Essays on Biochemistry

Chloroplasts: the plant's capture and production modules

Increasing metabolic potential: C-fixation

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Abstract

Due to a growing world population, crop yields must increase to meet rising demand. Crop plants also require adaptation to optimise performance in the changing environments caused by climate change. Improving photosynthetic carbon fixation is a promising, albeit technically challenging, strategy whose potential has only just begun to be considered in breeding programs. Rubisco, a fundamental enzyme of carbon fixation, is extremely inefficient and many strategies to improve photosynthesis focus on overcoming the limitations of this enzyme, either by improving Rubisco activity and regulation or by improving the supply of substrates. Although progress is being made, the need to tailor solutions for each crop and their respective environments has been highlighted. Even so, continuing research will be required to achieve these objectives and to grow crops more sustainably in the future.

Key words CO_2, Rubisco, Rubisco activase, RuBP regeneration, Sedoheptulose-1,7-bisphosphatase, specificity factor, Carbon concentrating mechanisms, Carboxysomes

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Photosynthetic CO_2 fixation in the Calvin-Benson-Bassham (CBB) cycle is the source of almost all the organic carbon in the biosphere. There is compelling evidence from a plethora of free air CO_2 enrichment experiments that increasing CO_2 fixation will increase crop biomass and yield [1]. The experimental evidence that increasing photosynthesis will increase crop yields is reinforced by modelling studies [2,3,4]. Increasing photosynthetic CO_2 fixation is widely recognized as a key objective in efforts to meet the demand for increased crop production and to secure global food security. Despite this, there have been very few attempts to consider, let alone incorporate, photosynthetic traits in breeding programmes [5,6,7]. Wider recognition of the potential benefits have recently resulted in several major international initiatives, focused on increasing photosynthetic CO_2 fixation in specific crops (e.g. C4 Rice, https: //c4rice.com/; RIPE, http://ripe.illinois.edu/; International Wheat Yield Partnership, http://iwyp.org/). Improving carbon fixation has been the subject of a considerable number of detailed reviews [e.g., 8,9,10,11]. Here we focus on the most recent developments in this area.

The rate of the CBB cycle is co-limited by the regeneration of the CO_2 acceptor, ribulose-1,5-bisphosphate (RuBP) and by the reaction of CO_2 with RuBP, catalysed by Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39). The extent to which each of these processes limits the rate of CO_2 assimilation is dependent on the relative abundance and activity of the underpinning molecular machinery, which is strongly influenced by environmental conditions.

Overcoming the inefficiencies of Rubisco continues to be a major focus of research (Figure 1). In addition to catalysing the productive carboxylation of RuBP in carbon assimilation, Rubisco also catalyses a competing and wasteful reaction with oxygen which necessitates the operation of the photorespiratory cycle, to return the diverted carbon to the CBB cycle. In addition, Rubisco from higher plants (and particularly those which lack a CCM) conducts successive rounds of catalysis (or turnover) at relatively low rates compared to the other enzymes of the CBB cycle. This necessitates the synthesis of large amounts of the enzyme (accounting for 30-50% of the soluble protein in crop leaves [12], in order to sustain the observed photosynthetic rates. Furthermore, the complex reaction mechanism of Rubisco results in not infrequent abortive side reactions, causing the production of tight binding inhibitors that prevent further catalysis. Despite these inefficiencies, Rubisco remains the only enzyme able to deliver the net assimilation of carbon on a global scale. Some alternative natural and synthetic carbon fixation pathways have been identified [13] and may be useful in future approaches to increase carbon fixation. As elaborated below, research into overcoming the limitations to photosynthetic CO_2 assimilation has focused on both improving the supply of CO_2 and RuBP to Rubisco, as well as improving the efficiency, speed and regulation of this enzyme.

Rubisco

To become catalytically competent the catalytic sites of Rubisco must assume a specific active site geometry. In this process of activation – which is reversible - the carbamylation of an essential lysine residue within the catalytic site, followed by stabilization of the resulting carbamate by a Mg^{2+} ion [14] gives rise to a catalytically active ternary complex ($E.CO_2.Mg^{2+}$). The carboxylase and oxygenase activities of Rubisco both occur at this activated catalytic site. Neither gaseous substrate binds directly to Rubisco prior to catalysis, but they compete for reaction with an enzyme-bound enediol isomer of RuBP. The chemistry of the carboxylase mechanism is reasonably well understood and entails a sequence of five partial reactions: enolization, carboxylation, hydration, carbon–carbon cleavage, and protonation with a stereochemical inversion resulting in the two molecules of D-3-phosphoglycerate (3PGA, [15]). However, some uncertainty remains as to whether the chemical mechanism is stepwise or concerted [16]. The oxygenase activity is initiated by the reaction between oxygen and the enediol of RuBP, and is followed by hydration and carbon–carbon cleavage to produce 2-phosphoglycolate (2PGA) and 3PGA [15] following a reaction pathway which is likely to be

singlet O_2-independent [17] and so unlikely to play a role in the removal of reactive oxygen species. Rubisco also catalyses periodic 'misfire' reactions, in which a peroxy-intermediate of the oxygenase pathway decomposes to yield hydrogen peroxide and pentodiulose-1,5-bisphosphate (PDBP, [18]). PDBP can further rearrange to 2-carboxytetritol 1,4-bisphosphate [19]. Both these bisphosphate compounds can bind with high affinity to activated Rubisco, preventing CO_2 fixation [20]. Furthermore, mis-protonation of the enediol of Rubisco-bound RuBP also occurs, forming D-xylulose-1,5-bisphosphate (XuBP, [21]) which is another potent, competitive inhibitor of catalysis. It was recently shown that a ubiquitous CbbY protein possessed XuBP phosphatase activity, with the potential to eliminate such inhibition and the consequent efficiency penalty [22].

