action in the guard cells are highly regulated by several compounds from ROS levels, hormones, antioxidants and peptides produced in another tissues (Watkins et al., 2014; Takahashi et al., 2018). In general, ABA is considered an important factor in the decline of both conductances. For example, exogenous ABA application promptly decreases to a similar extent g_m and g_s in several species (Vrábl et al., 2009; Mizokami et al., 2015, 2019; Sorrentino et al., 2016) (Table 1). However, in poplar, the sensitivity of g_m to ABA is probably lower than that of g_s, as g_s in well-watered poplar changed concomitantly with accordingly with slight daily variations in ABA but g_m did not (Brunetti et al., 2019). This may indicate a different pathway for ABA sensitivity, which is also suggested in *Arabidopsis* mutant lines lacking OST1 and SAC1, where the g_m-g_s coupling is hindered (Mizokami et al., 2019). Aquaporins could be responsible for the ABA-mediated g_m decrease. ABA mediates decreases in membrane water permeability via aquaporin activity down-regulation, resulting in a reduction in K_{leaf} (Shatil-Cohen et al., 2011). Indeed, exogenous ABA application affects the expression of several aquaporins members of the PIP subgroup in *Arabidopsis* (Jang et al., 2004). Recently, light has been brought in this matter, it was published that stomata closure required the activity of the PIP2;1, especially in response to ABA (increases osmotic water permeability in guard cells) in *Arabidopsis* (Grondin et al., 2015). Interestingly, authors observed that normal stomata functionality was recovered by the overexpression of Open stomata 1 (OST1) / Snf1-related protein kinase 2.6 (SnRK2.6) (a kinase involved in guard cell ABA signalling) in the knock-out plants mediated by a phosphorylation mechanism in the cytosolic PIP2;1 peptide at Ser-121.

Further connections can be established also with the role of CAs related to the interplay between both conductances. While the role of CAs in g_m has been discussed in previous sections and recently reviewed (Momayyezi et al., 2020), additionally *Arabidopsis* lacking several CAs showed limited g_s response to CO_2 changing concentrations, higher g_s and density compared to wild type (Hu et al., 2010, 2015; Engineer et al., 2014). On the other hand, it has been also suggested that CAs can act as CO_2 sensors for guard cells supplying HCO_3^- for OST1 kinase that regulates S-type anion channels related to CO_2 stomata closure (Hu et al., 2010, 2015; Xue et al., 2011).

In the previous section, important differences in stomatal responsiveness to abiotic factors among phylogenetic plant groups has been highlighted. In this sense given that mesophyll photosynthetic capacity differs substantially between angiosperms and basal lineage of plants (Tosens et al., 2016; Gago et al., 2019), it is reasonable to hypothesize that mesophyll metabolism may play a central role in the regulation of stomatal movements across plant evolution. This idea is strengthened by the fact that guard cell metabolism cannot sustain stomatal movements by itself (Messinger, 2006; Mott, 2009), but depending, from mesophyll-derived metabolites such as sugars and organic acids that are charged into the apoplast and transported to guard cells through the transpiration stream (Fujita et al., 2013; Nunes-Nesi et al., 2011; Daloso et al., 2016; Santelia and Lawson, 2016; Daloso et al., 2018). What still remains unknown is whether and to which extent signals from mesophyll cells contribute to the differential velocity of stomatal responses observed among angiosperms and other plant groups, and how it is possible to use this knowledge to optimize water use efficiency.

It seems reasonable to speculate that another possible link between both conductances is the redox status of leaves and the whole plant. ROS were traditionally considered as toxic byproducts of aerobic metabolism, i.e. photosynthesis, and cells should deal with them to avoid excessive oxidation and irreversible damage; however, currently, ROS are being recognized as essential signalling molecules for several processes regulation (Mittler, 2017; Foyer et al., 2018). In agreement, evidence suggesting the association of NAD+ metabolism with stomatal function (Hashida et al., 2010), as well as the NAD+ transport between organelles regulating guard cell biogenesis have been found in *Arabidopsis* (de Souza Chaves et al., 2019). For instance, it was discovered the contribution of the aquaporin AtPIP2;1 to the intracellular accumulation of H_2O_2 (facilitating water efflux required for stomatal closure), that is also related with superoxide dismutases and CW peroxidases that drive the production of apoplastic ROS and CW remodeling (Wu and Cosgrove, 2000; Khokon et al., 2011; Kadota et al., 2014; Rodrigues et al., 2017). ROS can not only alter g_s, but also major described components of g_m as the functionality of diverse enzymes related to g_m as AQPs and CAs (see sections 4.3 and 4.4), cell and chloroplast membranes lipidic profile (changes in the lipid species, saturation and length of their chains) that will alter their permeability and the activity of transmembrane proteins as AQPs (Lee et al., 2004; Phillips et al., 2009; Burgos et al., 2011) and drive major changes into the CW composition (Tenhaken, 2015; Clemente-Moreno et al., 2019). Under stress conditions, ROS signaling and process regulation can become even more essential (before oxidative burst will promote physiological damage), and thus the altered redox status can drive a pool of mechanisms in order to deal with abiotic stress.

