Prospects for enhancing leaf photosynthetic capacity by manipulating mesophyll cell morphology

Running title: Photosynthetic capacity and leaf cell morphology

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Highlight: Cell morphology in leaves affects photosynthesis by controlling CO₂ diffusion and light distribution. Recent work has uncovered genes that control cell size, shape, and number paving the way improved photosynthesis.

29 Abstract

30 Leaves are beautifully specialized organs designed to maximize the use of light and
 31 CO₂ for photosynthesis. Engineering leaf anatomy therefore brings great potential to
 32 enhance photosynthetic capacity. Here we review the effect of the dominant leaf
 33 anatomical traits on leaf photosynthesis and confirm that a high chloroplast surface
 34 area exposed to intercellular airspace per unit leaf area (S_c) is critical for efficient
 35 photosynthesis. The possibility of improving S_c through appropriately increasing
 36 mesophyll cell density is further analyzed. The potential influences of modifying
 37 mesophyll cell morphology on CO₂ diffusion, light distribution within the leaf, and
 38 other physiological processes are also discussed. Some potential target genes
 39 regulating leaf mesophyll cell proliferation and expansion are explored. Indeed, more
 40 comprehensive research is needed to understand how manipulating mesophyll cell
 41 morphology through editing the potential target genes impact leaf photosynthetic
 42 capacity and related physiological processes. This will pinpoint the targets for
 43 engineering leaf anatomy to maximize photosynthetic capacity.

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45

46 **Key words:** chloroplast surface area exposed to intercellular airspace, light
 47 distribution, mesophyll cell density, mesophyll conductance, photosynthesis, target
 48 genes

49

50 **Abbreviations:** A_n , photosynthesis rate; f_{ias} , fraction of intercellular airspace; g_m ,
 51 mesophyll conductance; g_s , stomatal conductance; L_{chl} , chloroplast length; LMA, leaf
 52 mass per unit leaf area; S_c , chloroplast surface area exposed to intercellular airspace
 53 per unit leaf area; S_{mes} , mesophyll surface area exposed to intercellular airspace; T_{chl} ,
 54 chloroplast thickness T_{cw} , mesophyll cell wall thickness; T_L , leaf thickness; T_{mes} ,
 55 mesophyll layer thickness.

56

57 Introduction

58 Increasing the conversion efficiency of intercepted radiation into biomass through
 59 enhancing photosynthesis has emerged as a promising focus to enhance crop
 60 production for meeting needs of the increasing population and for plant resilience in
 61 the face of the changing global climate (Long *et al.*, 2015b; Ort *et al.*, 2015; Souza *et al.*, 2017). Some current approaches to improving photosynthesis in C₃ crops, include
 62 conversion to C₄ metabolism, engineering a photorespiratory bypass, optimizing
 63 regeneration of RuBP etc. It is estimated that these could increase the light conversion
 64 efficiency by 5%-60% compared with today's best cultivars (Long *et al.*, 2015b).
 65 Using the rice GRCROS model, Yin and Struik (2017) simulated nine genetic
 66 engineering strategies for enhancing C₃ leaf photosynthesis to improve crop
 67 production under the present climate and the climate predicted for 2050. Although the
 68 knowledge of photosynthesis has exploded recently, there is no doubt that it is still a
 69 major challenge to understand the mechanisms, vulnerabilities, and potentials for
 70 improvement of photosynthesis (Niinemets *et al.*, 2017).

72 Leaves are beautifully specialized organs evolved for efficient photosynthesis. Their
 73 morphological differences can explain substantial variations in photosynthetic
 74 properties among different species or the same species under different environmental
 75 constraints (Oguchi *et al.*, 2005; Terashima *et al.*, 2011; Peguero-Pina *et al.*, 2017;
 76 Tsukaya, 2018). For example, alterations in photosynthetic performance of sun and
 77 shade leaves of the same plant are intimately connected with leaf anatomy (Terashima
 78 *et al.*, 2001, 2006; Oguchi *et al.*, 2005; Théroux-Rancourt and Gilbert, 2017). The
 79 inseparable relationship between leaf architecture and photosynthesis thus brings
 80 great potential to enhance photosynthetic efficiency by modifying leaf anatomy.
 81 Tholen *et al.* (2012) described how altering leaf anatomy could significantly influence
 82 light distribution and CO₂ diffusion in the leaf. Nonetheless, precise targets for
 83 engineering leaf anatomy are still unknown because of poor understanding of the
 84 molecular mechanisms controlling leaf anatomy.

85 Leaf mass per unit leaf area (LMA), one important leaf morphological trait that
 86 affects photosynthesis, is also related to environmental stress and longevity of plants
 87 (Niinemets, 2001; Poorter *et al.*, 2009; de la Riva *et al.*, 2016). There exists
 88 considerable variation regarding the correlation between LMA and photosynthesis.
 89 Negative relationships between LMA and photosynthesis are associated with higher
 90 investment in nonphotosynthetic tissue, such as cell walls (Onoda *et al.*, 2004, 2017;
 91 Hassiotou *et al.*, 2010) and lower mesophyll conductance (Flexas *et al.*, 2008;
 92 Niinemets *et al.*, 2009; Weraduwege *et al.*, 2016). Positive relationships are associated
 93 with improvement of chloroplast exposure to intercellular airspace (Peguero-Pina *et al.*
 94 *et al.*, 2017) and chloroplast CO₂ concentration, which ultimately enhances the
 95 carboxylation rate (Cai *et al.*, 2014; Flexas *et al.*, 2014) under higher LMA owing to an
 96 increase in leaf thickness. The influence of LMA on photosynthesis depends in part on
 97 how LMA impacts mesophyll conductance. An increase in LMA related to increased
 98 leaf density might cause mesophyll cells to be densely packed, which will inevitably
 99 increase mesophyll CO₂ resistance (Flexas *et al.*, 2008; Niinemets *et al.*, 2009;
 100 Weraduwege *et al.*, 2016). However, if the mesophyll cell space is large enough to
 101 avoid excessive cell-to cell contact, it is possible to increase mesophyll and
 102 chloroplast surface area exposed to intercellular airspace per unit leaf area and
 103 improve mesophyll conductance (Peguero-Pina *et al.*, 2017). Recently, John *et al.*
 104 (2017) wrote that LMA is determined by leaf cell numbers, dimensions and mass
 105 densities. Optimizing LMA through regulating mesophyll cell morphological features
 106 thus might be a good target to enhance leaf photosynthetic capacity.

