



## Review

## Opinion: Prospects for improving photosynthesis by altering leaf anatomy

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## ABSTRACT

Engineering higher photosynthetic efficiency for greater crop yields has gained significant attention among plant biologists and breeders. To achieve this goal, manipulation of metabolic targets and canopy architectural features has been heavily emphasized. Given the substantial variations in leaf anatomical features among and within plant species, there is large potential to engineer leaf anatomy for improved photosynthetic efficiency. Here we review how different leaf anatomical features influence internal light distribution, delivery of CO<sub>2</sub> to Rubisco and water relations, and accordingly recommend features to engineer for increased leaf photosynthesis under different environments. More research is needed on (a) elucidating the genetic mechanisms controlling leaf anatomy, and (b) the development of a three dimensional biochemical and biophysical model of leaf photosynthesis, which can help pinpoint anatomical features required to gain a higher photosynthesis.

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## 1. Introduction

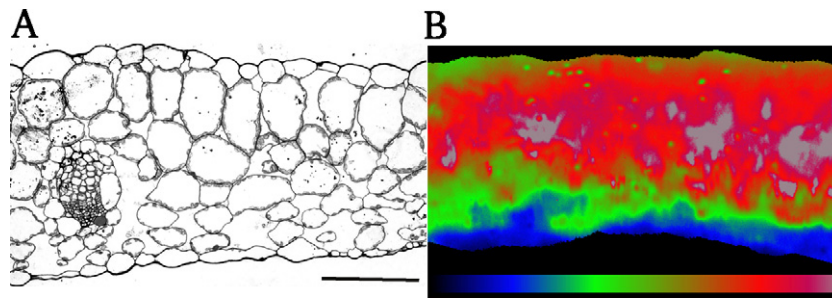
The improvement of crop yields during the last 50 years can be largely attributed to better crop architecture, an increased harvest index, and increased application of fertilizers and pesticides [1–3]. Recently, improving photosynthesis has been recognized as an additional option to achieve increased yields [1,3]. A number of metabolic targets and canopy architectural features have

been identified that can be modified to achieve enhanced rates of photosynthesis, e.g. altering Rubisco kinetics [4,5], manipulation of photoprotection [6,7], rebalancing the carbon metabolic processes [8], introducing photorespiratory bypass pathways [9,10], C<sub>4</sub> photosynthetic pathways [11,12], introducing inorganic carbon transporters in mesophyll cells [13] and selecting for crops with more erect leaves [1]. Surprisingly, the potential of manipulating leaf anatomy to gain increased photosynthetic properties has so far received less attention.

Is modifying leaf anatomy relevant for improving photosynthesis? Evidence suggests that this is indeed the case. Firstly, leaf anatomy varies substantially among species and even within species under different environments, and variation of the leaf anatomy is closely related to photosynthetic properties [14]. Secondly, leaf anatomy is closely linked to leaf mass per unit area,

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**Fig. 1.** A cross-section of a leaf of *Arabidopsis thaliana* (A) and a profile of chlorophyll fluorescence in a comparable leaf (B) obtained as described by Vogelmann and Evans [29]. The false color scales show an increase in relative fluorescence intensity from blue to lilac. The light levels inside a leaf are highly heterogeneous, which is in direct contrast to scenarios where light is assumed either as homogeneous or as described by an exponential function.

which is a product of leaf thickness and leaf density and correlates well with plant growth rates [15]. In this article, we summarize our current understanding of the mechanisms through which leaf anatomy influences photosynthesis and hence plant growth [14,16,17]. These mechanisms include the effects of the anatomy on the distribution of light in the mesophyll, the  $\text{CO}_2$  diffusion processes [18,19], leaf temperature and leaf water relations [20], and leaf toughness [21,22]. Leaf structure and photosynthetic biochemistry also interact beyond the direct effect of anatomy on the leaf internal environment, as indicated by the change of leaf structure upon decreased expression of Rubisco [23,24]. The structural determinants of leaf photosynthetic efficiency have been extensively examined before [25]. Therefore, in this review we mainly highlight factors that are of specific importance to limiting photosynthesis in crop species, discuss the genetic regulation controlling the formation of specific leaf structural features, suggest several options that may help to improve photosynthetic efficiency, and finally advocate a new approach to identify anatomical features related to photosynthetic efficiency that can be targeted for improvement by genetic engineering or selective breeding.

## 2. Anatomy and the leaf internal environment

### 2.1. Influence of leaf anatomy on the internal light distribution

Similar to the light environment inside a canopy, the distribution of light inside the mesophyll is highly heterogeneous. The photosynthetic photon flux density tends to decrease with depth into a leaf, and the steepness of this gradient depends on the specific mesophyll structure [26–29]. However, the light received by some mesophyll cells may be higher than the light intensity at the surface of the leaf as a result of focusing of incident light by lens-shaped epidermal cells [30]. These observations highlight the important role of the leaf anatomy in determining the light levels that are available for photosynthesis at different locations within a leaf (Fig. 1).

The photosynthetic properties of the upper cell layers in the mesophyll are similar to those of sun-adapted leaves whereas the lower layers are more close to shade-adapted leaves [31], suggesting that the photosynthetic machinery acclimates to the internal light environment. However, the gradient of the photosynthetic capacity through the leaf tends to be more gradual than the gradient of light absorption [32] and this relative over-investment in the photosynthetic machinery lower in the leaf results in a sub-optimal photosynthetic efficiency. The heterogeneity of the leaf internal light environment is attributed to a number of mechanisms that are discussed below.