The high degree of amino acid sequence and structural homology between forms of Rubisco from diverse species, imply a conserved catalytic mechanism [23]. Even so, significant interspecies variation in the magnitude of common catalytic parameters has been reported [24,25]. Despite its abundance *in planta* and the vast database of Rubisco sequence information spanning the plant kingdom, the extent to which the associated catalytic parameters vary *per se* has only just begun to be addressed. Until recently, our knowledge of the catalytic variability of Rubisco was comparatively small, because only a few species had been examined and even amongst these a complete kinetic characterisation - including rate constants and substrate affinities for both carboxylase and oxygenase activities (k_{cat}^c, k_{cat}^o, K_c and K_o, respectively) as well as estimates of Rubisco abundance - was rare. The catalytic properties of Rubisco isolated from this very small number of species had suggested an inverse relationship [26] between the carboxylase capacity (k_{cat}^c) of Rubisco and the corresponding specificity factor ($S_{c/o}$), which reflects the ability of the enzyme to discriminate between CO_2 and O_2 [27]. This in turn led to the hypothesis that there was an obligatory trade-off between optimisation for speed on the one hand, and optimisation for substrate affinity on the other [28] and thence to the suggestion that Rubisco may already be optimized to fulfill its role in any specific photosynthetic organism [29] on account of the prevailing evolutionary constraints [30]. Since then, Rubisco has been extracted from a much larger and more diverse variety of sources and the kinetic properties fully determined, often over a range of temperatures [25,31,32,33,34]. All these studies have demonstrated significant variability in the kinetic properties of Rubisco from different species. Importantly, no correlation between k_{cat}^c and $S_{c/o}$ (inverse or otherwise) was detected amongst the 11 types of diatom Rubisco of Form ID (or 'red'-type lineage) which were studied by Young et al [35]. Even more surprising were the highly significant positive correlations between k_{cat}^c and $S_{c/o}$ demonstrated between Form IB Rubisco from 75 diverse plant species at 20 and 25°C [25]. Together, these more comprehensive studies demonstrate that any evolutionary constraints imposed by an inverse relationship between the speed and affinity of Rubisco, have important exceptions, giving renewed hope that both might be favourably engineered within the same holoenzyme.

The observed variations in kinetic properties probably arise from differences in the enolization equilibrium and a change in the energy barriers associated with CO_2 and O_2 addition. But how specific amino acid sequences determine this variation remains unclear [17]. Sequence analysis of many of the species investigated, has identified some amino acid residues that alter catalytic activity and could be targeted in attempts to engineer improved Rubisco efficiency in crop systems [25,36].

In higher plants the (Form I) Rubisco holoenzyme is composed of 8 chloroplast-encoded large subunits (L) arranged as an L8 (4 x L2) core, which is capped at opposite, structurally equivalent, poles by 2 tetrads of nuclear-encoded small subunits (2 x S4) [37]. Whilst the large subunits contain the catalytic sites and are the primary determinants of the catalytic properties of Rubisco, there is evidence that Rubisco kinetic properties are also affected by the small subunits [38,39]. Within a species, the L subunits of Rubisco have identical amino acid sequences, but the small subunits are encoded by a multi-gene family for which individual members are differentially expressed. The S subunit gene sequences within this family are generally so highly conserved that

the amino acid sequences of the S subunits they encode are identical [40] (Spreitzer 2003). However, a distinct S subunit gene sequence with lower homology, present on a distinct chromosome and encoding a distinct S subunit, was recently demonstrated in rice. The expression of this divergent sequence was tissue specific and the corresponding Rubisco holoenzyme had distinct catalytic properties[41].

Integral to the biochemical models of photosynthetic CO_2 assimilation of Farquhar et al [42] are the relative contributions of RuBP regeneration and the role played by Rubisco in the rate of CO_2 assimilation. Specifically, the way in which Rubisco abundance, carboxylase capacity, activation state, specificity and affinity for oxygen, impact upon CO_2 assimilation are all taken into account, making the model a powerful predictive tool. In this way, the theoretical outcome of substituting a native Rubisco with that from other plant species can be assessed across a range of CO_2 concentrations. Modelling indicates that the direct replacement of native Rubisco from major crops with specific high-performing Rubiscos, would support significant increases of leaf photosynthesis at current atmospheric CO_2 levels and high irradiance [2,10]. Typically, the predicted improvements were most pronounced at relatively low internal CO_2 concentrations, where leaf photosynthesis is typically limited by Rubisco activity; this may be advantageous under conditions where water is limiting, since attempts to reduce water loss by stomatal closure would result in lowered internal CO_2 concentrations [25,32,43]. However, the extent of the predicted improvement was found to depend on the specific crops and their growth environment. In addition, as the Rubisco kinetic constants also have different thermal responses, it has become apparent that any engineering solutions will need to be tailored to each crop and environment [25,32,33,34].

