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Modifying mesophyll conductance to optimise photosynthesis in crops

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1 Introduction

The need to feed a growing global population, combined with a changing climate and diminishing resources, has driven researchers towards improving crop productivity through a variety of approaches (Bailey-Serres et al., 2019; Ray et al., 2013). Recently, this effort has begun to focus on improving photosynthesis as a key driver of crop yields. The process of photosynthesis is dependent upon acquisition of carbon dioxide (CO₂) from the atmosphere, which is converted into carbohydrates by the enzyme Rubisco within the chloroplast. In C₃ plants,

the diffusion of atmospheric CO₂ into leaf chloroplasts is a limiting step in this process, and improving the conductance to CO₂ diffusion within leaves, termed mesophyll conductance (g_m), is predicted to increase photosynthetic rates and improve water use efficiency (Long et al., 2015; Lundgren and Fleming, 2020).

Modelling has suggested that improving the conductance of CO₂ into chloroplasts would increase photosynthetic assimilation rates due to several mechanisms (Wu et al., 2019). Rubisco efficiency would be improved as higher concentration of available CO₂ increases the carboxylation rates of Rubisco, resulting in less photorespiration. With more CO₂ available for fixation, the nitrogen cost of synthesising and maintaining photosynthetic machinery is also reduced due to increased efficiency in the system (Evans and Clarke, 2019), allowing for grain protein content to be maintained under higher assimilation rates without additional fertilisation.

An increase in g_m would also allow higher photosynthetic rates to be achieved without greater water loss through an adjustment of stomatal conductance. At the surface of the leaf, the boundary layer and stomatal properties influence both water vapour transpiration and gas diffusion into the sub-stomatal cavity. Thus, a trade-off exists between preventing water loss through transpiration and maintaining CO₂ diffusion to allow for photosynthesis, which is regulated dynamically by stomatal conductance. With higher g_m , stomatal conductance could be reduced while still maintaining the same chloroplastic CO₂ concentration, reducing water losses due to transpiration through the stomata.

The partial pressure of CO₂ within the chloroplast is highly variable due to the rate of CO₂ assimilation by Rubisco and the total conductance to CO₂. Higher assimilation rates consume more CO₂, tending to decrease CO₂ concentration in the chloroplast, while increases in g_m tend to increase it. Therefore, the combination of improved g_m with other complementary photosynthetic improvements will be essential to offset reductions in chloroplast CO₂ partial pressure, further enhancing the impact of increased g_m .

The path of CO₂ to the chloroplast is different in C₃ and C₄ photosynthesis plants, with the specialised anatomy of C₄ plants creating a spatial separation that concentrates CO₂. This impacts the way in which mesophyll conductance is estimated and interpreted. In this chapter, we will focus on strategies to improve mesophyll conductance in C₃ plants only. For a comprehensive discussion of recent advances in mesophyll conductance in C₄ (and CAM photosynthesis) plants see Cousins et al. (2020).

On its journey from the atmosphere into chloroplasts in C₃ plants, CO₂ faces several barriers that impose resistance to diffusion, including the cell wall, plasma membrane, cytosol, chloroplast envelopes and chloroplast stroma (Fig. 1, Clarke et al., 2021). These resistances together determine the overall mesophyll conductance. This book chapter will describe each factor contributing

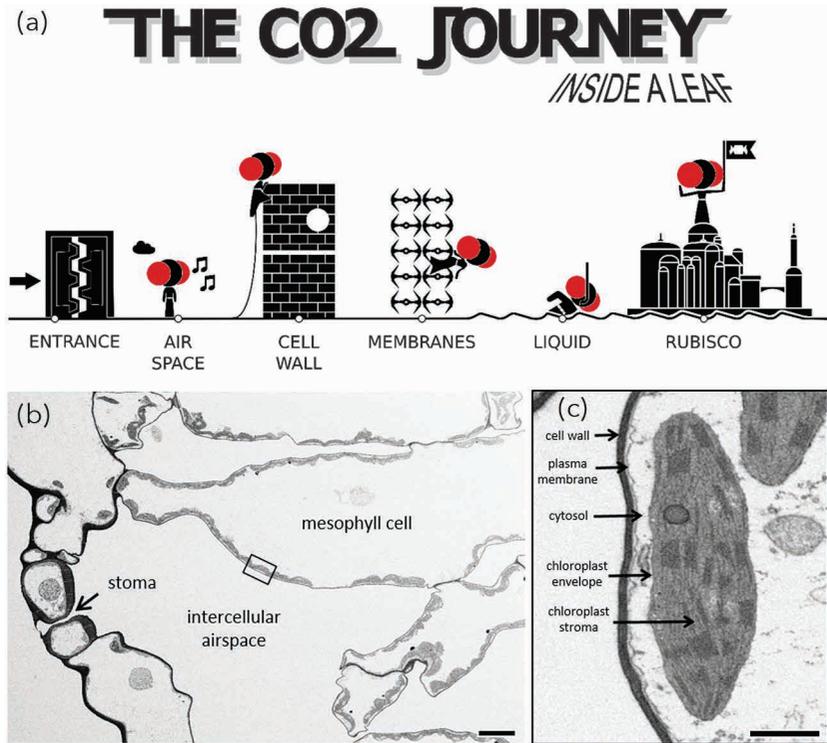


Figure 1 A Star Wars-inspired cartoon depicting the CO₂ diffusion journey inside the leaf with Princess Leia representing a CO₂ molecule (a) and the corresponding electron micrographs illustrating the actual CO₂ diffusion pathway within a tobacco leaf (b and c). For photosynthetic CO₂ assimilation to occur, CO₂ has to diffuse through stomata (entrance), intercellular airspace, cell wall, membranes (plasma membrane and chloroplast envelope) and the liquid phase in the cytosol and chloroplast stroma. Once inside the chloroplast, CO₂ is then fixed by Rubisco into energy-rich sugars as part of the photosynthesis process. Bar in *b* = 10 μm, bar in *c* = 1 μm. Adapted from Clarke et al. (2021).

to the resistance to CO₂ diffusion within C₃ leaves and discuss research efforts to overcome these resistances and improve the CO₂ concentration at the site of carboxylation.