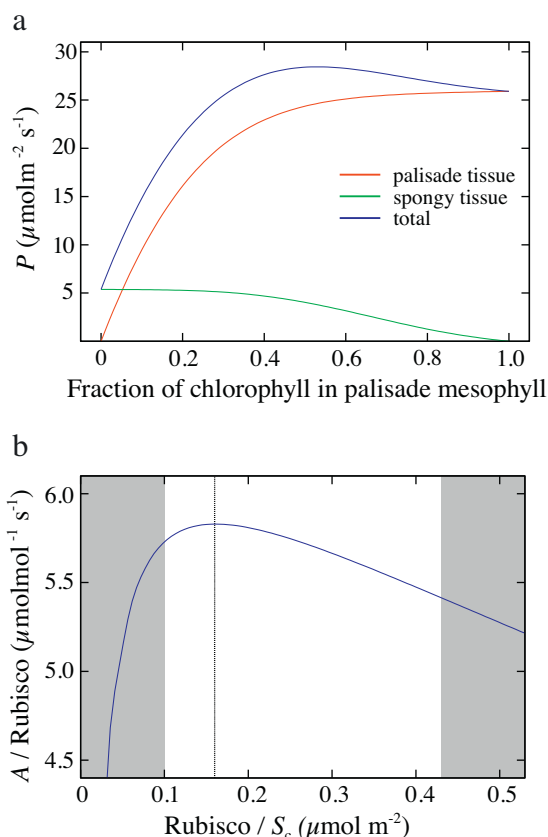
In many flat, broadleaf species the columnar palisade tissue facilitates the penetration of light deeper into the mesophyll. Light scattering is higher in the spongy mesophyll and increases the probability of light absorption [29]. The low absorbance of green relative to red and blue light by chlorophyll generates a differential

absorption spectrum throughout the leaf, with red and blue light being absorbed predominantly by the palisade tissue. Combined with the increased propensity for light scattering in the spongy cells, green light will be mainly absorbed by chloroplasts lower in the leaf [27,32]. Such a differential absorption spectrum may also help decrease photoinhibition by avoiding the absorbance of large amounts of green light by the top cell layers of the leaf. In this respect it is worth noting that there is a gradient of photoinhibition, demonstrated by analyzing chlorophyll fluorescence throughout the leaf [33,34].

In addition to the lower photosynthetic capacity of chloroplasts in the lower layers of the leaf, the spongy tissue contains more airspaces and therefore has a much lower photosynthetic capacity per unit leaf area. Why then do plants invest a significant portion of their chlorophyll in these spongy cells? It is possible to calculate the optimal distribution of chlorophyll between spongy and palisade tissue based on a model for the light-transmission in the mesophyll [32]. This analysis shows that an optimal photosynthetic rate can be sustained by green light if around half of the total chlorophyll is invested in the spongy tissue (Fig. 2A). Note that this analysis neglects several other aspects of the relation between photosynthesis and the light gradient, such as the possible occurrence of photoinhibition at high light intensities [33].

Bundle-sheath extensions, parenchyma or sclerenchyma cells without chloroplasts extending from the vascular bundle to the epidermis in so-called heterobaric leaves [35,36], may guide light deeper into the mesophyll of thick sun-adapted leaves [37,38]. In addition, various biominerals (such as calcium carbonate bodies) present in the leaves of some plant species can scatter the light within the leaf, leading to a more equally distributed light flux [39]. The potential impact on photosynthesis of the light heterogeneity caused by the presence of bundle-sheath extensions and biominerals in the leaf has not yet been quantitatively evaluated.

In addition to the effect of the mesophyll structure, chloroplast distribution influences the leaf internal light environment. For example, in several species chloroplasts move from a horizontal arrangement to a vertical arrangement after exposure of the leaf to high light. This response is thought to prevent the exposure of most chloroplasts to light intensities that would cause photo-damage [33,40] and decreases light absorption by cells near the upper surface of a leaf [32]. The latter would result in a more equal distribution of light throughout the leaf, which increases photosynthesis before the point where the light intensity becomes saturating [41]. This differential absorption allows the total light intensity to be more evenly distributed throughout the leaf and correspondingly increase whole-leaf photosynthetic  $\text{CO}_2$  uptake, similar to the improvement of canopy photosynthesis with more erect leaves [1]. Under low light, chloroplast move into a more horizontal arrangement that maximizes light absorption [42], and is expected to increase light limited photosynthetic rates, although the evidence for this remains scarce [43,44].



**Fig. 2.** (A) Modeled relation between the fraction of chlorophyll present in the palisade mesophyll and the photosynthetic rate that can be sustained by green light. Gross photosynthesis ( $P$ ) was calculated using a typical light response curve, i.e.  $\theta P^2 - (\alpha\phi I + P_{\max})P + \alpha\phi I P_{\max} = 0$  [32], where the curvature factor  $\theta = 0.965$ , maximum quantum yield  $\phi = 0.1$ , and light intensity  $I = 600 \mu\text{mol m}^{-2} \text{s}^{-1}$ .  $P_{\max}$  was set at  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ , but the average photosynthetic capacity of the spongy tissue was assumed to be only 20% of that in the palisade tissue [32]. Light absorbance  $\alpha$  was calculated using the Kubelka–Munk theory, with the absorption parameter  $k$  and scattering parameter  $s$  for green light in the palisade tissue being 3400 and 1100, and for the spongy tissue 5300 and 3500, respectively [32]. The total chlorophyll content of the leaf was kept constant at  $0.5 \text{ mmol m}^{-2}$  and the reflectance of the abaxial epidermis was set at 25%. (B) Relationship between the amount of Rubisco enzyme per unit exposed chloroplast surface ( $\text{Rubisco}/S_c$ ) and the rate of photosynthesis per unit Rubisco ( $A/\text{Rubisco}$ ) as predicted by a three-dimensional diffusion model. We assumed a near-saturating light intensity and the intercellular  $\text{CO}_2$  concentration was 30 Pa. Additional model parameters are described in [68]. Because the respiration rate was kept constant, an increase in Rubisco initially increases the net photosynthesis per unit Rubisco, because respiration per unit Rubisco decreases. Investing in more Rubisco per unit chloroplast area (thicker or more dense chloroplasts) increases the draw-down of the  $\text{CO}_2$  concentration in the chloroplasts and this leads to higher oxygenation rates and lowers photosynthetic efficiency. The gray area indicates values for  $\text{Rubisco}/S_c$  that are outside of the observed experimental values [66]. The dotted vertical line indicates the optimal Rubisco content per chloroplast surface assuming 100% of the Rubisco is activated.  $A/\text{Rubisco}$  depends on Rubisco activity, light level, temperature and  $\text{CO}_2$  concentration, but the values presented here are in the same range as those measured for tobacco leaves ( $5.6 \text{ mol mol}^{-1} \text{s}^{-1}$  for plants grown in a growth cabinet and assuming the molecular mass for Rubisco is 560 kDa [24]).