Although it may be possible to introduce novel Rubiscos by conventional breeding (e.g. wide crosses [32]), in most cases a biotechnological approach appears essential if the benefits of the most promising (and more distantly related) forms of Rubisco are to be realised. Thus far, attempts to introduce novel forms of Rubisco by nuclear transformation have had limited success. In contrast, the introduction of novel Rubisco via plastid transformation has been much more successful [24,36,43,44] although this technology is not yet available for any of the major food crops (i.e. maize, wheat and rice). Optimising the catalytic properties via the Rubisco S subunit offers a potentially exciting alternative or complementary avenue to enhance crop photosynthesis [24]. However, even when plastid transformation becomes more widely applicable, it cannot be assumed that any non-native or modified Rubisco would be assembled and be functional *in planta*. When novel Rubiscos have been expressed in tobacco, only those from higher plant species most closely related to tobacco are functional. Although it has been possible to express functional cyanobacterial Rubisco in transplastomic tobacco [44], the amount of functional enzyme was low [45]. In general, Rubisco from more distantly related species (e.g. green algae and monocots with respect to a dicot recipient) is not assembled into functional enzyme [24, 36] due to chaperone incompatibilities [43].

Regulation

The activity of Rubisco *in vivo* is determined by carbamylation and/or by the tight binding of inhibitors [12]. In daylight the catalytic misfire product PDBP and other inhibitors can lock active sites into an unproductive form [46]. In some species during periods of low light or darkness 2-Carboxy-D-arabitinol 1-phosphate (CA1P), a naturally occurring transition state analogue of the carboxylase reaction is synthesized and binds tightly to Rubisco, preventing catalysis [47]. Whilst inhibitors prevent catalysis they do also protect Rubisco from proteolytic breakdown and oxidative damage under stress [20,48]. The removal of such inhibitors from Rubisco requires the action of the ancillary protein Rubisco activase (Rca, EC 4.1.1.39) in an ATP-dependent manner.

Rca is a member of the AAA+ super family whose members perform chaperone like functions [49]. The rate of inhibitor removal by Rca determines the duration of photosynthetic

induction, i.e. the time required to reach a constant steady-state rate of net CO_2 assimilation upon transition from low to high irradiance [50,51,52,53,54]. This is especially important considering that plants in an agronomic context will frequently experience fluctuations in the light environment and rarely operate under steady-state conditions of photosynthesis. Taylor & Long [54] have recently demonstrated in wheat that the lag in photosynthetic induction associated with Rubisco activation during shade-to-sun transitions could diminish the carbon assimilation potential by 21%. While this lag in photosynthetic induction occurs in most plant species studied to date [55], in Arabidopsis plants engineered to express only a non-regulated form of Rca, photosynthetic induction in response to increases in irradiance was faster than in wild type (wt), and biomass accumulation was enhanced relative to wt when grown under a periodically fluctuating irradiance regime[52]. These observations make it realistic to explore variation in the response of Rubisco activation to irradiance increases when attempting to optimise the efficiency of carbon assimilation under agronomic environments characterised by fluctuations in irradiance.

The ubiquitous presence of promiscuous sugar phosphatases, such as CA1P and XuBP phosphatases, which dephosphorylate a range of Rubisco inhibitors rendering them non-inhibitory [22,56] suggests a role for Rubisco inhibition in nature. The potential for Rubisco inhibition to protect Rubisco from proteolytic breakdown under conditions of environmental stress [20,48] remains to be tested *in planta*. However, in agronomical settings where attempts are made to reduce the effects of stress, there is potential to realise increased biomass production by accelerating the adjustment of photosynthetic CO_2 assimilation in shade-to-sun transitions, as recently demonstrated in field-grown tobacco engineered to adjust more rapidly during shade-to-sun transitions [57].

Increasing RuBP supply

The regeneration of RuBP co-limits the CBB cycle and the share of this control is predicted to increase as atmospheric CO_2 concentrations continue to rise. Modelling studies [58] have suggested that the CBB cycle is not optimised, but that over expression of the CBB cycle enzymes: sedoheptulose-1,7-bisphosphatase (SBPase: EC.3.1.3.37), fructose-1,6-bisphosphate aldolase (FBPA: EC 4.1.2.13) and Rubisco would increase carbon assimilation. However, in most cases, any such optimisation would need to be tailored to the specific crop and growth environment and would itself require further modification in response to continuing climate change [59].

SBPase catalyses the dephosphorylation of sedoheptulose-1,7-biphosphate (SBP) to sedoheptulose-7-phospate in the first committed step of RuBP regeneration. SBPase alone exerts substantial control over RuBP regeneration. Glasshouse experiments with transgenic plants have confirmed that over expression of SBPase can increase photosynthetic rate and biomass in several species: tobacco [60], rice [61] and wheat [62] although the magnitude of the response was variable. However, in field trials with the same transgenic tobacco and rice lines, increases in biomass were only seen at elevated CO_2 concentrations or under temperature stress.

FBPA promotes the condensation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P), forming fructose-1,6-bisphosphate F1,6P). Glasshouse experiments with transgenic tobacco plants have confirmed that over expression of FBPA can increase photosynthetic rate and biomass in tobacco although the stimulation was greatest when the plants were grown with variable square wave lighting and at elevated CO_2 concentrations rather than at ambient CO_2 [63].