2 Points of resistance to diffusion of CO₂

The CO₂ diffusion path within the leaf and its encompassing sequence of barriers directly determines mesophyll conductance. These barriers to CO₂ movement can be described as a series of resistances in the leaf, referred to as mesophyll resistance (r_m), which is inverse to g_m ($r_m = 1/g_m$). Although existing experimental methods cannot measure the individual components of r_m or g_m ,

these components can be studied separately using computational models, and understanding each component is critical to understand g_m as a whole.

Mesophyll resistance is generally divided as follows (Lundgren and Fleming, 2020; Ren et al., 2019):

- gas phase resistance;
- liquid phase resistance.

Gas phase resistance encompasses CO_2 movement through intercellular airspaces of the leaf, while liquid phase resistance is made up of the following series of resistances (Cousins et al., 2020; Evans et al., 2009):

- cell wall;
- plasma membrane;
- cytosol;
- chloroplast envelope;
- chloroplast stroma.

Liquid phase resistance accounts for up to 90% of mesophyll resistance to CO_2 diffusion through the leaf (Lu et al., 2016; Tosens et al., 2012) and is the primary focus of the following sections which discuss the roles of cell anatomy and biochemical processes in determining g_m .

3 The interaction between mesophyll cell anatomy, light and g_m

The constraints on CO_2 diffusion within the mesophyll are determined to a large extent by the shape, size and density (packing) of mesophyll cells and the position and orientation of the chloroplasts within, since these factors determine the liquid path length that CO_2 must travel from the intercellular air spaces to the chloroplasts. First, the arrangements of cells within the mesophyll in relation to CO_2 diffusion and light are discussed. In particular, the mesophyll can be considered a porous medium, where CO_2 diffuses through intercellular airspace and into the cells containing the chloroplasts. Some of the largest physical determinants of g_m are the surface area of the cells exposed to the intercellular airspace (S_m), the surface area of the chloroplasts exposed to the intercellular airspace (S_c) and the ratio S_c/S_m . S_c/S_m expresses the relative fraction of available area that is occupied by chloroplasts through which CO_2 can diffuse towards the site of Rubisco.

The most straightforward improvement of g_m would be through an increase of S_m and S_c . The presence of many small cells within the mesophyll

increases both S_m and S_c , which has been proposed as a potential target for improving g_m . Indeed, several studies have shown that this strategy increases g_m , with a positive effect on photosynthetic capacity. In *Arabidopsis*, increased cell number and packing increased g_m (Lehmeier et al., 2017) and in rice, leaves with lobed cells had an increased S_m , increased g_m and photosynthetic capacity (Sage and Sage, 2009). The lobed cell shape of rice leaves is an example of a mesophyll cell anatomy that can increase cell surface to volume ratio and enhance S_m as well as light absorption. Cell shape as well as cell number and packing are therefore both important to consider when improving g_m and the effects on S_m and S_c are reviewed below.

Increasing mesophyll cell number and packing does increase S_m but cannot be increased indefinitely, as it comes at a higher construction cost (cell wall material) and subsequently smaller leaves (Lundgren and Fleming, 2020; Ren et al., 2019). Increasing cell number and packing would logically increase S_m , but there is a trade-off between the number, packing and size of cells, since cell size is negatively related to cell number within a given space. Following the simplified model of Ren et al. (2019), S_m is related to the circumference of the cell when considered as a spherical object. If a single large cell has an S_m of π , then nine smaller cells within the same space would have an S_m of 3π . However, the nine cells have more areas where chloroplasts overlap and this would decrease S_c . Therefore, an increase in S_m by higher cell density is only beneficial for increasing g_m if S_c is not reduced proportionally due to cell surface overlapping.

S_c is also influenced by chloroplast number, movement and distribution in the mesophyll. A larger number of chloroplasts per cell can enhance S_c (Miyazawa and Terashima, 2001). But, as for a larger cell number, this cannot be increased without limit. Some species have a ratio of S_c to S_m (S_c/S_m) that is far below 1, where there could be benefit in an increased S_c . Still, a larger effect is expected from greater S_m to allow for further increases of S_c . Therefore S_c and S_m should be increased simultaneously to realise the greatest enhancement of g_m (Ren et al., 2019). The re-distribution and movement of chloroplasts is also known to change S_c (Hanba et al., 2002; Oguchi et al., 2003). Under high light intensity, the chloroplasts can be arranged vertically along the anticlinal cell walls (profile position) to avoid damage by excess light intensity. Under low light intensity, they can move in a perpendicular position (face position) to maximise light capture. The possibility of chloroplasts to move and still face the mesophyll airspace is limited when chloroplast number is high. For example, in rice, the chloroplasts are very tightly packed in the lobed mesophyll cells (Sage and Sage, 2009). However, under high light intensity, tightly packed chloroplasts and limited chloroplast movement can increase the risk of high light damage and may be more disadvantageous than gains in g_m .