Given that light levels rapidly decrease with depth in the leaf, an optimal use of the available light puts constraints on the maximum thickness of leaves. Indeed, even at high light intensities, thicker leaves do not necessarily result in an enhanced photosynthetic capacity on a mass basis [45,46]. However, to ensure efficient use of all light energy captured by a leaf, a minimal leaf thickness is required to contain the necessary photosynthetic machinery and other cellular components. Furthermore, as indicated above, a certain thickness allows for a more efficient absorption of solar energy, by absorbing red and blue light predominantly in the top layers of

the leaf, and absorbing green light in the lower layers. Lastly, after canopy closure, there may simply not be enough space for increasing light capture by enlarging leaf area and the only gain possible is to further enhance the light absorption of all individual leaves. These reasons may explain why a low leaf mass per unit area correlates less well with high growth rates when irradiance increases [47,48] (but see [17]), suggesting that plants with thick or dense leaves have some advantages under high light.

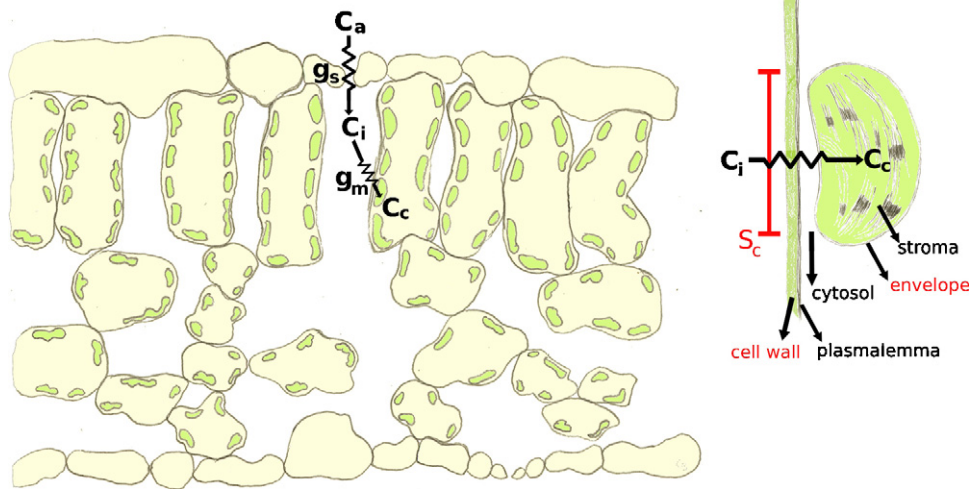
## 2.2. Influence of leaf anatomy on delivery of $\text{CO}_2$ to Rubisco

Atmospheric  $\text{CO}_2$  enters the leaf through stomata on the leaf surface. Under saturating light conditions, photosynthesis may be limited by the supply of  $\text{CO}_2$  from the atmosphere, and crop yields in several species have been found to correlate well with stomatal conductance [49–51]. From the substomatal cavities,  $\text{CO}_2$  diffuses through intercellular airspaces and subsequently through the cell wall, cytosol, chloroplast envelope, and finally into the chloroplast stroma. Each of these components has a certain diffusion resistance to  $\text{CO}_2$ . The total diffusion resistance between substomatal cavities and  $\text{CO}_2$  fixation by Rubisco is termed internal or mesophyll resistance and is commonly quantified by its reciprocal, the mesophyll conductance ( $g_m$ ) [52,53]. The leaf photosynthetic rate may be increased by up to 20% if mesophyll resistance can be eliminated [3].

Leaf anatomy has a large influence on several of the components that underlie  $g_m$  (Fig. 3). The intercellular airspace forms the first layer of resistance to  $\text{CO}_2$ . A long  $\text{CO}_2$  diffusion pathway through the intercellular airspace of thick or very dense leaves would result in a relatively large resistance [54]. The length of the diffusion pathway depends on the distance between stomata on the surfaces of the leaf, and will be greater if stomata are present on only one side of the leaf (hypostomatous leaves) [54]. In addition, the size and shape of the mesophyll cells will determine the effective diffusion path-length to the chloroplasts (porosity and tortuosity) [54]. However, given that the diffusion of  $\text{CO}_2$  in air is  $10^4$  times faster than that in solution, the contribution of resistance by diffusion through airspaces is usually relatively small, especially in species with stomata on both sides of the leaf [55–57].  $\text{CO}_2$  diffusion in the mesophyll of thick leaves indeed correlates with airspace volume [58–60], suggesting that in such cases, intercellular airspace does have an effect on  $\text{CO}_2$  diffusion rates. The presence of extensive bundle-sheath extensions effectively divides the mesophyll into compartments in so-called heterobaric leaves, and such a leaf morphology may limit lateral diffusion [35,61–63]. By contrast, homobaric leaves lack such bundle-sheath extensions and allow faster lateral movements of gases in the leaf [54,61–63]. The latter may benefit photosynthetic efficiency under conditions where the light intensity on the surface is highly heterogeneous [64].