107 Early in the 1960s and 1970s there were many attempts to correlate leaf anatomical
 108 characteristics with photosynthesis. Mesophyll cell size was found to be an important
 109 factor (Jellings and Leech, 1984). Wilson and Cooper (1970) reported that selection
 110 for a smaller cell size could be used to improve photosynthesis and yield in ryegrass
 111 (*Lolium perenne*). But at that time, it was not entirely known how mesophyll cell size
 112 impacts leaf photosynthesis rate; possible factors include cell volume, cell surface

area, or the effect of cell shape on cellular packing within the leaf (Jellings and Leech, 1984). In the last two-decades, extensive investigations of leaf anatomical traits and photosynthesis among different species or same species under various environmental conditions have reemerged (Kogami *et al.*, 2001; Hanba *et al.*, 2002; Oguchi *et al.*, 2005; Peguero-Pina *et al.*, 2012, 2016; Sáez *et al.*, 2017). Although the typical characteristics of leaves with high photosynthetic efficiency among different species or environmental factors are not always consistent, a higher proportion of chloroplast surface area exposed to intercellular airspace per unit leaf area (S_c) is generally associated with high rates of photosynthesis. High S_c reduces the CO_2 diffusion resistance in the leaf and improves CO_2 concentration at the sites of carboxylation. Higher S_c values associated with more and smaller mesophyll cells could lead to selection for smaller cells. Achieving higher S_c values through manipulating mesophyll cell morphological traits might be a crucial spotlight of engineering leaf anatomy to enhance leaf photosynthetic efficiency.

Furthermore, increasing explorations of the key target genes regulating cell proliferation and expansion provides more possibilities for engineering leaf architecture to improve photosynthetic efficiency. Takai *et al.* (2013) revealed the gene *GREEN FOR PHOTOSYNTHESIS* (*GPS*) increases mesophyll cell number between vascular bundles, which leads to enhanced photosynthesis rates. Lehmeier *et al.* (2017) manipulated mesophyll cell density through promoting or repressing *KIP-RELATED PROTEIN1* (*KRP1*) and *RETINOBLASTOMA RELATED PROTEIN 1* (*RBR1*) gene to increase photosynthetic capacity. Our recent work also found that the leaves of *ROOT HAIR DEFECTIVE 3* (*RHD3*) defective mutants have more and smaller mesophyll cells, and their photosynthetic rate was much higher than wild type. All these indicate that regulation of mesophyll cell division, expansion and arrangement in leaves is a potential target of engineering leaf anatomy by genetic approaches to enhance photosynthetic efficiency. Therefore, this manuscript reviews the effects of the dominant leaf anatomical traits on leaf photosynthesis, analyzes the

possibility of improving S_c through re-arranging mesophyll cells, and further discusses the potential influences of modifying leaf anatomy on CO_2 diffusion, light utilization and other physiological processes. Finally, some potential target genes are explored.

2. Enhancing leaf photosynthetic capacity by altering leaf anatomy

2.1 Effects of leaf anatomical characteristics on photosynthesis

A typical C_3 leaf cross-section, comprising two epidermal layers surrounding a tight array of columnar cells (palisades) and irregularly shaped cells (spongy), provides powerful tool to explore various morphological features associated with leaf physiological functions. Here we summarize 43 published research papers and extracted the major leaf anatomical and photosynthetic parameters (Table S1), including leaf thickness (T_L), mesophyll layer thickness (T_{mes}), fraction of intercellular airspace (f_{ias}), LMA , mesophyll cell wall thickness (T_{cw}), chloroplast length (L_{chl}), chloroplast thickness (T_{chl}), mesophyll surface area exposed to intercellular airspace (S_{mes}), S_c , stomatal conductance (g_s), mesophyll conductance (g_m) and photosynthesis rate (A_n) (Fig. 1). There are huge variations in leaf morphological characteristics of different species in different environments, and the coefficient of variance (c.v.) values vary from 22% to 79%. The smallest coefficient of variation is the length of the chloroplast. The average chloroplast length is $4.81 \mu\text{m}$, varying from $1.58 \mu\text{m}$ to $6.86 \mu\text{m}$. The coefficient of variation of LMA is highest, averaging 76 g m^{-2} and ranging from 11 g m^{-2} to 285 g m^{-2} .

(Fig. 1)

There is no significant correlation between LMA and A_n (Table 1), as reported in previous studies (Hassiotou *et al.*, 2010; Veromann-Jürgenson *et al.*, 2017). Moreover, the long distance between these two parameters indicates wide variability between LMA and A_n (Fig. 2). As described in many reports (Poorter *et al.*, 2009; de la Riva *et al.*, 2016; Veromann-Jürgenson *et al.*, 2017; John *et al.*, 2017), leaf thickness, mesophyll

layer thickness, mesophyll cell wall thickness and intercellular airspace are the dominant factors influencing LMA . When the LMA value is less than 100 g m^{-2} , an increase in LMA significantly promotes S_{mes} and S_c values. However, when it exceeds 100 g m^{-2} , higher LMA value plays only a minor role in the improvement of the S_{mes} and S_c value. On the contrary, higher LMA reduces the f_{ias} and S_c/S_{mes} values, possibly because of densely packed mesophyll cells, further decreasing g_m (Table 2).

Despite different species and environmental conditions, significantly positive correlations between S_c and A_n and g_m are observed; S_c is the closest positive parameter to A_n and g_m of all summarized leaf morphological parameters. From our results, the average S_c value is $11.1 \text{ m}^2 \text{ m}^{-2}$, ranging from $1.2 \text{ m}^2 \text{ m}^{-2}$ of *Deschampsia antarctica* Desv in Lagotellerie island (Sáez *et al.*, 2017) to $32.3 \text{ m}^2 \text{ m}^{-2}$ of *Abies pinsapo* needles in the Mediterranean area (Peguero-Pina *et al.*, 2012). High variations and significant positive correlation between S_c and A_n confirm that S_c is a potential target of engineering leaf anatomy to enhance leaf photosynthetic capacity.