The distribution of light strongly depends on the 3D anatomy and arrangement of mesophyll cells and has an influence on the so-called 'apparent' g_m : the effective g_m at leaf level that can vary with light intensity and CO_2 concentration, even though the underlying conductance at cell level remains the same (Evans, 2021). This is because within the mesophyll, the assimilation of CO_2 is distributed among many chloroplasts. These chloroplasts are all spatially distributed at different depths and consequently experience different light environments depending on the vertical attenuation of light in the mesophyll (Earles et al., 2019). Light attenuation is affected by the colour and intensity of the incoming light as well as the shape of the mesophyll cells. For instance, vertical columnar palisade cells allow light to penetrate deeper into the leaf (Terashima and Saeki, 1983; Vogelmann and Evans, 2002; Vogelmann and Martin, 1993). Irregularly shaped spongy mesophyll cells and air spaces increase reflection and refraction of light, causing optical path lengthening (Terashima et al., 2009; Vogelmann and Martin, 1993). This is also known as the detour effect and, apart from cell shape, is due to the higher refractive index of cells versus air. Thus, the spatial distribution and shape of cells and chloroplasts influence light attenuation within the mesophyll. This becomes evident at low light where the chloroplasts in the lower layers of mesophyll cells may operate at the light compensation point and do not contribute much to the total g_m , while at high light, this contribution becomes substantial (Th eroux-Rancourt and Gilbert, 2017). Indeed, 'apparent' g_m has been shown to respond to light intensity at both steady state (Busch et al., 2020; Xiong et al., 2018; Yin et al., 2020) and under dynamic conditions (Carriqu  et al., 2019; Liu et al., 2022; Sakoda et al., 2021). So although cell wall and membrane properties like S_m and S_c are important, the 3D structure and arrangement of mesophyll cells determine to a large extent how the 'apparent' g_m reacts to environmental changes such as light.

4 Leaf age and g_m

An example of the link between leaf anatomy and g_m can be observed in leaves of different age and canopy position. Several studies have examined the modulation of g_m by the mesophyll cell architecture in relation to leaf age, canopy position and light intensity. During the development of a leaf, it has been shown that g_m can limit photosynthesis considerably (Carriqu  et al., 2021; Clarke et al., 2021; Hanba et al., 2001; Hoshika et al., 2020; Marchi et al., 2008; Miyazawa et al., 2003; Miyazawa and Terashima, 2001; Niinemets et al., 2012; Tosens et al., 2012). In most species, g_m increases with leaf expansion and reaches a maximum with full expansion and then declines with leaf age (Loreto et al., 1994; Niinemets et al., 2012). The increase in g_m during expansion

is largely due to the growth and expansion of mesophyll cells and the increase of leaf thickness both of which increase S_m and S_c . The subsequent decline in g_m after expansion has been attributed to the increase in cell wall thickness (Miyazawa et al., 2003; Miyazawa and Terashima, 2001; Sugiura et al., 2020; Tosens et al., 2012) and reduction of cell wall effective porosity (Niinemets et al., 2012). In tobacco, g_m reductions with leaf age were accompanied by both cell wall thickening and a decrease in S_c/S_m (Clarke et al., 2021).

Another observed effect of leaf age was that of reduction of the thickness of chloroplasts. The thickness of the chloroplast determines the diffusion path length of CO_2 inside the chloroplast stroma and, in theory, can influence the conductance of CO_2 in the liquid phase; an important component of g_m . A decrease in chloroplast stroma thickness with leaf age has been observed in *Arabidopsis* (Carriquí et al., 2021), while other studies have not seen a decrease with age (Clarke et al., 2021) or have not found a correlation between chloroplast stroma thickness and g_m (Tosens et al., 2012). It is noteworthy that chloroplast stroma thickness was negatively affected by the growth light intensity in some species (Tosens et al., 2012) but not in others (Oguchi et al., 2003; Vidal et al., 1990). Still, chloroplast stroma thickness can potentially be an important factor for g_m . This was best illustrated in a study on transgenic tobacco with reduced Rubisco content that had lower chloroplast stroma thickness compared with the wild type and was accompanied by a lower CO_2 drawdown (Evans et al., 1994).

The observed changes in g_m with leaf age highlight that an increase in g_m can be realised by:

- an increase in S_m and S_c by mesophyll cell expansion and increased leaf thickness;
- a reduction in cell wall thickness.

However, this will need to be accomplished at a cell density that allows maximal exposure of cell walls to the intercellular airspace. In that regard, an arrangement of mesophyll cells that are more loosely packed together may allow for a more homogenous distribution of light throughout the mesophyll. This would not only increase S_m and S_c but also the light level for chloroplasts at lower layers in the mesophyll that would enhance the 'apparent g_m ' (Fig. 2).

The effects of leaf age on g_m illustrate that mesophyll anatomy influences g_m to a large extent and that it can be exploited. There is a variation in the extent to which these leaf age effects are observed not only between species, but also in response to growth light intensity. Especially in canopies with steep light gradients, the effect of light intensity may potentially be stronger than that of leaf age but has not been extensively studied in relation to g_m in C_3 plants.