After diffusion through intercellular airspaces,  $\text{CO}_2$  dissolves in the apoplastic water of the cell walls. The slow diffusion of  $\text{CO}_2$  through cell wall forms a major limitation to the rate of photosynthesis [65,66] and the conductance through the liquid phase scales well with wall thickness across a range of different species [65,66]. Both theoretical and experimental data suggest that the cell wall is responsible for 20–50% of the total diffusion resistance [60,65–67].

Given that the diffusion of  $\text{CO}_2$  is much faster in air than in liquid, the fastest way to diffuse through the cytosol is to ensure that a large chloroplast surface area is immediately adjacent to the cell wall. The cytosol is more viscous than pure water, which may further limit the conductance [68]. On the other hand, the pH is slightly above neutral and there is sufficient carbonic anhydrase present in the cytosol to facilitate diffusion [66,68]. Diffusion from “free” cell wall areas not directly facing chloroplasts has been assumed negligible [66,69]. Indeed, mesophyll conductance,  $g_m$ , correlates well with the chloroplast surface area facing intercellular airspaces



**Fig. 3.** The various factors influencing the delivery of  $\text{CO}_2$  to Rubisco. The flux of atmospheric  $\text{CO}_2$  ( $C_a$ ) into the leaf is limited by the stomatal conductance ( $g_s$ ). Inside the leaf, the flux between  $\text{CO}_2$  inside the intercellular airspace ( $C_i$ ) and the  $\text{CO}_2$  concentration at the site of carboxylation in the chloroplast ( $C_c$ ) is limited by the mesophyll conductance ( $g_m$ ). A larger amount of chloroplast surface that is directly exposed to intercellular airspace ( $S_c$ ) would increase  $g_m$  by increasing the surface area available for diffusion. In addition, the  $\text{CO}_2$  flux can be enhanced by lowering the resistance of individual components of the diffusion pathway. Potential targets that can be engineered to gain increased delivery of  $\text{CO}_2$  to Rubisco are indicated in red.

[19,69,70]. However, if there are only small gaps between individual chloroplasts, the path length for diffusion through the cytosol from free cell walls would not be too large and the diffusive flux through this surface area can be significant [67]. Such a chloroplast arrangement is common in many crop species and allows for a more efficient use of the complete wall surface area with a limited number of chloroplasts. Nevertheless, a more complete coverage of the cell wall surface with chloroplasts will still further minimize the total diffusion resistance, and maximally utilize the available wall surface for diffusion. For example, rice mesophyll cells are small and heavily lobed, and chloroplasts cover nearly the entire periphery of the mesophyll cells, with small stroma-filled protrusions that fill the gaps between chloroplasts along the cell walls [71].

To estimate the effect of cell shape and size on the mesophyll surface area available for diffusion, it is helpful to represent the lobed mesophyll cells by multiple overlapping spheres. The surface to volume ratio of a sphere with radius  $r$  equals  $3/r$ . Thus, assuming the ratchet-shaped mesophyll cells of rice consist of multiple overlapping spheres reducing the effective radius of each roughly spherical lobe by about 20%, this would increase the surface to volume ratio by 25% compared to isolated spherical cells. The small size of the mesophyll cells in rice (ca.  $1 \times 10^3 \mu\text{m}^3/\text{cell}$ ) compared to  $30 \times 10^3 \mu\text{m}^3/\text{cell}$  for barley [72,73] results in an even larger surface area available for  $\text{CO}_2$  diffusion. Interestingly, selection for a smaller cell size (at the same ploidy level) has been reported to increase photosynthetic capacity and yield in ryegrass (*Lolium perenne*) [74]. In wheat (*Triticum* spp.), photosynthetic capacity correlated negatively with the ratio between cell size and genome size over a range of ploidy levels [75]. However, it is unknown whether the positive effect of a smaller cell size in these studies was related to a reduction in mesophyll diffusion resistance.

The organelle arrangement in the cytosol may also influence  $\text{CO}_2$  diffusion by limiting the escape of respiratory and photorespiratory  $\text{CO}_2$  out of the cell. In rice mesophyll cells, mitochondria are located more towards the center of the cell, within an encasement of chloroplasts along the wall surface. Such an arrangement maximizes  $\text{CO}_2$  refixation, as any  $\text{CO}_2$  released by the mitochondria would have to cross the stroma before exiting the cells [67,71]. These typical features of rice leaf anatomy may explain why rice has nearly 80% of the photosynthetic capacity of wheat, despite having only half the mesophyll thickness [71].

Current best estimates of the individual resistances of the components of the diffusion pathway suggest that the chloroplast envelope may well form the largest resistance to diffusion, but accurate estimates of in vivo membrane permeabilities are difficult to obtain [65,68,76]. Overexpression of aquaporins, which may facilitate  $\text{CO}_2$  transfer across the chloroplast envelope and/or the plasma membrane, can increase mesophyll conductance, and lead to an up to 20% increase in photosynthesis, in both rice (*Oryza sativa*) and tobacco (*Nicotiana tabacum*) [77,78, and X.-G. Zhu, unpublished results]. Alternatively, carbon transport across the chloroplast membranes could also be facilitated by expression of inorganic carbon transporters from either cyanobacteria or algae in higher plants [13, Steve Long, Pers. Comm.].