(Fig.2)

(Table 1)

(Table 2)

2.2 Increase S_c by manipulating mesophyll cell morphology

S_c is the most important leaf structural parameter in photosynthetic studies during the last decades. von Caemmerer and Evans (1991) showed that higher S_c could explain the relatively large CO_2 transfer conductance of rice. Almost all subsequent studies have confirmed the importance of having a high S_c value for maximizing photosynthesis (Terashima *et al.*, 2011). The S_c value is influenced by chloroplast number, distribution in the mesophyll and mesophyll surface area exposed to intercellular airspace. An increase in the number of chloroplasts in mesophyll cells is beneficial to improve S_c (Miyazawa and Terashima, 2001). Additionally, changes in light intensity and quality can induce movement and re-distribution of chloroplasts

within cells, further affecting S_c (Hanba *et al.*, 2002; Oguchi *et al.*, 2003). The maximum value of S_c is determined by S_{mes} , which is the closest positive parameter to S_c (Fig. 2). In our analysis the average S_c/S_{mes} value is 0.71, implying that nearly 3/4 of mesophyll cell surface area facing intercellular airspace is occupied by chloroplast. For *Castanopsis sieboldii*, the S_c/S_{mes} ratio could be 1.0 after 20 days of full leaf area expansion (Hanba *et al.*, 2002). Oguchi *et al.* (2003) described that transferring the mature leaf from low to high light significantly increased photosynthesis rate, associated with improving S_c . The photosynthesis rate in the low-light leaf was significantly lower than the leaf exposed to high light because of restricted S_{mes} value. Greater S_{mes} is a prerequisite for a larger S_c . Therefore, simultaneously raising the S_{mes} and S_c is the key to enhance leaf photosynthetic efficiency.

Nobel *et al.* (1975) first reported in that the mesophyll cell surface area per unit leaf area (A_{mes}/A in their notation), calculated assuming that mesophyll cells were cylindrical with hemispheres at each end, was correlated with CO_2 assimilation rate. Evans *et al.* (1994) optimized the calculation depending on the shape of palisade and spongy tissue. According to our literature review, the average S_{mes} value is $15.9 \text{ m}^2 \text{ m}^{-2}$, ranging from $2.8 \text{ m}^2 \text{ m}^{-2}$ of *Arabidopsis cgr2/3* mutant leaf (Weraduwanage *et al.*, 2016) to $41.6 \text{ m}^2 \text{ m}^{-2}$ of sun *Camellia japonica* leaf (Terashima *et al.*, 2006). To facilitate CO_2 diffusion in the leaf, most of the surface area of mesophyll cells are exposed to intercellular airspaces (Nobel, 2009). Greater total length of mesophyll cells exposed to intercellular airspaces thus is prerequisite to obtain the greater values of S_{mes} . Terashima *et al.* (2011) illustrated four strategies of improving mesophyll surface area, including (1) cell elongation, (2) cell elongation accompanied by cell division, (3) decrease in cell size, (4) armed cells of grass species having lobes. For lobed cells or armed cells, high surface area exposure of mesophyll cells and chloroplasts to intercellular spaces and enhanced mesophyll conductance was found in *Oryza* spp and *Viburnum* spp (Sage and Sage, 2009; Chatelet *et al.*, 2013). Adachi *et al.* (2013) and He *et al.* (2017) showed two rice lines named BTK-a and BTK-b to have

223 20-50% higher photosynthetic rate than the parents because of highly developed lobes
 224 of mesophyll cells. Although there are slight differences in the strategies to improve
 225 mesophyll surface area, the key of the other three approaches is the trade-off between
 226 cell size and cell density because the mesophyll cell size is negatively correlated with
 227 cell number when the leaf thickness is constrained (Pyankov *et al.*, 1999).

228 To understand S_{mes} easily, here some geometrical idealization is adopted. Let us
 229 assume that all mesophyll cells in a cross-section are spherical; cells are tightly
 230 packed and all cell walls are exposed to the intercellular airspaces (Fig. 3). Thus, S_{mes}
 231 is equal to the total circumference of the spherical cells divided by the width of the
 232 cross-section according to the 2D model. If there is only a single uniform sphere in
 233 cross-section, S_{mes} is π . If cell diameter is reduced to one-third, three layers with nine
 234 uniform spherical cells would produce a S_{mes} of 3π . Increasing cell density would
 235 significantly improve S_{mes} when the leaf thickness remains unchanged. Indeed,
 236 mesophyll cell density cannot be increased indefinitely. Smaller cell volume would
 237 result in over-lapping chloroplasts in mesophyll cells because of the strong correlation
 238 between the number of chloroplasts in a cell and the mesophyll cell area (Pyke and
 239 Leech, 1991, 1992). Therefore, the optimum is the minimum volume of mesophyll
 240 cell in which all the chloroplasts are tightly attached to cell wall and avoid
 241 over-lapping of chloroplasts. We have not rigorously examined the published
 242 photomicrographs of leaf cross-section, however, the number of chloroplasts in
 243 individual mesophyll cells seen in 2D cross-section is usually about 10-30. Compared
 244 with other leaf anatomical features, the variation in length and thickness of
 245 chloroplasts is smaller, with an average of $4.81 \mu m$ and $2.46 \mu m$, respectively. The
 246 typical spherical mesophyll cell area containing 10-30 chloroplasts occupying all
 247 mesophyll surface area facing intercellular airspace is between $241-1780 \mu m^2$. If the
 248 mesophyll surface area is less than that value, chloroplasts might overlap in the
 249 mesophyll cell, which would result in the decrease of S_c . In addition, adequate
 250 mesophyll cell space is a prerequisite to achieve an increase in cell density. From Fig.

3, it could be found that f_{ias} is still 0.215, despite many more mesophyll cells. From the literature, the average f_{ias} is 0.241 regardless of species and environment, varying from 0.059 of *Oryza sativa* cslf6-2 mutant (Ellsworth *et al.*, 2018) to 0.675 of *Asplenium scolopendrium* (Carriqui *et al.*, 2015). Great variations in f_{ias} produce the possibility to utilize internal volume of the leaf to assemble more mesophyll cells to improve S_{mes} and S_c . Authentic mesophyll cells are not uniformly spherical and are more complex, nevertheless, for most species the intercellular space is sufficient to be equipped with more mesophyll cells. Appropriate manipulation of mesophyll cell density to increase mesophyll and chloroplast surface area exposed to intercellular air space is a potential approach for enhancing leaf photosynthetic capacity by altering leaf anatomy.