5. Concluding remarks

Now, global food security is a major worldwide concern attempting to the predicted climate change scenarios for the most important agricultural regions on the planet and the growing human population. The current food and fuel requirements are obtained based on large water consumption and nutrients, is the new challenge for society and the scientific community to ensure or even increase productivity in these predicted scenarios.

Diffusional limitations (i.e. g_s and g_m) to photosynthesis can impose easily about two-thirds of the total photosynthetic limitation under optimum conditions, but under mild and severe abiotic stress conditions they can reach >85% for most of the studied species already characterized (Flexas et al., 2018; Gago et al., 2019). Thus, knowledge about both conductances is essential to improve WUE, targeted crop breeding programs and sustainable, resource-efficient and environmental-friendly agriculture strategies. Curiously, the knowledge acquired from decades of research from g_s and g_m mainly differs in their origin. Biochemical regulation and signaling pathways are highly defined for g_s -and there is still a lot of controversy regarding the molecular mechanisms-, however, an important lack of knowledge is observed for its structural determinants. The opposite is true for g_m, known as a highly-complex parameter, anatomical determinants are much more well-characterized than its biochemical regulation and signaling pathways that still remains mostly unknown. Even more scarce is the information in the literature discussing the known coupled behaviour that frequently shows both conductances under most of the conditions (Flexas et al., 2013) and, moreover, establishing the mechanisms that could be the common determinants linking their dynamics.

Given the complexity of this challenging research focus, integrative multi-disciplinary studies are needed to join ecophysiology with molecular biology using systems biology approaches (Gago et al., 2016; Flexas and Gago, 2019; Medeiros et al., 2019). What remains as key targets for the future would be 1) to complete the mentioned lack of information for each of the conductances, 2) to determine the putative common molecular mechanisms driving the coupled behaviour between both conductances and 3) to use this knowledge for improving crop breeding strategies to increase maximal photosynthetic capacity and WUE in the global change scenario.

Author Statement

J.G., D.M.D and J.F. conceived and designed the idea of this review and wrote the first draft of the paper with subsequent inputs from all co-authors. M.N. developed the dataset compilation. D.M.D, W.A.L, A.N. drive the writing of section 3. J.F, M.N., A.P., M.C.M, M.C develop section 4. M.M, M.C.M. and J.G. lead section 5. M.M., J.G., and J.F. design the figures.

Conflict of interest

The authors declare no conflicts of interest.

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Table 1. Coordination of the stomatal (g_s) and mesophyll (g_m) conductances short-term responses (min-hours) to several environmental factors and ABA application. The responses are highlighted in blue when g_s and g_m display the same trend and in red when they differ. [a] Only high nitrogen plants included. [b] Lines studied were CML333, IL14H, Ki11, Mo17 and W22; all five presented the same g_s and g_m response.

Treatment	Species	Genotype/line	g_s	g_m	Reference
Low CO_2	*Triticum aestivum*	Yecora 70	↓	=	Tazoe et al. (2009)
(C_a < 400 ppm)	*Eucalyptus globulus*		↑	↑	Douthe et al. (2011)
	Eucalyptus saligna		↑	↑	Douthe et al. (2011)
	Eucalyptus sieberi		↑	↑	Douthe et al. (2011)
	Oryza sativa	Heshengwanyou	↑	↑	Xiong et al. (2015)[a]
	Zea mays	Various[b]	↑	↑	Kolbe and Cousins (2018)
High CO_2	*Nicotiana tabacum*	Samsun	↓	↓	Flexas et al. (2007)
(C_a > 400 ppm)	*Nicotiana sylvestris*		↓	↓	Flexas et al. (2007)
	Triticum aestivum	Yecora 70	=	=	Tazoe et al. (2009)
	Eucalyptus globulus		=	=	Douthe et al. (2011)
	Eucalyptus saligna		=	=	Douthe et al. (2011)
	Eucalyptus sieberi		↓	=	Douthe et al. (2011)
	Oryza sativa	Heshengwanyou	↓	↓	Xiong et al. (2015)[a]