Before  $\text{CO}_2$  is finally carboxylated, inorganic carbon diffuses from the inner chloroplast membrane to the site of fixation at Rubisco. If Rubisco is equally distributed throughout the stroma, the effective diffusion path-length through the chloroplast is much less than the chloroplast thickness if Rubisco. However, the high protein content results in a quite viscous stroma medium that would still result in a relatively low diffusion conductance [68]. The pH in the stroma in light is around 8, and the available carbonic anhydrase rapidly equilibrates  $\text{CO}_2$  and  $\text{HCO}_3^-$ , which enhances the diffusion rate and maintains a high  $\text{CO}_2$  concentration around Rubisco [65,66,68]. Since the CA concentration in the stroma is already relatively high,  $\text{CO}_2$  and  $\text{HCO}_3^-$  concentrations are thought to be close to the chemical equilibrium, it is therefore unlikely that a further increase in CA would significantly improve the rate of photosynthesis [65,68].

Rubisco not only catalyzes the reaction of ribulose 1,5-bisphosphate (RuBP) with  $\text{CO}_2$ , i.e. carboxylation, but also the reaction of RuBP with oxygen, i.e. RuBP oxygenation. The ratio of RuBP carboxylation to oxygenation varies depending on the concentration of  $\text{CO}_2$  around Rubisco. Normally, it is assumed that the oxygen concentration inside stroma is equivalent to the outside oxygen concentration, though this assumption has not been rigorously tested. The  $\text{CO}_2$  concentration at the site of carboxylation in the stroma is usually only about 50% of the ambient  $\text{CO}_2$  concentration at 25 °C [19,53]. Thus, at current ambient  $\text{CO}_2$  concentrations the RuBP oxygenation rate is around 40% of the carboxylation rate. With decreasing  $[\text{CO}_2]$ , the ratio of RuBP oxygenation to carboxylation gradually increases, effectively lowering



the rate of photosynthesis per unit Rubisco. Given this, it is advantageous to minimize the draw-down of the  $\text{CO}_2$  concentration by Rubisco in the stroma. If a high Rubisco content would be present in a small chloroplast volume, this would strongly decrease the  $\text{CO}_2$  concentration at the carboxylation site, which would subsequently increase the ratio of RuBP oxygenation to carboxylation and therefore decrease the amount of photosynthesis per unit Rubisco [66]. Instead, under saturating light conditions, the photosynthesis per unit Rubisco can be enhanced by increasing leaf thickness [57]. This allows for a large amount of Rubisco per unit leaf area, while at the same time keeping the amount of Rubisco per stroma volume low enough to prevent an excessive draw-down of the stroma  $\text{CO}_2$  concentration (Fig. 2B). Therefore, a thicker leaf can be advantageous for achieving a high photosynthetic efficiency under high light.

### 2.3. The relationship of leaf anatomy with water relations, temperature and leaf toughness

The slow decline in atmospheric  $\text{CO}_2$  since the Cretaceous era is correlated with a steady increase in the density of stomata on leaves of vascular plants, effectively countering the negative effect of the diminishing  $\text{CO}_2$  concentrations on leaf photosynthesis by raising stomatal conductance [79]. This increase in stomatal conductance resulted in additional water demands that were supported by a greater investment in vascular leaf tissue as indicated by the increase in vein density during the same period [80]. Currently, more than 25% of the total resistance for the water movement through the plant resides in the leaves [81], showing the importance of the leaf venation system for maintaining adequate transpiration rates.

The close relationship between  $\text{CO}_2$  delivery and water transportation in a leaf puts constraints on physiological and anatomical features of a leaf under a specific environment. For example, the xylem architecture imposes a limit on the maximum transpiration rates that can be achieved without dehydration of the mesophyll [81,82]. Thus, a high hydraulic conductance avoids inhibitions on the leaf biochemistry that can be expected when the volume of the protoplast in the mesophyll is reduced as a result of a water deficit [83]. Moreover, without sufficient evaporation, leaves would rapidly become too hot for many biological functions [20] and a minimum leaf thickness is required to allow for an adequate heat capacity. Some specific leaf anatomical features, such as the shape of the lamina, can help maintain leaf temperature by allowing for more efficient convective cooling [20]. It has been suggested that an increased stomatal conductance improves yield by lowering canopy temperatures, benefiting the activity of photosynthetic enzymes and reproductive development [49,51].

Although wider veins are more efficient for water transport, they are also more susceptible to freezing-induced cavitation (the formation of air bubbles inside xylem vessels), which would result in a lower hydraulic conductivity [84]. This may explain the prevalence of vessels with a narrow diameter at higher latitudes [84]. The hydraulic resistance of the leaf mesophyll is relatively high compared to that of xylem vessels, and as a result the hydraulic resistance of a leaf scales well with the path length of water through the mesophyll [85]. Because, as mentioned above, hydraulic conductivity constrains the photosynthetic capacity, maximum rates of photosynthesis increase with both vein density and with the distance from vein to epidermis [85]. In addition, an increased vein density may facilitate a higher photosynthetic capacity by allowing for more efficient photosynthate export from mesophyll cells [86].

In gymnosperms, lignification and apoptosis of part of the mesophyll tissue results in the formation of so-called transfusion tissue that greatly increases the conductivity to water [87]. Similarly,

bundle-sheath extensions in angiosperms have been suggested to act as a water conduit between the vasculature and the epidermis [35,36]. These extensions are more prevalent under conditions of high vapor pressure differences, warm temperatures or high light. Bundle-sheath extensions may also contribute to support and protection of the leaf blade against collapse after severe dehydration or other stresses [35,36,88]. In addition, they may be responsible for rapid stomatal responses to drought signals by facilitating the transportation of such signals from the transpiration stream to the leaf surface [36].