In transgenic tobacco plants the over expression of SBPase and FBPA together increased the CO_2 assimilation rate at intercellular CO_2 concentrations above 300 μmol mol^{-1} compared to both the non-transformed controls and to lines over expressing the SBPase alone [64]. At ambient CO_2,

plants over-expressing SBPase and FBPA also had a significantly higher light saturated rate of photosynthesis than the wt. Over-expression of FBPA together with SBPase had a positive effect on growth in high light when compared with either non-transformed plants or plants over-expressing SBPase alone, but this cumulative effect of co-expression did not occur when plants were grown in low light [64].

The 2PGA generated by the oxygenase activity of Rubisco is converted into 3PGA during photorespiration (two molecules of 2PGA are required to form one 3PGA), and subsequently into RuBP by the CBB Cycle. A pivotal, multifunctional enzyme complex of photorespiration is mitochondrial glycine decarboxylase (GDC). Over expression of the H protein of GDC in transgenic Arabidopsis plants, increased both net carbon fixation and growth [65]. This result contradicted earlier models, which had predicted that at current atmospheric CO_2 concentrations there was an over investment in the enzymes of this cycle [58]. The results of Timm et al [65], indicate that the apparent overinvestment may actually have been due to an underinvestment in this CN carrier (the H-protein). Simkin et al [66] recently demonstrated that over expression of GDC-H in tobacco in combination with SBPase and FBPA over expression, resulted in cumulative positive effects on leaf area and biomass accumulation. The value of such multigene manipulations in plants grown under field conditions awaits demonstration [59], although this technology has the potential to deliver greater benefits than single gene approaches.

An alternative approach has been to diminish the penalties (i.e. energy consumed and loss of fixed carbon and nitrogen) of photorespiration by modifying the photorespiratory cycle. More efficient alternative metabolic pathways that incorporate enzyme catalysed reactions only found in bacteria, algae and Archeae and which complement those already present in higher plants have been designed and tested. Whilst some did not appear effective [67], others were accompanied by increases in biomass [68,69]. Modelling studies have predicted that the impact of the photorespiratory bypasses vary widely depending on the species and the growth environment [70,71]. The value of such manipulations for the growth of crop plants under field conditions must now be confirmed.

Increasing CO_2

Increasing the concentration of CO_2 at the catalytic site of Rubisco will increase the proportion of carboxylation relative to oxygenation, thereby increasing overall carbon assimilation and biomass production. This has been achieved in some species (e.g. C_4 plants, CAM plants) through the evolution of carbon concentrating mechanisms (CCMs). One approach to increasing photosynthesis in C_3 crop plants such as rice or wheat, would be to introduce one of these mechanisms.

As C_4 photosynthesis has evolved independently many times before [72,73], it may be possible to introduce a functional C_4 pathway into crops that lack one. In the C_4 pathway, the initial fixation of CO_2 catalysed by mesophyll phosphoenolpyruvate carboxylase, is followed by product translocation and decarboxylation to release CO_2 in the vicinity of bundle sheath Rubisco. Establishing an analogous process in a C_3 plant would necessitate the introduction of multiple anatomical and biochemical changes (https://c4rice.com/). Substantial progress is being made in understanding the genetic mechanisms involved in the development of Kranz (C_4) anatomy [74] but introduction of a functional C_4 system has yet to be accomplished. If successful, the introduction of a C_4 system into hitherto C_3 recipients would decrease the amounts of Rubisco - and thus nitrogen fertiliser - required and would also promote water use efficiency [75]. However, running any C_4 system requires an additional energy input, and so the resulting crops may be better suited to high light environments.

A simpler approach would be to introduce a CCM that does not require major anatomical changes. The cyanobacterial CCM which concentrates CO_2 within Rubisco-containing carboxysomes is relatively straight forward. Introduction of such a CCM into a higher plant would require relatively modest changes to the chloroplasts, namely, the introduction of functional carboxysomes, the removal of stromal carbonic anhydrase and the introduction of HCO_3^- transporters within the chloroplast envelope. Good progress has been made in the expression of carboxysome-like structures [76] and of a faster cyanobacterial Rubisco [44] within higher plant chloroplasts, but further progress is needed to integrate these two processes; to promote HCO_3^- accumulation in the stroma; and to ensure the synthesis of adequate amounts of functional Rubisco. In parallel with these developments, rapid progress is also being made in identifying and understanding the formation and function of the analogous pyrenoid CCM found in green alga [e.g. 77], which offers an alternative approach for engineering more efficient plants [78].