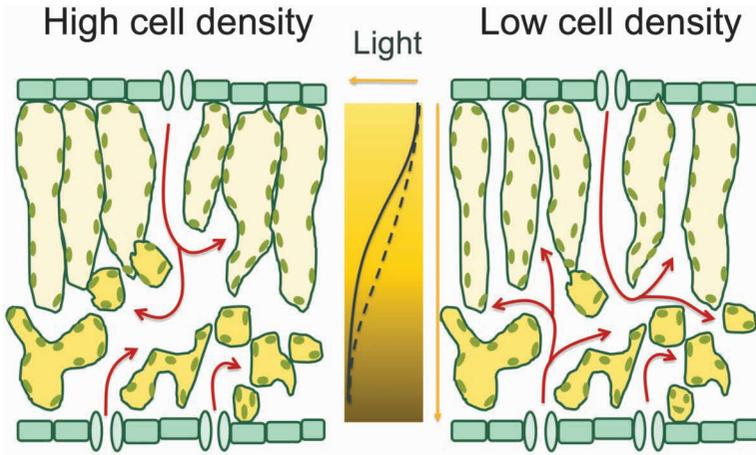


Figure 2 A diagram of mesophyll tissue shown in cross section through the leaf. The arrangement of mesophyll cells can be more closely packed (high cell density) or more loosely packed (low cell density), influencing both the gas diffusion pathways (red lines) and light distribution throughout the mesophyll (black lines).

5 Cell wall diffusion

Diffusion through the cell wall is the first step in the liquid phase of CO_2 diffusion through the leaf. Cell walls are structurally complex barriers that function to protect plants from biotic and abiotic stresses, maintain structural integrity and support cell division and plant growth (Cosgrove, 2005; Houston et al., 2016). The walls contain pores filled with apoplastic fluid; these pores form the interface between the gas and liquid phases of diffusion and provide routes for CO_2 to move from the intercellular airspaces into mesophyll cells. Several model studies of g_m have suggested that the cell wall is one of the main constraints on mesophyll conductance (Evans et al., 1994; Gago et al., 2020; Terashima et al., 2011; Tomas et al., 2013; Xiao and Zhu, 2017; Yin and Struik, 2017).

The path through the cell wall is complicated, and the overall resistance depends on the number of channels or pores, the effective length of each channel and the rate of diffusion along each channel (see Fig. 3 in Evans et al., 2009). These considerations can be expressed mathematically using four components: cell wall thickness, tortuosity of the path through cell wall pores, cell wall porosity and diffusivity of CO_2 through the apoplastic fluid (Evans, 2021). Although one cannot definitively calculate cell wall resistance and each of its components, it can be estimated with the help of plant models. The impact of cell wall thickness on g_m is relatively well studied being that it is the only component affecting cell wall conductance to CO_2 that can be easily and reliably measured. Cell wall thickness has been measured in hundreds of

species using transmission electron microscopy (Gago et al., 2020). Several studies have reported that cell wall thickness is negatively correlated with mesophyll conductance, suggesting selection or genetic modification for thinner cell walls could be a good target to enhance mesophyll conductance (Clarke et al., 2021; Onoda et al., 2017; Ren et al., 2019; Terashima et al., 2011; Tosens et al., 2012). However, one must consider how thin a mesophyll cell wall can be made without jeopardising its structural integrity (Cosgrove, 2018). The ideal solution will likely be complicated and depend on factors such as the specific plant in question, and its mesophyll cell size and cell wall composition (Lundgren and Fleming, 2020). Additionally, the lack of data on the regulation of cell wall thickness and genes that may be involved make targeted changes quite challenging. Moreover, much uncertainty exists with the other three components of cell wall resistance partly due to the fact that there are currently no good methods available to experimentally measure these parameters (Evans, 2021).

The composition of the cell wall is another element which may influence g_m by modifying CO_2 diffusion. Primary cell walls are composed of a complex carbohydrate matrix of cellulose, hemicellulose and pectin (Cosgrove and Jarvis, 2012; Cosgrove, 2005; Tenhaken, 2015). It is not difficult to envision that altering the absolute amounts or ratios of these components will affect CO_2 diffusion through the cell wall. These changes could potentially modify cell wall thickness or alter porosity and the liquid path length through the cell wall (Ellsworth et al., 2018). However, how changes in cell wall composition affect tortuosity and porosity remains relatively unexplored (Carriqui et al., 2020). Pectins are known to have a role in regulating cell wall porosity but little is known about their direct effect on CO_2 diffusion (Fleischer et al., 1999; Rondeau-Mouro et al., 2008). The ability to directly measure these parameters is essential in order to answer these questions.

A small number of recently published studies have started to examine the relationships between cell wall composition and mesophyll conductance using cell wall mutants, stress treatments and interspecific variation. Ellsworth et al. (2018) observed that rice mutants unable to synthesise mixed-linkage glucans (a cell wall hemicellulose) had reduced photosynthetic rates, g_m , S_c and leaf mass per area. In addition, the authors calculated the effective porosity of the mesophyll cell walls, which was estimated to be significantly reduced in the mutants relative to the control. From this it was concluded that mixed-linkage glucans in cell walls improve effective porosity and CO_2 diffusivity in rice (Ellsworth et al., 2018). Another study looking at pectin methylesterase and pectin acetylerase mutants in *Arabidopsis* reported reductions in CO_2 assimilation and g_m associated with reduced galacturonic acid content (the main component of pectin; Roig-Oliver et al., 2021). Two other studies looking at water stress in grapevines and tobacco found that decreases in photosynthesis and g_m were associated with changes in

cellulose and pectin content in the cell wall (Clemente-Moreno et al., 2019; Roig Oliver et al., 2019). Furthermore, to assess whether cell wall composition plays a role in existing differences in g_m between species from common environments, Carriquí et al. (2020) measured cell wall composition, CO_2 assimilation and g_m in seven gymnosperm species. The study found g_m to be correlated with cell wall composition, likely signifying a change in the diffusivity of the cell wall. The authors conclude that higher ratios of pectin to hemicellulose and/or pectin to cellulose was correlated with increased g_m , suggesting pectin has a role in regulating CO_2 diffusivity in the cell wall of gymnosperms.