Leaf anatomy also plays an important role in maintaining the structural integrity of leaves. Large leaves require a disproportionately greater investment in supporting tissue to counter leaf bending [25,89] and the leaf mass per unit area of such large leaves is generally higher [21,22]. This is especially important in cereals as a greater investment in leaf mass area also allows for more erect leaves, which would optimize the light gradient in the canopy [90]. Reduced cell size would increase the mechanical strength of the leaf, allowing for thinner walls that may benefit  $\text{CO}_2$  diffusion [57]. Bundle-sheath extensions in heterobaric leaves can potentially provide additional mechanical support to leaf blade [88]. Leaves with a high leaf mass area (in particular as a result of a high tissue density) usually have a longer lifespan, which may ensure sufficient return of the carbon and energy investment that is required for constructing such leaves [14,91].

### 3. Genetic regulation of leaf anatomical characteristics

To allow the engineering of a specific leaf anatomy that would achieve a more homogeneous internal light distribution, more efficient  $\text{CO}_2$  delivery and improved water transport capacity, more effort is required to study the genetic mechanisms underlying different leaf structures. Here we describe the current status of the knowledge on such genetic controls of a few key photosynthesis-related leaf anatomical features, i.e. establishment of the dorsiventral polarity, vasculature formation, laminar expansion, shape morphogenesis, and the establishment of palisade and spongy tissues. The role of hormone signaling in leaf development is also be briefly described.

Leaves arise from a group of cells located at the flank of the shoot apical meristem. These cells differentiate along three axes to form a three-dimensional leaf primordium [92,93]. The establishment of the dorsiventral polarity is a critical event in the development of the leaf structure. The differentiation into ad- and abaxial domains is first induced by a yet unknown signal originating from the meristem [94]. Several small RNAs and a number of transcription factors have now been identified that play a role in determining dorsiventral identity [94,95]. Leaf vasculature develops on the interface between the ad- and abaxial domains, with the xylem differentiating adaxially and the phloem abaxially. The differentiation of mesophyll cells into veins is induced by auxin transport through cells and the auxin distribution depends on the specific localization of several auxin efflux carriers [96]. Although the mechanisms underlying differential expression of auxin carriers in the mesophyll are not clear, genes involved in the development of the ad/abaxial polarity appear to affect the spatial distribution of such carriers [97]. The distribution of auxin also plays an important role in the outgrowth of the lamina and leaf margin formation [94], which subsequently contributes to determining the shape of the leaf.

The regulatory network related to lamina outgrowth after the establishment of the dorsiventral domains has recently been reviewed [94]. Little is known about how these regulatory networks bring about the remarkable diversity of leaf shapes and mesophyll structure. At the start of leaf development, the plane of

the cell division is predominantly anticlinal, and after this initial division phase, lamina growth proceeds via cell expansion that is frequently associated with endoreduplication cycles [93,98]. A number of genes involved in leaf lamina growth have now been identified from *Arabidopsis* mutant studies. These genes are related to brassinosteroid synthesis, microtubule arrangement, cytoskeleton organization and cell cycle regulation (reviewed in [99,100]).

The morphogenesis of mesophyll cell shape is still poorly understood, but recently a comprehensive mechanism has been proposed for the formation of lobed cells [101]. According to this model, microtubule bundles determine the deposition of local cell wall thickenings, which prevent local growth resulting in lobes and constrictions. Actin filaments at the tips of these lobes may play a role in cell wall differentiation for lobe elongation. A number of candidate genes that are involved in these processes have been identified [101,102]. The most common differences in mesophyll cell shape are between the cells of the palisade and spongy tissues that occur in many leaves. Usually palisade parenchyma develops on the adaxial side, whereas the spongy parenchyma develops on the abaxial side of the leaf. Leaves that develop in high light conditions show an early onset of periclinal divisions at the expense of anticlinal divisions, leading to the typical sun leaf structure with an increased number of palisade cell layers [103]. The genetic factors controlling these differences in the direction of the cell division have not yet been identified.

In contrast to the unifacial leaves that develop in some monocots [104], members of the  $C_3$  grass family, including wheat and rice, retain an ad- and abaxial identity in the leaf blade. Although a clear differentiation between palisade and spongy parenchyma cannot be distinguished in such grass leaves, leaf size and thickness acclimates to the light environment [105]. The stomatal density, epidermal cell shape, the number of cell layers in the palisade tissue and the leaf mass per unit area in young developing leaves are affected by the light environment or  $CO_2$  concentration that was perceived by more mature leaves [106–110], suggesting that a systemic signal affects the development of shade or sun-acclimated leaves. Furthermore, the accumulation of photosynthetates in mature leaves may also trigger the development of sun or shade-type leaves in younger leaves [103,111,112]. In this regard, carbohydrate metabolism plays an important role in the acclimation of the photosynthetic machinery to the light intensity [113]. Although mutants with impaired photoassimilate export retain the ability to increase leaf thickness when grown under high light, such leaves contain fewer mesophyll cells [113].

Plant hormones are obvious candidates for both local and systemic signals that affect the development of a specific leaf structure. As described above, auxin plays a key role in the establishment of the leaf polarity and venation patterning [114]. Giberellin application decreases leaf mass per unit area [115], whereas mutants with impaired gibberellin synthesis or perception have an increase [15,116]. Cytokinin application increases leaf mass area, and the distribution of cytokinins in a leaf by the transpiration stream can be an effective mechanism that adjusts the leaf mass area and photosynthetic capacity to the local light intensity in the canopy [117,118]. Other hormones may only have indirect effects on the leaf structure. For example, although mutants with lower abscisic acid levels have a relatively high leaf mass area, this effect may be mainly due to a deteriorated water status [15,119]. Depending on the concentration, ethylene can promote or inhibit leaf elongation rates [120], though little is known about its effect on leaf and cell morphology. Plants with an impaired sensitivity to ethylene have a lower leaf mass area, which was linked to a lower organic nitrogen content per unit area, and a reduced photosynthetic capacity [121,122].