In C_3 plants, the concentration of CO_2 in the intercellular spaces is typically 30% lower than in the atmosphere [79] and the average ratio of chloroplastic to intercellular CO_2 is 0.7 [80]. This means that the CO_2 concentration in the immediate vicinity of Rubisco is typically 50% of that in the atmosphere. Since even small increases in CO_2 concentration will directly increase photosynthetic carbon assimilation, any trait that can increase this is a potential target for crop improvement. CO_2 enters the leaves via the stomata, whose conductance is determined by their size, distribution and regulation. Even traits that only indirectly influence stomatal conductance (e.g. root architecture, facilitating access to soil moisture) may promote CO_2 assimilation and should be considered [81]. Several different genes that affect stomatal conductance have been identified and successfully manipulated to increase photosynthetic rates [82]. But as CO_2 enters leaves, water escapes by transpiration and so improving photosynthetic assimilation by manipulating stomata alone will inevitably increase water consumption, which may be disadvantageous in water-limited environments [82]. However, by increasing the conductance of CO_2 from the sub-stomatal spaces to Rubisco, i.e. mesophyll conductance, it should be possible to increase photosynthesis without adversely affecting water use [83]. Whilst the factors involved in mesophyll conductance are not fully characterised, both anatomical (e.g., mesophyll structure, cell wall thickness and chloroplast distribution) and biochemical factors (e.g., patterns of carbonic anhydrase and aquaporin expression) have been implicated [82]. Whereas the anatomical traits are probably the strongest determinants of constitutive mesophyll conductance the biochemical factors are more important in responding to environmental changes [84].

Conclusions

Free air CO_2 enrichment experiments have convincingly shown that increasing photosynthesis does increase crop yields. Whilst photosynthetic carbon fixation is a complex trait, several strategies to increase it have been identified both from *in silico* and experimental research. However, further targets, for example related to Rubisco biogenesis, regulation and stability, may yet be identified. A number of target traits have already been tested which, using glass house conditions, delivered positive results. Under such conditions it has also been possible to combine several traits for even greater gains. However, the results from a very limited number of field trials using similar material, have been more variable with increases only occurring under certain test conditions (e.g., elevated CO_2 or stress). This serves to highlight the need to tailor solutions to specific crops and to the environments in which they will be grown, to deliver the greatest impact. GM approaches will be needed to deliver this, but this will also require overcoming societal concerns over the risks of deploying biotechnology [85]. The relevance of trade-offs between higher carbon fixation capacity and stress tolerance also await evaluation [86], and may equally depend on wider acceptance of these emerging technologies before being satisfactorily implemented.

Summary

- Increasing photosynthesis will increase crop yields provided that other constraints are not limiting
- Much of the focus is on directly or indirectly overcoming the limitations of Rubisco
- There is abundant significant variation in Rubiscos isolated from different species and Rubisco activase that could be exploited to increase crop yields
- Increasing the capacity to regenerate RuBP or decreasing the costs of photorespiration have worked well in model species
- Ambitious synthetic biology approaches are in progress to create novel CO_2 concentrating mechanisms in crop plants

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References.

1. Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytologist, **165**, 351–371.
2. Long SP, Zhu X-G, Naidu SL, Ort DR (2006) Can improvement in photosynthesis increase crop yields? Plant, Cell & Environment, **29**, 315–330
3. Song Q, Chu C, Parry MAJ, Zhu X-G (2016) Genetics-based dynamic systems model of canopy photosynthesis: the key to improve light and resource use efficiencies for crops. Food and Energy Security **5**, 18-25.
4. Yin X, Struik PC (2017) Can increased leaf photosynthesis be converted into higher crop mass production? A simulation study for rice using the crop model GECROS Journal of Experimental Botany, **68**, 2345–2360
5. Driever SM, Lawson T, Andralojc PJ, Raines CA, Parry MAJ (2014) Natural variation in photosynthetic capacity, growth and yield in 64 field grown wheat genotypes. Journal of Experimental Botany **65**, 4959-4973
6. Gaju O, Desilva J, Carvalho P, Hawkesford M, Groffiths S, Greenland A, Foulkes MJ (2016) Leaf photosynthesis and associations with grain yield, biomass and nitrogen-use efficiency in landraces, synthetic-derived lines and cultivars in wheat. Field Crops Research **193**, 1-15
7. Carmo-Silva E, Andralojc PJ, Scales JC, Driever SM, Mead A, Lawson T, Raines CA, Parry MAJ (2017) Phenotyping of field-grown wheat in the UK highlights contribution of light response of photosynthesis and flag leaf longevity to grain yield. Journal of Experimental Botany **68**, 3473-3486
8. Zhu X-G, Long SP, Ort DR (2010) Improving photosynthetic efficiency for greater yield. Ann Rev Plant Biol **61**, 235-261
9. Raines (2011) Increasing photosynthetic Carbon assimilation in C3 plants to improve crop