3. Potential changes of photosynthetic capacity by manipulating mesophyll cell morphology

3.1 The mechanism of enhancing photosynthesis

CO₂ diffusion

After arriving at substomatal cavities, atmospheric CO₂ diffuses through intercellular airspaces and then passes through the cell wall, cytosol, chloroplast envelope, finally reaching the sites of carboxylation in the chloroplast. The total CO₂ diffusion resistance between substomatal cavities and carboxylation sites is termed mesophyll resistance and its reciprocal is defined as mesophyll conductance (g_m), which is sufficiently small to restrict photosynthesis (Warren, 2008; Flexas *et al.*, 2008, 2012; Evans *et al.*, 2009). Mesophyll resistance can be divided into the gas-phase and liquid-phase resistance, in which liquid-phase resistance could account more than 90% of mesophyll resistance (Parkhurst, 1994; Tosens *et al.*, 2012; Lu *et al.*, 2016). In the liquid-phase, many CO₂ diffusion parameters have not been accurately quantified (Evans *et al.*, 2009; Xiao and Zhu, 2017), however, cell wall thickness and S_c are considered the most important anatomical parameters influencing g_m (Syvertsen *et al.*,

1995; Tomás *et al.*, 2013). Summarized data confirmed the significantly negative correlation between g_m and cell wall thickness (Fig. 2). By contrast, g_m is positively correlated with S_c because a large chloroplast surface area adjacent to the cell wall is beneficial for fast diffusion through the cytosol and minimizes the total diffusion resistance. He *et al.* (2017) reported an increase in the number of lobed mesophyll cells induced much higher total surface area of mesophyll cells in the BTK lines, further resulting in greater mesophyll conductance. As shown in Fig.3, without increasing leaf thickness, increasing mesophyll cell number improves the mesophyll surface area, thereby increasing the number of chloroplasts per unit leaf area and S_c . Higher S_c improves CO_2 mesophyll conductance, increases CO_2 concentration at carboxylation site and enhances photosynthetic capacity.

289 **Light interception and distribution in leaves**

290 Leaf anatomy is well designed to capture solar and maximize light absorption; leaves
291 often absorb more than 85% of the photosynthetically active radiation that hits them
292 (Evans and Poorter, 2001). Lens-shaped epidermal cells can concentrate incident light
293 severalfold, columnar palisade cells facilitate the penetration of light into the leaf, and
294 randomly arranged spherical spongy cells and intercellular airspaces intensely scatter
295 and reflect light, which increases the probability of light absorption for photosynthesis
296 (Vogelmann *et al.*, 1996a,b; Ustin *et al.*, 2001; Smith *et al.*, 2004). With depth into a
297 leaf, the light and chlorophyll fluorescence intensity, especially for red and blue light,
298 decline remarkably due to absorption and scattering, depending upon pigmentation as
299 well as the mesophyll structure (Vogelmann, 1993; Vogelmann and Evans, 2002).

300 Cui *et al.* (1991) showed more and elongate palisade cells within sun leaves facilitated
301 the light transmission in leaves and spread the light through the depth of the leaf while
302 less compact palisade cells within shade leaves helps increase light absorption
303 through greater scattering. Thus, altering leaf anatomy through regulating mesophyll
304 cell geometry and packing would greatly influence the distribution of internal light.
305 More and slightly-stacked mesophyll cell layers could efficiently facilitate the

penetration of light into the leaf reducing light absorption in the top most layer that might be saturated by light allowing light to penetrate to the lower layers that may not be saturated by light. On the other hand, smaller intercellular airspace with small pockets could scatter light more (Smith *et al.*, 2004), increase the light pathlength and enhance light absorption. Besides reducing CO₂ diffusion resistance, higher S_c accompanied by more chloroplasts exposed to intercellular airspace aids efficient light absorption and helps satisfy the electron requirement for CO₂ assimilation associated with high CO₂ conductance (Terashima *et al.*, 2009). The transgenic line *ATML1_{pro}*:*KRPI* leaves exhibited an increase in palisade cell density improving air channel circularity and density, promoting the proportion of light energy used and operational electron transport rate (Lehmeier *et al.*, 2017).

3.2 Other potential changes

Leaf growth is determined by the amount of photosynthetic C partitioned between leaf area growth and *LMA* (Weraduwaage *et al.*, 2015). An alteration in C investment to leaf area or *LMA* could influence plant growth. Weraduwaage *et al.* (2016) showed that overexpression of the *Cotton Golgi-related (CGR)* genes *CGR2* and *CGR3*, which mediate pectin methylesterification, increased C partitioned to leaf area growth and improved whole-plant photosynthesis and plant growth. Greater numbers of mesophyll cells would result in higher *LMA* (Pyankov *et al.*, 1999; Poorter *et al.*, 2009; John *et al.*, 2017), which requires more C invested in *LMA* and reduces the available C for leaf area growth. Additionally, mesophyll cells make more than 90% of total leaf respiration owing to more mitochondria in mesophyll cells (Long *et al.*, 2015a). More mesophyll cells mean more mitochondria and higher leaf respiration, reducing photosynthetic C available for plant growth (Weraduwaage *et al.*, 2016). Manipulating mesophyll cell density could be used to enhance photosynthetic capacity, nonetheless, it might change the allocation of photosynthetic C, further affecting plant growth.