In summary, much progress has been made during the last decade in our understanding of the regulatory mechanisms that

**Table 1**

Ideal leaf anatomical features for water relations, light absorption and  $CO_2$  diffusion under three different environmental conditions.

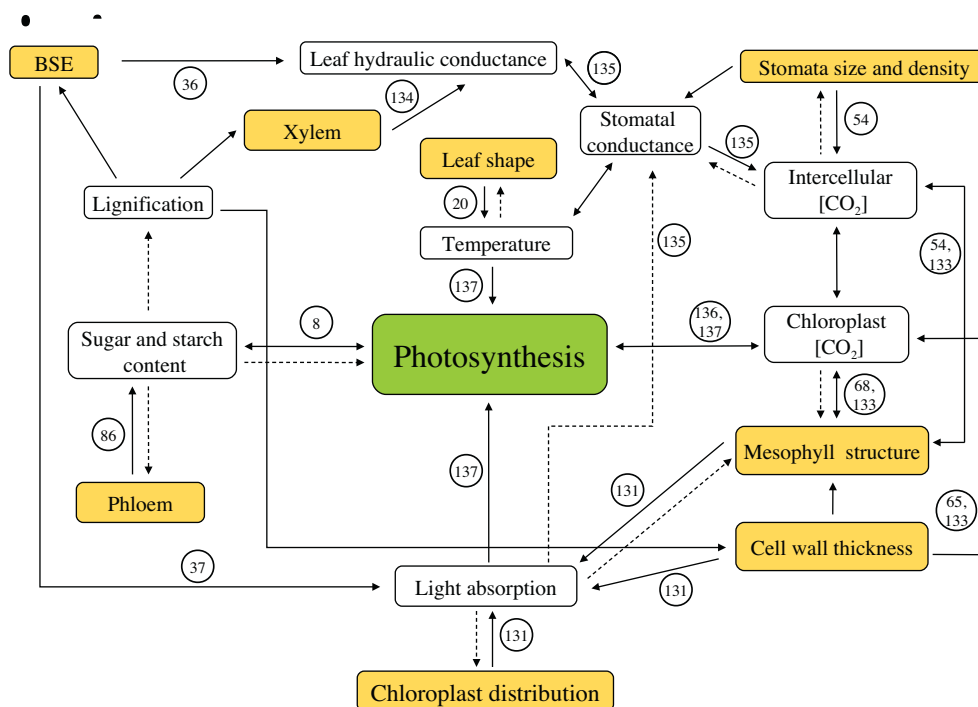
| Environment  | Leaf ideotype  |
|--|--|
| Warm (temperate or tropical), high light, with ample water supply                    | <i>Thick leaves</i> with many thin chloroplasts allow more light absorption in a dense canopy and enlarge the surface area available for $CO_2$ diffusion. A <i>high stomatal density</i> on both sides of the leaf facilitates $CO_2$ diffusion. <i>Thin cell walls</i> and <i>small mesophyll cells</i> further reduce diffusion resistances. <i>Elongated palisade cells</i> are necessary for a homogeneous light distribution within a leaf. <i>Erect leaves</i> allow for more leaves in a dense canopy, no specific palisade cells are necessary and all cells can be <i>thin walled, heavily lobed</i> and <i>small</i> resulting in a large surface area for diffusion. The erect leaves are usually thin and have a low heat capacity. Without high transpiration rates such leaves can become too hot under direct sunlight, therefore a high hydraulic conductance is necessary for maintaining a high stomatal conductance. This further requires a <i>high vein density, large veins</i> and <i>bundle-sheath extensions</i> . Extended lignification of the vasculature and bundle-sheath extension are necessary to maintain leaf shape. |
| Warm, high light, semi-arid environments   | The ideal leaf architecture will be constrained by water relations. More <i>stomata on the lower side of the leaf</i> . The leaf may be somewhat thinner than under wet conditions to minimize $CO_2$ diffusion, but this might lead to more water losses. <i>Thick cell walls</i> are necessary to maintain the cell structure under dry conditions. <i>Elongated palisade cells</i> homogenize the light within the leaf. A $C_4$ metabolism and anatomy would be most beneficial under these conditions   |
| Cool, with lower light or more diffuse light (at high latitudes or overcast weather) | <i>Thin leaves</i> are sufficient to capture all available light. Occasional periods of frost require <i>small veins</i> . Cells should be <i>small</i> and possibly <i>lobed</i> to increase the surface area available for diffusion.  |

control leaf growth. Still, the molecular mechanisms controlling leaf-level traits affecting the photosynthetic biochemistry (such as thickness, airspace volume, chloroplast surface areas) are still largely unknown. These traits reflect emerging properties of a complex network, and are not controlled by any particular single gene. Mathematical and computer modeling can help to link these networks with leaf level physiological traits, and systems models aiming for an improved understanding of leaf growth are being developed [123,124]. Such models can potentially be used to explore mechanisms controlling leaf architecture, and may also be used to identify genetic targets that regulate leaf traits related to an enhanced photosynthetic capacity.

#### 4. Perspectives on options to modify leaf anatomy for increased photosynthesis

A specific leaf anatomy often offers adaptive advantages to the native environment of a species [25]. For example, the occurrence of bundle-sheath extensions in rice increases the hydraulic conductance and can support a high stomatal conductance at the cost of reducing the amount of mesophyll cells per unit leaf area. Such a leaf anatomy may be advantageous under conditions where plenty of water is available but it may be less suitable under more dry conditions. Although an ideal environment for achieving improved yields would provide ample supply of light, water and nutrients at optimum growth temperatures, in practice it will be necessary to breed cultivars that are best adapted to a specific set of conditions.