yield: current and future strategies. Plant Physiology **155**, 36-42
10. Parry MAJ, Reynolds M, Salvucci ME, Raines C, Andralojc PJ, Zhu X-G, Price GD, Condon AG, Furbank R (2011) Raising Yield Potential of Wheat: (II) Increasing photosynthetic capacity and efficiency. Journal of Experimental Botany **62**, 453-468.
11. Ruan C-J, Shao H-B,Teixeira da Silva JA (2012) A critical review on the improvement of photosynthetic carbon assimilation in C3 plants using genetic engineering. Critical Reviews in Biotechnology **32**, 1-21
12. Carmo-Silva E, Scales JC, Madgwick PJ, Parry MAJ (2015) Optimizing Rubisco and its regulation for greater resource use efficiency. Plant Cell & Environment **38**, 1817-1832
13. Bar-Even A, Noor E, Lewis NE, Milo R. (2010) Design and analysis of synthetic carbon fixation pathways. Proceedings of the National Academy of Sciences (USA) **107**, 8889-8894.
14. Lorimer GH, Miziorko HM (1980) Carbamate formation on the ε-amino group of a lysyl residue as the basis for the activation of ribulose bisphosphate carboxylase by CO_2 and Mg^{2+}. Biochemistry **19**, 5321-5328.
15. Cleland WW, Andrews TJ, Gutteridge S, Hartman FC, Lorimer GH (1998) Mechanism of Rubisco: The carbamate as general base. Chemical Reviews **98**, 549-561.
16. Tcherkez G. (2013) Modelling the reaction mechanism of ribulose-1,5-bisphosphate carboxylase/oxygenase and consequences for kinetic parameters. Plant Cell and Environment **36**, 1586-1596
17. Tcherkez G. (2016) The mechanism of Rubisco catalysed carboxylation. Plant Cell and Environment **39**, 983-997
18. Kane H, Wilkin J, Portis AR, Andrews TJ (1998) Potent inhibition of ribulose bisphosphate carboxylase by an oxidized impurity in ribulose-1,5-bisphosphate. Plant Physiology **117**, 1059-1069
19. Harpel MR, Serpersu EH, Lamerdin JA, Huang Z-H, Gage DA, Hartman FC (1995) Oxygenation mechanism of ribulose-bisphosphate carboxylase/oxygenase. Structure and origin of 2-carboxytetritol 1,4-bisphosphate, a novel O_2-dependent side product generated by a site-directed mutant. Biochemistry **34**, 11296-11306.
20. Parry MAJ, Keys AJ, Madgwick PJ, Carmo-Silva EA, Andralojc PJ (2008) Rubisco regulation: a role for inhibitors. Journal of Experimental Botany, **59**, 1569–1580
21. Pearce FG (2006) Catalytic by-product formation and ligand binding by ribulose bisphosphate carboxylases from different phylogenies. Biochemical Journal **399**, 525-534.
22. Bracher A, Sharma A, Starling-Windhof A, Ulrich Hartl F & Hayer-Hartl M (2015) Degradation of potent Rubisco inhibitor by selective sugar phosphatase. Nature Plants **1**, Article number: 14002 doi:10.1038/nplants.2014.2
23. Andersson I, Taylor TC (2003) Structural framework for catalysis and regulation in ribulose-1,5-bisphosphate carboxylase/oxygenase. Archives of Biochemistry and Biophysics **414**, 130-140.
24. Parry MAJ, Andralojc PJ, Scales JC, Salvucci ME, Carmo-Silva AE, Alonso H, Whitney SM (2013) Rubisco Activity and regulation as targets for crop improvement. Journal of Experimental Botany **64**, 709-715.
25. Orr DJ, Alcântara A, Kapralov MV, Andralojc PJ, Carmo-Silva E, Parry MAJ (2016) Surveying Rubisco diversity and temperature response to improve crop photosynthetic efficiency. Plant Physiology **172**, 707-717
26. Bainbridge G, Madgwick P, Parmar S, Mitchell R, Paul M, Pitts J, Keys AJ, Parry MAJ (1995) Engineering Rubisco to change its catalytic properties. Journal of Experimental Botany **46**, 1269-1276.
27. Laing WA, Ogren WA, Hageman RH (1974) Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2, O_2, and ribulose 1,5-diphosphate carboxylase. Plant Physiology **54**, 678-685.
28. Zhu X-G, Portis AR, Long SP (2004) Would transformation of C3 crop plants with foreign