Previous studies have described strong correlations between leaf hydraulic conductance and CO₂ diffusion and photosynthesis rate (Flexas *et al.*, 2013; Scoffoni

334 *et al.*, 2016; Barbour, 2017). Both leaf water transport and CO₂ delivery have
 335 similarities in leaf anatomical traits. The leaf hydraulic system is composed of the
 336 major and minor veins, bundle sheaths, and the mesophyll cells outside the vein
 337 xylem. Mesophyll cell characteristics, such as mesophyll thickness, mesophyll cell
 338 porosity and connectivity, mesophyll cell surface area for evaporation, which
 339 significantly affect CO₂ diffusion in leaf, are key parameters determining hydraulic
 340 conductance (Sack *et al.*, 2015). Greater porosity of mesophyll cells contributes more
 341 to a high leaf hydraulic conductance, and broken connections between cells and fewer
 342 pathways of water flow would cause a lower aquaporin activity (Scoffoni *et al.*, 2014).
 343 Small leaves with smaller cells tend to have greater minor vein length per unit leaf
 344 area, conferring high leaf hydraulic conductance (Scoffoni *et al.*, 2011). Changes in
 345 gas exchange traits associated with altering mesophyll cell morphology would
 346 influence leaf hydraulic conductance and transpiration. Theoretically, increasing
 347 mesophyll cell density would not affect intercellular airspace (Fig. 3), nevertheless, an
 348 increase in mesophyll cell density inescapably decreases intercellular air space
 349 (Lehmeier *et al.*, 2017). Hanba *et al.* (2004) documented that the leaves of transgenic
 350 rice Tr6322-H had higher mesophyll cell density and more developed collenchyma
 351 coupled to a higher mesophyll conductance, however, its leaf development might be
 352 influenced by water deficit because of increased water loss. The regulation of
 353 mesophyll cell morphology has potential to enhance photosynthetic capacity, however,
 354 it is worth studying whether it will increase hydraulic conductance simultaneously or
 355 change the composition of hydraulic conductivity.

356 Terashima *et al.* (2006) have discussed the importance of having sufficient S_c by
 357 decreasing cell size for efficient photosynthesis and compared the merits and demerits
 358 of different mesophyll cell sizes. Different species can have considerably distinct
 359 mesophyll cell sizes as a result of natural selection in various environments. Annual
 360 herbaceous plants often have large mesophyll cells, while ever-green trees tend to
 361 have smaller mesophyll cells. The leaves with small mesophyll cells have strong

362 mechanical strength, long longevity, small heat capacity, and lower expansion rate.
363 Additionally, they exhibit higher information cost (nuclear N- and P and chloroplast N
364 and P). From this perspective, plants in natural environments may not benefit from
365 very small mesophyll cells.

366 However, for agroecosystems, there might be more advantages to select small
367 mesophyll cells. High mechanical strength would be beneficial to enhance crop pest
368 resistance. The maintenance of leaf morphology depends on the cell wall, which is
369 helpful to increase resistance to drought stress. For intensive agricultural production,
370 N and P inputs are always sufficient or even excessive. If manipulating mesophyll cell
371 morphology could improve photosynthetic capacity and increase crop yield without
372 additional inputs, then N and P utilization efficiency would be higher. Selecting for
373 smaller mesophyll cells does not mean completely changing the shape and size of the
374 mesophyll cells. Instead, depending on the current size and arrangement of mesophyll
375 cells, it may be possible to reduce mesophyll cell size appropriately by altering cell
376 proliferation and expansion to improve S_c and reduce CO₂ diffusion resistance,
377 thereby enhancing photosynthetic efficiency. Indeed, comprehensive studies
378 estimating the potential for regulating mesophyll cell morphology and the related
379 changes on physiological and biochemical should be made.

380 **4. Target genes could be used to regulate mesophyll cell morphology**

381 Leaf growth has been divided into three phases: (1) leaf primordia occur at the shoot
382 apical meristem, (2) cells proliferate and form specific structures during primary
383 morphogenesis, (3) leaf cells begin to expand at secondary morphogenesis (Donnelly
384 *et al.*, 1999; Andrianakaja *et al.*, 2012). In the transition from primary to secondary
385 morphogenesis, a retrograde chloroplast signal induces the onset of cell expansion and
386 the leaf becomes photosynthetically active at the same time (Andrianakaja *et al.*, 2012),
387 indicating that leaf growth as well as leaf morphology is inseparably correlated with
388 photosynthesis. Although leaf morphology is regulated in response to different growth
389 stages and environments, genetic control is fundamental to produce various leaves in

390 nature. Thus, leaf morphogenesis and the related genes are hotspots in plant biology
 391 research. Numerous *Arabidopsis* mutants with altered leaf size and/or shape have been
 392 isolated (Horiguchi *et al.*, 2006) and a key aspect of genes affecting leaf
 393 morphogenesis also have been identified during the past years. Several articles have
 394 reviewed in detail the critical genes regulating leaf shape, size, cell proliferation and
 395 expansion (Beemster *et al.*, 2003; Micol, 2009; Horiguchi and Tsukaya, 2011;
 396 Gonzalez *et al.*, 2012; Rodriguez *et al.*, 2014, 2016; Hepworth and Lenhard, 2014).
 397 However, leaf development is a complex biological process and is determined by
 398 many genes and pathways. The molecular mechanisms underlying leaf size and shape
 399 are only starting to be unraveled. Although extensive research is still needed, key
 400 genes can provide some mechanisms for regulating leaf morphology.

401 Cell number, which plays a more important role in determining the final size of plant
 402 organs rather than cell size (Gázquez and Beemster, 2017), mainly depends on cell
 403 proliferation. Based on the existing research results, we divide the principal genetic
 404 factors regulating cell proliferation into three groups:

405 (1) **Direct regulators.** Overexpression or loss of these genes can directly regulate cell
 406 proliferation. such as GROWTH-REGULATING FACTORs (*GRF*) family, which has
 407 been widely studied (Omidbakhshfard *et al.*, 2015; Tsukaya, 2016). There are nine
 408 members in the *Arabidopsis thaliana* GRF family (*AtGRF*), of which *AtGRF1*, 2, 3, 4,
 409 5, and 9 are involved in the regulation of cell proliferation. However, while other
 410 family members have partly overlapping roles, the function of *AtGRF5* is to regulate
 411 cell proliferation. When *AtGRF5* and *AN3* (the transcription coactivator) were
 412 overexpressed, plants developed larger leaves as a result of increasing cell number
 413 rather than cell size (Horiguchi *et al.*, 2005). For *AtGRFs*, expression is regulated by
 414 miRNA miR396 and ectopic expression of miR396 repressed the *AtGRF* activity and
 415 resulted in smaller leaves with reduced cell number (Rodriguez *et al.*, 2010).
 416 Additionally, overexpressing other genes, such as *AINTEGUMENTA* (*ANT*)
 417 (Mizukami and Fischer, 2000), *Arabidopsis SKP1-LIKE1* (*ASK1*) (Zhao *et al.*, 1999),

418 *Arabidopsis thaliana* *NGATHA* (*AtNGA*) (Lee *et al.*, 2015), *G-PATCH DOMAIN*
 419 *PROTEIN1* (*gdp1*) (Kojima *et al.*, 2018), *KLU* (Anastasiou *et al.*, 2007),
 420 *OLIGOCELLULA1*, 4, 6 (*oli1*, 4, 6) (Fujikura *et al.*, 2009), *POINTED FIRST LEAD 2*
 421 (*PFL2*) (Ito *et al.*, 2000), *ROTUNDIFOLIA4* (*ROT4*) (Narita *et al.*, 2004),
 422 *STRUWWELPETER* (*SWP*) (Autran *et al.*, 2002) and *TCP4* (Schommer *et al.*, 2014)
 423 could prolong cell proliferation and produce large leaves with more cells.