These novel studies have started to provide critical information about the relationship between g_m and cell wall properties. However, additional work is needed to explore new variations on cell wall composition such as increasing or decreasing the amounts of individual primary cell wall components. Analyses comparing species in other land plant groups besides gymnosperms will also be helpful since differences in main cell wall components exist between plant types (Popper et al., 2011). The information gained from such studies can be used to directly target changes in cell wall composition through genetic engineering or selection. Based off our current knowledge, we propose some potential targets for modification with the goals of gleaning a better understanding of and enhancing CO_2 diffusivity across the cell wall:

- Genes coding for enzymes that catalyse cellulose, hemicellulose or pectin biosynthesis
- Genes involved in cell wall loosening and cell expansion
- Genes that affect methylation and/or crosslinking of primary cell wall components

Overall, altering cell wall properties such as composition and thickness is a promising route for reducing CO_2 diffusion resistance across the cell wall and increasing mesophyll conductance. However, true engineering remains elusive because many fundamental questions about the genetic control of cell wall properties and the relationship between those properties and g_m remain unanswered. Future exploratory work, as well as the development of new measurement techniques for measuring diffusion and relevant cell wall parameters, will be crucial for successful genetic engineering of mesophyll conductance.

After crossing the mesophyll cell wall, a series of liquid phase resistances remains between CO_2 and the site of carboxylation. These are the plasma membrane, cell cytosol, chloroplast envelopes and the liquid stroma inside the chloroplasts. The resistance imposed by the membrane components (plasma and chloroplast envelope) are discussed together, followed by liquid cytosol and stroma components.

6 Cellular membranes and CO₂ diffusion

Cellular membranes are composed of a lipid bilayer and function to modulate movement of molecules in and out of the cell. CO₂ is hydrophobic and non-polar, and initial studies on membrane permeability in artificial lecithin-cholesterol bilayers concluded that biological lipid membranes offered little to no resistance to CO₂ (Gutknecht et al., 1977). These membrane models, however, did not account for the dense packing of proteins within the membrane and differing lipid composition. Unlike animal cells, plant membranes do not contain cholesterol but instead have a suite of related sterols (Reszczyńska and Hanaka, 2020).

Alteration of lipid content in plant membranes could be a target for improving g_m ; however, as the membranes are very protein-dense, the available free lipid membrane for CO₂ diffusion is likely quite limited. The lipid and protein composition of membranes can nevertheless be significantly remodelled in response to environmental cues, suggesting optimisation of this process could potentially improve overall g_m (Uemura et al., 1995).

Cellular membranes impose up to 50% of the resistance to CO₂ diffusion within mesophyll cells (Endeward et al., 2017; Evans, 2021). These membranes include the plasma membrane (PM) and the dual membranes of the chloroplast envelope (CE). The CO₂ permeability of the CE is assumed to be half that of the PM, as the envelope is composed of dual membranes, thus increasing the resistance (Evans, 2021). While some argue the Meyer–Overton rule (also known as the solubility-diffusion model) accounts for all gaseous CO₂ transfer across membranes (Missner and Pohl, 2009), the PM and CE contain channels and transporters that facilitate CO₂ transfer across the membrane and manipulation of these has become a target for understanding and improving mesophyll conductance in plants.

7 Improving g_m using aquaporins as CO₂ channels

Aquaporins are a prominent gene family involved in CO₂ transfer across membranes. While initially named for their water transfer capabilities, further studies have reported aquaporins to be multifunctional proteins, capable of facilitating the transfer of various solutes, ions and gases, including CO₂ (Groszmann et al., 2017). The functional aquaporin unit is composed of four monomer channels coming together to form a tetramer, which creates a fifth central pore. Solute specificity is influenced by individual monomer properties and their combinations in the tetramer (Otto et al., 2010). Aquaporins are dynamic proteins that can move in and out of the membrane in response to environmental stimuli, and the pores within the tetramer can also be gated, allowing close regulation of channel activity (see Groszmann

et al., 2017 for a detailed discussion of plant aquaporin structure, function and regulation).

Early work in mammalian systems first identified aquaporin proteins as CO₂ channels. The human red blood cell aquaporin, AQP1, when expressed in *Xenopus* oocytes, increased membrane permeability to CO₂ (Cooper and Boron, 1998; Nakhoul et al., 1998). In plants, members of the PIP aquaporin family are homologous to mammalian AQP1, and generally localise to the plasma membrane, with some isoforms also detected in chloroplast envelopes by Western blot and proteomic analysis (Beebo et al., 2013; Uehlein et al., 2008).

Expression of a tobacco AQP1 homologue, NtAQP1 (also known as NtPIP1;5s, De Rosa et al., 2020), in oocytes also conferred increased CO₂ permeability (Uehlein et al., 2003). A function in mesophyll conductance was predicted and *in vivo* ectopic or antisense expression study of NtAQP1 in tobacco altered mesophyll conductance, with ectopic expression increasing g_m by up to 20% (Flexas et al., 2006). Additional studies targeting aquaporins from other species and across environmental conditions have had mixed effects on mesophyll conductance, with some studies reporting effects on g_m (Ermakova et al., 2021; Hanba et al., 2004; Heckwolf et al., 2011; Mori et al., 2014; Uehlein et al., 2008; Xu et al., 2019), while in others studies, no effects were observed (Clarke et al., 2022; Kromdijk et al., 2020).