The ideal anatomy presented in Table 1 serves as a starting point for identifying leaf traits that can be modified to achieve a



**Fig. 4.** Schematic overview of the major relations between leaf anatomy and photosynthetic efficiency. Anatomical traits are indicated in yellow. Continuous arrows indicate direct biophysical relations whereas dotted arrows indicate regulatory relationships. Numbers refer to current literature that provides a quantitative description of that relation. A complete systems model of leaf photosynthesis needs to include all these different aspects.

higher photosynthetic efficiency under a specific set of conditions. We emphasize that these recommendations are based on empirical observations of the relationship between leaf anatomical features and photosynthetic efficiencies in a relatively limited number of plant functional groups. Given that leaves in nature have large variations in anatomical features [14], the effects of these recommended options to increase photosynthesis in more species still need to be tested. Furthermore, the optimal leaf anatomy varies with the environment and the developmental stage. For example, a more rapid closure of the canopy during early growth can be achieved by selecting for plants with a low leaf mass area early in the growth season [50,125], but it has also been suggested that the observed increase in leaf mass area during the later growth stages of rice and wheat [50,125] allows for a more efficient capture of light energy after canopy closure. Lastly, the optimal leaf anatomy depends on the specific light and  $\text{CO}_2$  conditions. Both leaf mass area and leaf thickness increase under high light or elevated  $\text{CO}_2$  concentrations [15,126]. Thicker leaves are preferred under high light in species with flat, horizontally oriented leaves [57]. However, the thin, erect leaves of rice result in a longer diagonally light-path across the leaf blade, reducing the effectiveness of an increased leaf thickness.

Domesticated rice has erect, thin leaves with a densely packed chlorenchyma tissue, bundle-sheath extensions and many small, heavily lobed chlorenchyma cells [71] that have relatively thin cell walls [127]. This leaf anatomy allows rice to achieve a high rate of photosynthesis in its natural habitat. It is difficult to envisage further improvements to the leaf structure without also changing biochemical traits. In this respect, it has been proposed to outfit rice with a  $\text{C}_4$  photosynthetic mechanism [11,128]. In many  $\text{C}_4$  plants, photosynthetic efficiency is enhanced by the accumulation of  $\text{CO}_2$  to above-ambient levels in bundle-sheath cells, that are more closely spaced, larger and contain more chloroplasts compared to those present in leaves of  $\text{C}_3$  plants [129]. Efforts are currently being undertaken to identify the possibility of realizing such a specialized anatomy in rice leaves [128,130].

Given the significant impact of these different anatomical features on photosynthesis, how can we expedite the progress of engineering a leaf anatomy that benefits photosynthetic efficiency? In addition to more research on the genetic mechanisms controlling leaf anatomical features, one aspect that needs substantial attention is the development of tools that enable us to pinpoint anatomical features of specific leaves in the canopy that could be altered in order to achieve a higher photosynthetic efficiency under a given set of environmental conditions. This is challenging because internal leaf microclimatic factors, e.g. light,  $\text{CO}_2$  and temperature, etc., are all influenced by leaf anatomy and these factors often interact with each other on several levels. As a result, the possible mechanisms underlying a particularly high or low photosynthetic rate in a leaf can be determined in retrospect, but we still lack an effective method to guide the design and engineering of leaf anatomy for increased photosynthetic  $\text{CO}_2$  uptake rate. In silico studies with comprehensive leaf photosynthesis models, which describe the major processes influencing photosynthesis, is a feasible option to study this question. Ideally, such a model needs to include a detailed leaf anatomical structure and describe light distribution,  $\text{CO}_2$  diffusion, metabolic reactions, plastid arrangement, stomatal responses and energetic balances in a leaf.

Is it realistic to develop such a model? Luckily, a number of basic modules required for developing a comprehensive model have already been constructed, e.g. models for the light distribution [131], leaf energy balance [132],  $\text{CO}_2$  diffusion [68,133], leaf hydraulics [134], stomatal conductance [135] and biochemical aspects of leaf photosynthesis [8,136,137]. The present knowledge and available quantitative models related to these modules are summarized in Fig. 4. Still, to combine these different modules into a robust, comprehensive photosynthesis model is a major challenge for plant systems biology research in the coming years. Once developed, combined with modern optimization algorithms, this model will provide a unique approach that will not only identify optimal engineering targets but also the quantitative features of engineering targets in a defined crop species and under a given set

of environmental conditions. Such a model will be a major tool to study the adaptation and evolution of various plant anatomical and biochemical features in previous and future climates.

## 5. Concluding remarks

Although leaf photosynthesis has been the focus of much research, crop yields are more related to whole canopy photosynthesis instead of leaf photosynthesis [138]. A clear relationship between crop yields and leaf photosynthetic capacities per se cannot always be identified [50,90], but leaf photosynthesis is amendable to genetic manipulation and forms a first step towards finding new approaches to improve crop yields. This review emphasizes that given the substantial variations in leaf anatomical features among different plant species, there is large potential to engineer leaf anatomy for improved photosynthetic efficiencies by homogenizing the leaf internal light distribution, and by minimizing CO<sub>2</sub> diffusion resistances in the leaf. Unfortunately, compared to metabolic engineering, engineering anatomy for increased photosynthesis is still in its infancy because the molecular mechanisms controlling leaf anatomical features are far from being understood. In addition, effective tools to pinpoint targets that can be engineered still need to be developed. A comprehensive systems model of leaf photosynthesis combined with optimization algorithms may provide a feasible strategy to achieve this goal.

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