424 (2) **Genes related to hormone signaling pathways.** Numerous hormones, such as
 425 auxins, brassinosteroids, cytokinin, gibberellins, jasmonic acid, etc, could act as
 426 signals for cell proliferation and expansion at the cellular level. The genes regulating
 427 hormone signals influence cell proliferation and expansion. For example, ectopic
 428 expression of *ARGOS* transduces auxin signals downstream of *AXR1*, which prolongs
 429 *ANT* expression and regulates cell proliferation, displaying enlarged leaves attributed
 430 to increase in cell number (Hu *et al.*, 2003). The *cro* mutants *det2* and *dwf1*, affected
 431 in brassinosteroid biosynthesis revealed fewer and smaller cells (Nakaya *et al.*, 2002).
 432 *AHK*-cytokinin receptor in *Arabidopsis thaliana* (Nishimura *et al.*, 2004),
 433 *PHYB*-involved in gibberellin biosynthesis (Chaiwanon *et al.*, 2016), and *aos* (*allene*
 434 *oxide synthase*) and *coil-16B* (*coronatine insensitive1*)-jasmonate receptor, could
 435 influence cell division and expansion by regulating related hormone signals.

436 (3) **Genes related to pathways synthesizing structural component.** Plant cells are
 437 encircled by a rigid cell wall and the biosynthesis and modification of cell wall would
 438 significantly influence cell expansion and proliferation. Cellulose, hemicellulose, and
 439 pectins are the dominant components of the plant cell (Ochoa-Villarreal *et al.*, 2012;
 440 Tenhaken, 2015) and the genes code for enzymes catalyzing cellulose, hemicellulose,
 441 and pectin synthesis could regulate cell expansion and proliferation. For example,
 442 overexpression of the *fras5*, a mutant allele of *AtCesA7* gene involved in catalyzing
 443 cellulose synthesis, reduced cellulose content, cell wall thickness, and cell elongation
 444 (Zhong *et al.*, 2003). The *ccr1* mutant has high ferulic acid content, an intermediate in
 445 lignin biosynthesis, and increased cell proliferation (Xue *et al.*, 2015). A knockout of

446 *CGR2* and *CGR3* genes showed reduced cell expansion and overall plant growth
 447 associated with reducing pectin methylesterification (Kim *et al.*, 2015).

448 The directly regulated genes, which might be involved in signaling, structural, and
 449 other aspects regulating cell and leaf shape and still require further research, mainly
 450 regulate cell proliferation. In most instances, overexpressing these genes results in
 451 larger leaves owing to increased cell number rather than cell size. However, genes
 452 related to hormone signaling and structural component synthesis always influence
 453 both cell proliferation and cell expansion. Generally, an increase in the number of
 454 cells leads to a reduction in the volume of the cells, which is called “compensatory
 455 effect”. Horiguchi and Tsukaya (2011) defined compensation as when “a decrease in
 456 cell number in a leaf caused by genetic defect leads to an enhanced cell expansion”.
 457 They thought altered cell proliferation triggered compensation rather than cell
 458 expansion. Moreover, compensation is induced only when cell proliferation is beyond
 459 a threshold level (Horiguchi *et al.*, 2006). It indicates increasing cell number within a
 460 certain range would not cause the changes in cell size, which provides the theoretical
 461 support of regulating mesophyll cell density to improve the S_{mes} and S_c values.

462 Besides manipulating mesophyll cell density, improving chloroplast density is helpful
 463 to increase the S_c values. Thus, the genes regulating chloroplast division will also be
 464 reviewed. The *ARC* (ACCUMULATION and REPLICATION of CHLOROPLASTS)
 465 mutants are an important genetic resource in the control of chloroplast division (Pyke
 466 and Leech, 1994; Gao *et al.*, 2003; Cho *et al.*, 2012; Zhou *et al.*, 2015). Gao *et al.* (2003)
 467 revealed that mutants of *ARC5* exhibit defects in chloroplast constriction and have
 468 enlarged dumbbell shaped chloroplasts. *GCI* (GIANT CHLOROPLAST) is another
 469 gene involved chloroplast division, which plays a positive role at an early stage of the
 470 division process, and *GCI* deficiency resulted in mesophyll cells harboring one to two
 471 giant chloroplasts (Maple *et al.*, 2004). Hymus *et al.* (2013) showed overexpression of
 472 *ATHB17* increases chloroplast numbers per unit cell size due to an increase in the
 473 number of proplastids per meristematic cell. As one of the critical genes regulating

cell proliferation, *AtGRF5* stimulates chloroplast division, resulting in a high chloroplast number per cell and a higher photosynthesis rate (Vercruyssen *et al.*, 2015). Additionally, cytokinin promotes chloroplast ultrastructure and chlorophyll synthesis. Cortleven and Schmulling (2015) summarized cytokinin signaling pathways of Arabidopsis regulating chloroplast development and function through transcription factors. As the cytokinin receptors, *AHK2* and *AHK3* genes not only trigger cell divisions (Nishimura *et al.*, 2004), but also regulate chloroplast development and chloroplast division. Recently, Larkin *et al.* (2016) described how the *REC* gene helps to establish the proportion of cellular volume devoted to chloroplasts; the *rec1* mutant has reduced size of the chloroplast compartment. Altered chloroplast coverage in cells might induce changes in S_c and g_m .