Questions remain about why manipulation of aquaporins does not produce consistent effects on mesophyll conductance, and several factors have been suggested that may be impacting the g_m phenotype in these studies. The underlying g_m capacity of a species may affect the ability to detect improvements in g_m . Many crop species already have relatively high g_m , driven in part by their thin cell walls (see 'Cell Walls' above). Modelling has suggested that when basal g_m is higher, increases will be subtle and harder to detect (Clarke et al., 2022). A study in tomato reported that ectopic expression of *NtAQP1* did not increase g_m until basal g_m levels had been sufficiently reduced through simultaneous ectopic expression of hexokinase (Kelly et al., 2014). Recent studies have also highlighted the dynamic effects of environmental factors on g_m . Conditions such as photoperiod, light intensity, day/night temperature, water availability, humidity and nutrient supply can all alter anatomical and biochemical components that influence g_m . The conditional effects of growth environment and growth stage between aquaporin loss-of-function mutants and g_m have been reported in rice (Huang et al., 2021), and in tomato, an aquaporin knockout mutant was only shown to reduce g_m when mutants were grown under high CO₂ levels (Zhang et al., 2021). Further studies to better understand these complex relationships will be essential to ensure manipulation of aquaporins can provide a consistent impact on g_m , and aquaporins remain a strong engineering target for enhancing g_m .

To further efforts to improve the membrane conductivity to CO₂, it may also be beneficial to look beyond aquaporins to find candidates from other systems where CO₂ transfer across membranes occurs. Red blood cells (RBCs), for example, require the ability to rapidly uptake CO₂ from the blood stream, and indeed AQP1 is present on these membranes (see Michenkova et al., 2021 for a comprehensive review). However, another protein, the Rhesus-AG protein (part of the Rhesus complex) is also present on the RBC membrane and also acts as a CO₂ channel, contributing half of CO₂ uptake in these cells (Endeward et al., 2008; Ripoche et al., 2006). Plants do not contain Rhesus proteins but do contain the somewhat homologous AmtB protein which is an ammonium channel (Marini et al., 1997). Rhesus and AmtB proteins are bidirectional channels, moving CO₂ or NH₃ respectively along a concentration gradient. Expression of a mammalian protein such as Rhesus-AG into plant membranes could be a strong target for increasing mesophyll conductance if the protein can be assembled and targeted correctly. No studies have reported success in expressing the related mammalian AQP1 *in planta* however, so this may not be a feasible approach. Identification of further CO₂ channels or transporters from plants or more closely related non-plant systems would provide new targets for engineering improved g_m . Expression of genes from non-plant photosynthesising organisms, such as green or red algae, may have more compatibility. Beyond CO₂ channels, several bicarbonate transporters have been identified in plants that may also support increased g_m if targeted correctly (Poschenrieder et al., 2018).

8 CO₂ solubility in liquids

On its journey to Rubisco, CO₂ must transverse two liquid compartments:

- the cell cytosol;
- the stroma within the chloroplasts themselves.

CO₂ gas can dissolve into liquids, but it can also be reversibly converted to bicarbonate which has a much higher solubility (Badger and Price, 1994). This reaction is catalysed by the enzyme carbonic anhydrase (CA), making the interconversion almost instantaneous. CAs are the second most abundant enzyme in plants (after Rubisco) and most of this activity is thought to support the diffusion of CO₂ through the cytosol and chloroplast stroma (Ogée et al., 2018). In plants, CAs have three forms, α , β and γ , that differ in their activity, expression and localisation patterns (Momayyezi et al., 2020). β -CA is the dominant isoform in plants and is found either free in the cytosol and stroma (Fabre et al., 2007) or bound to organelle membranes via interaction with aquaporin proteins (Wang et al., 2016).

The localisation of CA within the cell is critical for facilitating carbon concentrating mechanisms such as cyanobacterial carboxysomes or in C_4 photosynthesis, where CA localisation determines what form of CO_2 exists within each compartment. Here we discuss the role of CA in C_3 plants with respect to its impact on mesophyll conductance, and how it may be engineered for improvement.

9 Improving g_m with carbonic anhydrases

CA activity in the stroma is likely to have a large impact on g_m , with models predicting that removal of stromal CAs would decrease g_m by up to 44% and photosynthetic assimilation by 7% (Tholen and Zhu, 2011). Experimental work using anti-sense CAs confirmed carbon discrimination is altered (Price et al., 1994; Williams et al., 1996), or that photosynthesis was reduced (Sasaki et al., 1998), though g_m itself was not altered. More recent studies have shown evidence that manipulation of CA can alter g_m (Gillon and Yakir, 2001; Perez-Martin et al., 2014). With many forms of CA present within plants, it is possible that functional redundancy is compensating for CA knock-down effects, and more research is needed to determine what levels of CA are optimal in various compartments.

A key development in our understanding of the role CAs have in mesophyll conductance has been the discovery of CA interaction with aquaporin proteins (Perez-Martin et al., 2014; Wang et al., 2016). By coupling CA to a CO_2 channel, diffusing CO_2 is instantaneously converted to bicarbonate, ensuring a strong pull down effect for CO_2 into the cytosol. This relationship could potentially be further exploited through an engineered complex (metabolon) of aquaporin and CA at the plasma membrane that ensures their interaction remains constant and optimised. A similar metabolon of a CA coupled to a bicarbonate transporter at either the apoplast/plasma membrane boundary or chloroplast envelope/stroma boundary could also improve CO_2 delivery through the mesophyll cell to the stroma. Overall, more research is needed to better understand the relationships between CA isoforms, localisation and mesophyll conductance to help guide targeted improvements in CO_2 diffusion within mesophyll cells.