Rubisco increase productivity? A computational analysis extrapolating from kinetic properties to canopy photosynthesis. Plant, Cell and Environment **27**, 155–165.
29. Tcherkez GGB, Farquhar GD, Andrews TJ (2006) Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. Proceedings of the National Academy of Sciences (USA) **103**, 7246–7251
30. Savir Y, Noor E, Milo R, Tlusty T (2010) Cross-species analysis traces adaptation of Rubisco toward optimality in a low-dimensional landscape. Proceedings of the National Academy of Sciences (USA) **107**, 3475–3480
31. Galmes J, Kapralov MV, Copolovici LO, Hermida-Carrera C. Niinemets U (2015) Temperature responses of Rubisco maximum carboxylase activity across domains of life: phylogenetic signals, trade-offs, and importance for carbon gain Photosynthesis Research **123**, 183-201
32. Prins A, Orr DJ, Andralojc PJ, Reynolds MP, Carmo-Silva E, Parry MAJ (2015) Rubisco catalytic properties of wild and domesticated relatives provide scope for improving wheat photosynthesis. Journal of Experimental Botany **67**, 1827-1838.
33. Hermida-Carrera C, Kapralov MV, Galmés J (2016) Rubisco catalytic properties and temperature response in crops. Plant Physiology **17**, 2549-2561
34. Sharwood RE, Ghannoum O, Kapralov MV, Gunn LH, Whitney SM (2016a) Temperature responses of Rubisco from Paniceae grasses provide opportunities for improving C3 photosynthesis. Nature Plants **2**, 1-9
35. Young JN, Heureux AMC, Sharwood RE, Rickaby REM, Morel FMM, Whitney SM (2016) Large variation in the Rubisco kinetics of diatoms reveals diversity among their carbon concentrating mechanisms. Journal of Experimental Botany **67**, 3445–3456
36. Whitney SM, Houtz RL, Alonso H (2011) Advancing our understanding and capacity to engineer nature's CO_2-sequestering enzyme, Rubisco. Plant Physiology **155**, 27–35
37. Andersson I (2008) Catalysis and regulation in Rubisco. Journal of Experimental Botany **59**, 1555-1568.
38. Genkov T, Meyer M, Griffiths H, Spreitzer RJ (2010) Functional hybrid Rubisco with plant small subunits and algal large subunits. Journal of Biological Chemistry **285**, 19833-19841.
39. Ishikawa C Hatanaka T Misoo S Miyake C Fukayama H. (2011) Functional incorporation of sorghum small subunit increases the catalytic turnover rate of Rubisco in transgenic rice. Plant Physiology **156**, 1603–1611.
40. Spreitzer RJ (2003) Role of the small subunit in ribulose-1,5-bisphosphate carboxylase/oxygenase. Archives of Biochemistry & Biophysics **414**, 141–149
41. Morita K, Hatanaka T, Misoo S, Fukayama H. (2014) Unusual Small Subunit That Is Not Expressed in Photosynthetic Cells Alters the Catalytic Properties of Rubisco in Rice. Plant Physiology **379**, 164, 69–79
42. Farquhar GD, Caemmerer S, Berry JA. (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C3 species. Planta **149**, 78-90
43. Sharwood RE, Ghannoum O, Whitney SM (2016) Prospects for improving CO_2 fixation in C_3 grasses through understanding C_4 Rubisco biogenesis and catalytic diversity Current Opinion in Plant Biology **31**, 135-142
44. Lin MT, Occhialini A, Andralojc JP, Parry MAJ, Hanson MR (2014) A faster Rubisco with potential to increase photosynthesis in crops. Nature **513**, 547-550
45. Occhialini A, Lin MT, Andralojc PJ, Hanson MR, Parry MAJ (2016) Transgenic tobacco plants with improved cyanobacterial expression but no extra assembly factors grow at near wild-type rates if provided with elevated CO2. Plant Journal **85**, 148-160
46. Keys AJ, Major I, Parry MAJ (1995) Is there another player in the game of Rubisco regulation. Journal of Experimental Botany **46**, 1245-1251.
47. Gutteridge S, Parry MAJ, Burton S, Keys AJ, Mudd A, Feeney J, Servaites JC, Pierce J (1986) A nocturnal inhibitor of carboxylation in leaves. Nature **324**, 274-276
48. Khan S, Andralojc PJ, Lea PJ, Parry MAJ (1999) 2'-carboxy-D-arabinitol 1-phosphate (CA1P)

protects ribulose-1,5-bisphosphate carboxylase/oxygenase against proteolytic breakdown. European Journal of Biochemistry **266**, 840-847

49. Spreitzer, R.J. and Salvucci, M.E. (2002) Rubisco: Structure, Regulatory Interactions, and Possibilities for a Better Enzyme. Annual Review Plant Biology, **53**, 449-475.
50. Hammond ET, Andrews TJ, Mott KA, Woodrow IE (1998) Regulation of Rubisco activation in antisense plants of tobacco containing reduced levels of Rubisco activase. The Plant Journal **14,** 101-110.
51. Yamori W, Masumoto C, Fukayama H, Makino A (2012) Rubisco activase is a key regulator of non-steady-state photosynthesis at any leaf temperature and, to a lesser extent, of steady-state photosynthesis at high temperature. The Plant Journal **71,** 871-880.
52. Carmo-Silva E, Salvucci ME (2013) The regulatory properties of Rubisco activase differ among species and affect photosynthetic induction during light transitions. Plant Physiology **161**, 1645-1655
53. Soleh MA, Tanaka Y, Nomoto Y, Iwahashi Y, Nakashima K, Fukuda Y, Long SP, Shiraiwa T (2016) Factors underlying genotypic differences in the induction of photosynthesis in soybean [Glycine max (L.) Merr.]. Plant, Cell & Environment **39,** 685-693.
54. Taylor SH, Long SP (2017) Slow induction of photosynthesis on shade to sun transitions in wheat may cost at least 21% of productivity. Philosophical Transactions of the Royal Society B, **372,** 20160543.
55. McAusland L, Vialet-Chabrand S, Davey P, Baker NR, Brendel O, Lawson T (2016) Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. New Phytologist **211,** 1209-1220.
56. Andralojc PJ, Madgwick PJ, Tao Y, Keys A, Ward JL, Beale MH, Loveland JE, Jackson PJ, Willis AC, Gutteridge S, Parry MA (2012) 2-Carboxy-D-arabinitol 1-phosphate (CA1P) phosphatase: evidence for a wider role in plant Rubisco regulation. Biochemical Journal **442,** 733-742.
57. Kromdijk J, Głowacka K, Leonelli L, Gabilly ST, Iwai M, Niyogi KK, Long SP (2016) Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. Science **354**, 857-861.
58. Zhu X-G, de Sturler S, Long SP. 2007. Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. Plant Physiology **145**, 513-526
59. Parry MAJ, Pennacchi JP, Robledo-Arratia L, Carmo-Silva E. (2017) Photosynthetic Improvement in wheat plants. In Achieving sustainable cultivation of wheat Volume 1: Breeding, quality traits, pests and diseases (ed. Prof. Peter Langridge).
60. Lefebvre S, Lawson T, Fryer M, Zakhleniuk OV Lloyd JC, Raines CA (2005) Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. Plant Physiology **138**, 451-460
61. Feng L Wang K Li Y Tan Y Kong J Li H Li Y Zhu Y . 2007b. Over-expression of SBPase enhances photosynthesis against high temperature stress in transgenic rice plants. Plant Cell Reports **26**, 1635–1646.
62. Driever SM, Simkin AJ, Alotaibi S, Fisk SJ, Madgwick PJ, Sparks CA, Jones HD, Lawson T, Parry MAJ, Raines CA (2017) Increased SBPase activity improves photosynthesis and grain yield in wheat grown in greenhouse conditions. Proceedings of the National Academy of Sciences (USA) **372**, 20160384
63. Uematsu K Suzuki N Iwamae T Inui M Yukawa H . 2012. Increased fructose 1,6-bisphosphate aldolase in plastids enhances growth and photosynthesis of tobacco plants. Journal of Experimental Botany **63**, 3001–3009
64. Simkin AJ, McAusland L, Headland LR, Lawson T, Raines CA (2015) Multigene manipulation of photosynthetic carbon assimilation increases CO2 fixation and biomass yield in tobacco. Journal of Experimental Botany **66**, 4075–4090