	Zea mays	Various[b]	**↓**	**=**	Kolbe and Cousins (2018)
	Arabidopsis thaliana	Col-0	**↓**	**↓**	Mizokami et al. (2019)
	Arabidopsis thaliana	*ost1*	**=**	**↓**	Mizokami et al. (2019)
	Arabidopsis thaliana	*sac1-2*	**=**	**↓**	Mizokami et al. (2019)
Low light	*Triticum aestivum*	Yecora 70	**=[MNN12]**	**=**	Tazoe et al. (2009)
(*PPFD* < 4[MNN13] 00 μmol m^{-2} s^{-1})	*Eucalyptus globulus*		**↓[MNN14]**	**↓**	Douthe et al. (2011, 2[MNN15] 012)
	Eucalyptus saligna		**↓**	**↓**	Douthe et al. (2011, 2012)
	Eucalyptus sieberi		**↓**	**↓**	Douthe et al. (2011, 2012)
High light	*Triticum aestivum*	Yecora 70	**↑[MNN16]**	**=**	Tazoe et al. (2009)
(*PPFD* > 1000 μmol m^{-2} s^{-1})	*Eucalyptus globulus*		**=[MNN17]**	**=**	Douthe et al. (2011, 2012)
	Eucalyptus saligna		**=**	**=**	Douthe et al. (2011, 2012)
	Eucalyptus sieberi		**=**	**=**	Douthe et al. (2011, 2012)
	Oryza sativa	Heshengwanyou	**↑[MNN18]**	**↑**	Xiong et al. (2015)[a]
	Eucalyptus tereticornis		**↑[MNN19]**	**↑**	Campany et al. (2016)
	Phlebodium aureum		**=[MNN20]**	**=**	Xiong et al. (2018)
	Nephrolepis cordifolia		**=**	**=**	Xiong et al. (2018)

	Taxus baccata		↑	↑	Xiong et al. (2018)
	Ginkgo biloba		↑	↑	Xiong et al. (2018)
	Nerium oleander		↑	↑	Xiong et al. (2018)
	Populus nigra		↑	↑	Xiong et al. (2018)
	Gossypium hirsutum		↑	↑	Xiong et al. (2018)
	Helianthus annuus		↑	↑	Xiong et al. (2018)
	Centella asiatica		=	↑	Xiong et al. (2018)
	Oryza sativa	Shanyou 63	↑	↑	Xiong et al. (2018)
B[MNN21] lue light	*Arabidopsis thaliana*	Col	↑	↓	Tholen et al. (2008)
	Arabidopsis thaliana	*gl1*	↑	↓	Tholen et al. (2008)
	Arabidopsis thaliana	*phot2*	↑	=	Tholen et al. (2008)
	Arabidopsis thaliana	*chup1*	↑	=	Tholen et al. (2008)
ABA application	*Nicotiana plumbaginifolia*	WT	↓	↓	Mizokami et al. (2015)
	Nicotiana plumbaginifolia	*aba1*	↓	↓	Mizokami et al. (2015)
	Prunus avium		↓	↓	Sorrentino et al. (2016)
	Populus nigra		↓	↓	Sorrentino et al. (2016)
	Olea europaea		↓	↓	Sorrentino et al. (2016)

	Rosoidea rosa	Camp David	↓	↓	Sorrentino et al. (2016)
	Arabidopsis thaliana	Col-0	↓	↓	Mizokami et al. (2019)
	Arabidopsis thaliana	*ost1*	=	=	Mizokami et al. (2019)
	Arabidopsis thaliana	*sac1-2*	↓	=	Mizokami et al. (2019)

,Figure 1. A summary of current knowledge on the regulation of both stomatal and mesophyll conductance. The size of boxes and letters is proportional to the magnitude of current knowledge on each case. Hence, for stomatal conductance its structural determinants are largely unknown but there is abundant knowledge both on signalling pathways and biochemical regulation. The opposite is true for mesophyll conductance, for which there is large knowledge on its structural determinants and very little is known about both signalling and biochemical regulation. Abbreviations: ABA, abscicic acid; IAS, intercellular air spaces, SLAC1, Slow Anion Channel Associated 1 (in the plasma membrane); PP2Cs/SnRK2, phosphatases/kinases regulation (in response to ABA in guard cells).