10 Estimating g_m

Although mesophyll conductance cannot be measured directly, a handful of techniques have been developed to indirectly estimate g_m and thorough reviews are available (Cousins et al., 2020; Pons et al., 2009). Two techniques

have proven to be most reliable and are based on gas exchange measurements either (i) in conjunction with carbon isotope discrimination ($\Delta^{13}\text{C}$); or (ii) in combination with chlorophyll fluorescence (Evans et al., 1986; Harley et al., 1992).

Carbon isotope discrimination methods are based on measuring the $^{13}\text{C}/^{12}\text{C}$ ratio of CO_2 remaining after photosynthetic CO_2 fixation. The heavier isotope of carbon, ^{13}C is discriminated against during diffusion through the leaf and the biochemical processes of photosynthesis (Farquhar et al., 1982). This is because $^{13}\text{CO}_2$ has lower diffusivity in air and liquids relative to the lighter isotope ($^{12}\text{CO}_2$) and the carboxylating enzyme Rubisco preferentially binds $^{12}\text{CO}_2$. Correspondingly, photosynthetic products are enriched in ^{12}C , while gas flowing over a photosynthesizing leaf is enriched in ^{13}C and exhibits an increased $\Delta^{13}\text{C}$ compared to the ambient air. Carbon isotope discrimination has generally been measured one of two ways: by the cryogenic trapping of CO_2 and subsequent measurement of $\Delta^{13}\text{C}$ using isotope ratio mass spectrometry (Evans et al., 1986) or by using tunable diode laser (TDL) absorption spectrometry (Barbour et al., 2007) which can obtain simultaneous measurements of carbon isotope discrimination and gas exchange.

Estimating mesophyll conductance from gas exchange combined with chlorophyll fluorescence relies on the modelled relationship between CO_2 assimilation (A), the electron transport rate (J) and the concentration of CO_2 at Rubisco (C_c ; FvCB model Farquhar et al., 1980). Two main modelling methods are used to estimate g_m : the constant J method and variable J method (Harley et al., 1992). In the constant J method, gas exchange measurements are made across a range of C_i values where J is assumed to be constant. In this method, fluorescence measurements are used solely to verify the assumption that J is constant under the measured range. Alternatively, the variable J method uses chlorophyll fluorescence to estimate J and then g_m (Harley et al., 1992). This method can be applied for any value of C_i , even when J is not constant.

Each of the aforementioned methods relies on different models and assumptions for the calculation of g_m and has corresponding limitations. They are all considered suitable for the determination of g_m values; however, several factors can and should be considered when determining which method is best suited for the planned experiment (Table 1). It is also common for values of g_m to vary depending on the estimation method applied, with the $\Delta^{13}\text{C}$ method often yielding higher estimates than chlorophyll fluorescence methods (Kromdijk et al., 2020). When possible, more than one method should be used to estimate g_m to increase confidence in the results due to uncertainties associated with each method.

Table 1 Factors to consider when choosing the most suitable method for estimating mesophyll conductance with regards to individual research questions

Factors to consider	Method			
	Carbon isotope discrimination and gas exchange	Tunable Diode Laser (TDL) absorption spectrometry	Constant J	Variable J
	Cryogenic trapping and isotope ratio mass spectrometry			
Equipment cost	\$\$\$\$	\$\$\$\$	\$\$	\$\$
Suitable for field measurements	No	No	Yes	Yes
Suitable for measuring at any CO_2 concentration	Yes	Yes	No	Yes
Suitable for measuring at non steady-state conditions	No	Yes	Yes	Yes
Measurement throughput	+	++	++++	++++
Accuracy	+++	++	+	+
Important requirements for accurate measurements	<ul style="list-style-type: none"> • large CO_2 draw down • large gas exchange chamber when possible • make measurements under non-photosynthetic conditions (2% O_2) 		<ul style="list-style-type: none"> • more suitable for small leaves with low CO_2 draw down • avoid measurements under conditions of very low stomatal conductance such as drought stress 	
Major sources of error	<ul style="list-style-type: none"> • uncertainties with assumed values of fractionation factors associated with carboxylation, respiration and photorespiration 		<ul style="list-style-type: none"> • assumes that J and g_m are constant across the CO_2 concentration range 	<ul style="list-style-type: none"> • uncertainties associated with estimating J from fluorescence measurements

11 Strategies for altering g_m

Strategies aimed at understanding and improving crop performance have changed over the years. During the Green Revolution, yields were increased by optimising light interception and carbon partitioning; however, these traditional methods for crop improvements are reaching a plateau, highlighting the need for novel approaches to ensure food production can keep up with the growing population (Ort et al., 2015). One promising target is the process of photosynthesis, which has not been fully optimised in food crops. Here, we discuss the use of selective breeding and synthetic biology via genetic engineering as possible approaches to enhancing photosynthesis by increasing mesophyll conductance.