5. Prospects to manipulate mesophyll cell morphology for enhancing photosynthesis

Regulation is disconnected from limitation (Sharkey and Weise, 2012). Light and CO_2 are the primary external constraints (limitations) on photosynthesis, therefore, regulating leaf morphology to take maximum advantage of light and CO_2 is key to maximize photosynthesis. During the past decades, considerable research has been conducted to study the influence of leaf morphology on photosynthetic capacity in different species and environments. These studies help interpret how plants achieve high photosynthetic efficiency by optimizing leaf anatomy under different environments. Nonetheless, for any given species, the leaf architectural traits with the highest photosynthetic capacity is not always clear. Small differences in leaf phenotypes and the large number of genes that affect leaf architecture make research in this area difficult. As the most widely used species in plant biology research, *Arabidopsis thaliana* has been screened and numerous mutants with different leaf size and/or shape have been isolated (Horiguchi *et al.*, 2006; Micol, 2009). A large number of the mutated genes determining cell proliferation, expansion and differentiation have been identified. However, these mutations have not typically been studied in the

context of the effects on photosynthesis. Therefore, the best targets for engineering leaf anatomy are unknown. More, and in-depth, studies should focus on (1) the differences in photosynthetic capacity under different leaf shape and size as well as leaf anatomy, (2) how to influence leaf photosynthesis through the potential target genes altering leaf morphology. Such research will pinpoint the targets for altering leaf architecture to maximize photosynthetic capacity.

Although light fluctuations significantly influence leaf photosynthesis (Kaiser *et al.*, 2018; Slattery *et al.*, 2018), CO₂ concentration is the dominant factor restricting photosynthesis and yield in crops under current conditions (Ainsworth and Long, 2005; Ellsworth *et al.*, 2012). In C₄ plants, the specialized Kranz anatomy helps concentrate CO₂ concentration and enhance photosynthetic efficiency. C₃ plants do not possess Kranz anatomy and CO₂ diffusion resistance in mesophyll cells significantly reduces the CO₂ concentration at the sites of carboxylation. If mesophyll resistance could be eliminated, photosynthesis rates would be improved by up to 20% (Zhu *et al.*, 2010). Increasing chloroplast area exposed to intercellular airspace as much as possible could reduce the CO₂ diffusion resistance and enhance mesophyll conductance and photosynthetic capacity. Terashima *et al.* (2001, 2006, 2011) have elaborated the importance of having sufficient S_c for efficient photosynthesis and have discussed the various strategies to increase the S_{mes} and S_c values by manipulating cell proliferation and expansion. They thought decreasing mesophyll cell size was an effective approach to increase S_c and decrease CO₂ diffusion resistance. Nevertheless, most plants do not tend to form small mesophyll cells as a result of natural selection. As early as the 1970s, selection for a smaller cell size have been considered as critical parameters to breed new variety with high photosynthesis and yield. Nevertheless, owing to poor understanding of molecular mechanisms controlling leaf anatomy as well as cell proliferation and expansion, the investigations into the influence of mesophyll cell size on photosynthesis have not been fully developed and the selection of more and smaller mesophyll cells is not currently considered a potential approach

530 to enhance photosynthesis capacity. Recent research shows increasing mesophyll cell
531 number through regulating genes related to the cell cycle are beneficial for improving
532 photosynthetic capacity (Takai *et al.*, 2013; Lehmeier *et al.*, 2017). It can be foreseen
533 that engineering leaf anatomy by manipulating cell proliferation and expansion using
534 gene regulation or gene editing technology will be a potential approach to enhance
535 plant photosynthetic capacity in future.

536 Indeed, many challenges, including photosynthesis and molecular mechanisms
537 controlling leaf anatomy, need to be solved. What is the potential for regulating cell
538 density to maximize photosynthesis? As mentioned in the compensation effect,
539 increasing in mesophyll cell number beyond a threshold value will trigger a decrease
540 in cell size (Horiguchi *et al.*, 2006). Smaller mesophyll cell size will restrict the
541 number and division of chloroplasts. Although the S_{mes} value is improved by
542 increasing mesophyll cell number, the S_c value might not be increased because of
543 over-lapping chloroplasts, which is unfavorable for enhancing photosynthesis. It is
544 therefore essential to explore the relationship between mesophyll cell traits and
545 mesophyll conductance and photosynthetic capacity. Additionally, an increase in leaf
546 mesophyll cell density would improve leaf mass density and LMA , which could
547 modify photosynthetic C partitioning to leaf area growth and growth in terms of LMA ,
548 further influencing plant growth. Weraduwege *et al.* (2016) revealed that in the *cgr2/3*
549 mutant greater C partitioning to LMA , associated with an increase in leaf cell density,
550 resulted in smaller leaf area and reduced whole-plant photosynthesis. A great number
551 of cells and organelles require more energy and hence incur higher maintenance
552 respiratory costs (Weraduwege *et al.*, 2016). What's more, alteration in mesophyll cell
553 number and size also would impact the interception of light, water transport and CO_2
554 assimilation. Comprehensive studies to analyze the effects of manipulating mesophyll
555 cell morphology on other physiological processes in leaves would be useful to
556 optimize the approach for enhancing photosynthetic capacity.

557 The selection of target genes for altering leaf anatomy also requires more accurate and

extensive research. As mentioned in Lehmeier *et al.* (2017), the suppression of *RBR1* removes some restrictions on cell division, leading to more and smaller mesophyll cells; however, the transgenic lines with higher photosynthetic capacity grew smaller than the wild type because of higher *LMA*. Although photosynthetic capacity is improved by regulating these genes, such plants do not achieve higher plant biomass and crop yield as result of the decrease of photosynthetic area. The genes coordinating the improvement of photosynthetic capacity and plant growth can meet the demand of modern agriculture for high yield.

Altering leaf anatomy to enhance leaf photosynthetic capacity must be achieved without influencing leaf area. From this perspective, the genes directly regulating cell proliferation might be considered first. For example, overexpression *AtGRF5* and *AN3* in *Arabidopsis thaliana* results in larger leaves with an increased cell number (Horiguchi *et al.*, 2005). Additionally, the genes controlling cell proliferation and expansion might change other physiological processes. For example, the *cgr2/3* mutant, double knockout of *CGR2* and *CGR3* genes, showed a reduced cell expansion and increased mesophyll cell density as result of a reduction in the degree of pectin methylation; however, it induced hardening of cell walls, and increased CO₂ resistance of the cell wall and reduced photosynthetic efficiency (Weraduwege *et al.*, 2016). Altering expression of ANT genes modified cell proliferation and regulated the number of higher order veins and minor tertiary veins (Kang *et al.*, 2007), which are closely related to leaf photosynthetic capacity (Sack *et al.*, 2013; Feldman *et al.*, 2017). It is therefore essential to understand the various functions of the target genes for accurate editing the genes to alter leaf morphology and enhance photosynthetic efficiency.