Selective breeding has successfully been used in agriculture to produce crops with desirable traits, such as disease resistance and larger grains, fruits and vegetables. For artificial selection of a trait to be successful, the trait must exhibit genetic variation and that variation must be heritable. Several studies have been published looking at existing interspecific and intraspecific natural variation of mesophyll conductance (Carriquí et al., 2015, 2020; Faralli and Lawson, 2020). A summary of published studies reveals significant variation of mesophyll conductance in some of the world's most important crops, where the measured values vary by as much as a factor of ten within a species (Table 2). These studies indicate a useful source of unexploited genetic diversity that could be used for selective breeding to improve g_m and potentially the photosynthetic efficiency of these crops.

Synthetic biology has also been used successfully in agriculture to produce disease-resistant and nutrient-enriched crops (Kumar et al., 2020). Genetic engineering of plants via the introduction of one or multiple transgenes can be used to express genes from other plants or organisms that may facilitate enhancement of mesophyll conductance, whether it is through altered cell morphology, cell wall structure, membrane composition or other biochemical processes. This strategy can also be used to stack multiple favourable traits

Table 2 Variation of mesophyll conductance (g_m) in different crops

Reference	Crop	Number of cultivars/ genotypes	Observed g_m range ($\text{mol m}^{-2} \text{s}^{-1}$)
Barbour et al. (2010)	Barley	6	0.05-0.50
Muir et al. (2014)	Tomato	8	0.16-0.28
Jahan et al. (2014)	Wheat	10	*0.51-1.05
Tomás et al. (2014)	Grapevine	7	0.14-0.21
Ouyang et al. (2017)	Rice	7	0.05-0.12
Tomeo and Rosenthal (2017)	Soybean	12	*0.039-0.24
De Souza et al. (2020)	Cassava	12	*0.20-0.50

*Values were converted to units of $\text{mol m}^{-2} \text{s}^{-1}$ assuming pressure of 1 standard atmosphere.

together, for example, g_m traits with other photosynthetic traits. Additionally genome-editing technology such as CRISPR can be used to knockdown, knockout or upregulate target genes of interest within the plant genome. Photosynthetic improvements via genetic engineering are a promising avenue that is currently underutilised partially due to insufficient knowledge and understanding about several aspects of mesophyll conductance.

At present, there are relatively few studies that have used genetic engineering approaches to alter mesophyll conductance. Studies utilizing RNAi technology to knockdown carbonic anhydrase activity in tobacco have shown little effect on mesophyll conductance to CO_2 (Price et al., 1994; Williams et al., 1996). Increases in mesophyll conductance and net photosynthesis were observed in rice through combined ectopic expression of cyanobacteria ICTB, a possible bicarbonate transporter and FBP/Sbpase, which plays an important role in ribulose-1,5-bisphosphate (RuBP) regeneration (Gong et al., 2015). This study illustrates the potential utility of using multigene constructs to engineer improved mesophyll conductance. Most success with engineering enhanced g_m has been limited to changes in aquaporin expression. Transgenic overexpression of tobacco aquaporin 1 (NtAQP1) increased mesophyll conductance along with photosynthetic rates and CO_2 concentrations in the chloroplasts of tobacco plants (Flexas et al., 2006), while RNAi knockdown of NtAQP1 decreased photosynthesis and CO_2 conductance within tobacco leaves (Uehlein et al., 2008). Ectopic expression of the barley aquaporin PIP2;1 and overexpression of the rice aquaporin PIP1;2 both resulted in increased mesophyll conductance in rice, with the latter also increasing plant biomass and grain yield (Hanba et al., 2004; Xu et al., 2019). Together these studies highlight the importance of aquaporins as key targets for improving CO_2 conductance within the leaf. However, in some species, stacking aquaporin overexpression with other CO_2 diffusion traits will be necessary to see measurable differences in g_m .

12 Conclusions and future trends

Mesophyll conductance to CO_2 is influenced by a complex combination of anatomical and biochemical traits within leaves. Engineering improvements to mesophyll conductance presents an appealing target for boosting photosynthesis, especially in limited water environments where improved CO_2 supply to Rubisco would allow stomatal conductance (and therefore water loss) to be reduced without a photosynthetic penalty. The stacking of improved g_m traits with improvements to Rubisco or RUBP regeneration efficiency should also deliver further enhancements to photosynthesis as CO_2 concentration at the site of fixation becomes limiting. Manipulation of individual resistances to CO_2 within leaves and cells can also be stacked together for further improvements to the overall mesophyll conductance.

In order to deliver this, further comprehensive studies are needed to facilitate engineering enhanced mesophyll conductance and photosynthetic efficiency, such as large-scale mutant screens to identify suitable genes for additional study and manipulation. Specifically, improvements in the fundamental knowledge of genetic controls in the following areas are required:

- cell size and shape;
- cell packing and leaf thickness;
- cell wall structure and function;
- membrane channel regulation.

13 Where to look for further information

Further useful information about mesophyll conductance and efforts to improve it to benefit plant productivity can be found in the following resources:

- Cellular perspectives for improving mesophyll conductance. Lundgren and Fleming 2020. <https://doi.org/10.1111%2Ftpj.14656>.
- Recent developments in mesophyll conductance in C3, C4 and crassulacean acid metabolism plants. Cousins et al. 2020. <https://doi.org/10.1111/tpj.14664>.
- Mesophyll conductance: the leaf corridors for photosynthesis. Gago et al. 2020. <https://doi.org/10.1042/BST20190312>.
- Mesophyll conductance: walls, membrane and spatial complexity. John R. Evans 2021. <https://doi.org/10.1111/nph.16968>.

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15 Author contributions

C. E.-S. S., S. M. D. and V. C. C. all wrote and edited the manuscript and are equal co-authors.

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