6. Conclusion

Natural selection allows for a range of leaf architectures to effectively capture light and exchange gases, resulting in high photosynthetic efficiency to grow and survive. Despite the variety of species and environmental conditions, S_c is the most important

586 indicator of leaf photosynthetic capacity. Having a sufficiently high S_c is a
 587 prerequisite of efficient photosynthesis. Chloroplast number and distribution in
 588 mesophyll cells and S_{mes} significantly influence S_c . Without overlapping mesophyll
 589 cells, increasing mesophyll cell density is an important strategy to improve S_{mes} and S_c .
 590 Depending on the manipulation of target genes involved in cell proliferation and
 591 expansion, such as *AtGRF* family, it should be possible to produce more mesophyll
 592 cells without influencing leaf area, further inducing higher S_c values and
 593 photosynthetic capacity. It is foreseen that the chain “gene regulation-leaf
 594 anatomy-photosynthetic capacity” will be an important strategy for enhancing
 595 photosynthetic capacity and crop yield. Indeed, there are many challenges that need to
 596 be thoroughly studied, such as the selection of target gene regulating cell proliferation
 597 and expansion, the potential of adjusting mesophyll cell number and size, and the
 598 effects of leaf anatomical traits on other physiological processes, including light
 599 utilization, water transport etc.

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Figure captions

Fig. 1. Scatterplot matrix of critical leaf morphology and photosynthetic parameters based on literature data. Note: The matrix was conducted by using the “car” package in R software. Diagonal panels show the distribution of each variable. Lower panels show scatterplot. *Tl*, leaf thickness; *Tmes*, mesophyll thickness; *fias*, fraction of intercellular air space; *LMA*, leaf dry mass per unit area; *Tcw*, mesophyll cell wall thickness; *Lchl*, chloroplast length; *Tchl*, chloroplast thickness; *Smes*, mesophyll surface area exposed to intercellular air space; *Sc*, chloroplast surface area exposed to intercellular air space; *Sc.Smes*, the ratio of *Sc/Smes*; *gm*, mesophyll conductance; *gs*, stomatal conductance; *An*, photosynthesis rate.

Fig. 2. Correlation between leaf morphology and photosynthetic parameters based on literature data. Note: The correlation was conducted by using the “corr” package in R software. The closer each variable is to another represents the higher relationship while the opposite is for widely spaced variables. The line color represents the direction of the correlation. The blue line is positive correlation and the red line is negative correlation. The line shade and thickness represent the strength of the relationship. The minimum correlation coefficient required to display a line between variables is 0.3.

Fig. 3. Representations of geometrical idealization of mesophyll cells showing how geometry affects the S_{mes} and f_{ias} value. *Smes*, mesophyll surface area exposed to intercellular air space; *fias*, fraction of intercellular air space.

Figures

Fig. 1.

Fig. 2.

Fig. 3.

Tables

Table 1 Matrix of correlation coefficient between leaf anatomical and photosynthetic parameters

	T _L	T _{mes}	f _{ias}	LMA	T _{cw}	L _{chl}	T _{chl}	S _{mes}	S _c	S _c /S _{mes}	g _m	g _s	A _n
T _L	1.00	0.97***	0.11	0.55***	0.60***	0.36**	-0.062	0.24**	0.085	-0.38***	-0.26**	-0.22*	-0.22**
T _{mes}		1.00	0.039	0.63***	0.52***	0.37**	0.15	0.37***	0.36***	-0.18*	-0.20*	-0.30**	-0.12
f _{ias}			1.00	-0.37***	0.033	0.42**	0.52***	0.19*	-0.021	-0.31***	-0.065	-0.22*	-0.23*
LMA				1.00	0.33***	0.19	-0.20*	0.33***	0.27***	-0.12	-0.17*	-0.13	-0.018
T _{cw}					1.00	0.06	0.35***	0.071	-0.27***	-0.48***	-0.44***	-0.45***	-0.52***
L _{chl}						1.00	0.50***	0.081	0.27*	0.32**	0.32*	0.16	0.26*
T _{chl}							1.00	-0.18	-0.26**	0.017	-0.24*	-0.28**	-0.34***
S _{mes}								1.00	0.80***	-0.099	0.22**	0.03	0.18*
S _c									1.00	0.46***	0.49***	0.24**	0.47***
S _c /S _{mes}										1.00	0.44***	0.41***	0.55***
g _m											1.00	0.75***	0.76***
g _s												1.00	0.78***
A _n													1.00

Note: ***, **, and * represents significant at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

Table 2 Changes of leaf anatomical and photosynthetic parameters under different LMA

LMA (g m ⁻²)	<50	50-100	>100	P value
T _L (μm)	145±61c	187±67b	320±172a	<0.001***
T _{mes} (μm)	127±54b	161±68b	306±151a	<0.001***
f _{ias}	0.276±0.165a	0.298±0.108a	0.186±0.128b	0.0076**
T _{cw} (μm)	0.242±0.135c	0.318±0.182b	0.409±0.171a	<0.001***
L _{chl} (μm)	4.38±1.53a	4.79±0.60a	4.91±0.88a	0.26
T _{chl} (μm)	2.38±0.88a	2.60±0.95a	2.09±0.78a	0.081
S _{mes} (m ² m ⁻²)	12.0±5.4b	19.6±4.5a	19.2±9.6a	<0.001***
S _c (m ² m ⁻²)	7.9±4.1b	12.6±5.1a	11.3±5.7a	<0.001***
S _c /S _{mes}	0.68±0.15a	0.72±0.18a	0.61±0.16b	0.013*
g _m (mol m ⁻² s ⁻¹)	0.119±0.104a	0.131±0.148a	0.062±0.042b	0.029*
g _s (mol m ⁻² s ⁻¹)	0.177±0.147a	0.123±0.080a	0.134±0.118a	0.073
A _n (μmol m ⁻² s ⁻¹)	10.9±8.3a	9.6±7.6a	9.8±5.5a	0.54

Note: ***, **, and * represents significant at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

Figures

Fig. 1.

Fig. 2.

Fig. 3.