65. Timm, S., Florian, A., Arrivault, S., Stitt, M., Fernie, A. R., and Bauwe, H. (2012). Glycine decarboxylase controls photosynthesis and plant growth. FEBS Lett. 586, 3692–3697.
66. Simkin AJ, Lopez-Calcagno PE, Davey PA, Headland LR, Lawson T, Timm S, Bauwe H, Raines CA. (2017) Simultaneous stimulation of sedoheptulose 1,7-bisphosphatase, fructose 1,6-bisphophate aldolase and the photorespiratory glycine decarboxylase-H protein increases CO2 assimilation, vegetative biomass and seed yield in Arabidopsis. Plant Biotechnology **15**, 805–816
67. Carvalho J, Madgwick PJ, Powers SJ Keys AJ, Lea PJ, Parry MAJ (2011) An engineered pathway for glyoxylate metabolism in tobacco plants aimed to avoid the release of ammonia in photorespiration. BMC Biotechnology **21**,11 111
68. Kebeish R, Niessen M, Thiruveedhi K, Bari R, Hirsch HJ, Rosenkranz R, Stäbler N, Schönfeld B, Kreuzaler F, Peterhänsel C. (2007). Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in Arabidopsis thaliana. Nature Biotechnology **25**, 593–599.
69. Maier A, Fahnenstich H, von Caemmerer S, Engqvist MKM, Weber APM, Flügge UI, Maurino VG. (2012) Transgenic introduction of a glycolate oxidative cycle into A. thaliana chloroplasts leads to growth improvement. Frontiers in Plant Science **3**, 38.
70. Xin, C-P, Tholen D, Devloo V, Zhu X-G. (2015). The benefits of photorespiratory bypasses: How can they work? Plant Physiology **167**, 574–85.
71. Basler G, Küken A, Fernie AR and Nikoloski Z (2016) Photorespiratory Bypasses Lead to Increased Growth in Arabidopsis thaliana: Are Predictions Consistent with Experimental Evidence? Front. Bioeng. Biotechnol. **4**:31. doi: 10.3389/fbioe.2016.00031
72. Sage RF (2004) The evolution of C4 New phytologist **161**, 341-370
73. Sage RF. (2016). A portrait of the C4 photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and Hall of Fame. Journal of Experimental Botany **67**, 4039–56.
74. Wang P, Khoshravesh R, Karki S, Tapia R, Balahadia CP, Bandyopadhyay A, Quick WP, Furbank R. Sage TL , Langdale JA. (2017) Re-creation of a key step in the evolutionary switch from C3 to C4 leaf anatomy. Current Biology doi.org/10.1016/j.cub.2017.09.040
75. Ghannoum O, Evans JR, von Caemmerer S. (2011) Nitrogen and water use efficiency of C4 plants. In: C4 photosynthesis and related CO_2 concentrating mechanisms (eds A.S. Raghavendra & R.F. Sage), pp. 129-146. Springer Science+Business Media B.V.
76. Lin MT, Occhialini A, Andralojc JP, Devonshire J, Hines KM, Parry MAJ, Hanson MR (2014) β-carboxysomal proteins assemble into highly organized structures in Nicotiana chloroplasts. The Plant Journal **79** 1-12
77. Mackinder LC, Meyer MT, Mettler-Altmann T, Chen VK, Madeline C. Mitchell MC, Caspari O, Freeman Rosenzweig ES, Pallesen L, Greeves G, Itakura A, Roth R, Sommer F, Geimer S, Mühlhaus T, Schroda M, Goodenough U, Stitt M, Griffiths H, Martin C. Jonikas MC (2016) A repeat protein links Rubisco to form the eukaryotic carbon-concentrating organelle. Proceedings of the National Academy of Sciences (USA) **113**, 5958–63.
78. Atkinson N, Feike D, Mackinder LC, Meyer MT, Griffiths H, Jonikas MC, Smith AM, McCormick AJ. 2016. Introducing an algal carbon-concentrating mechanism into higher plants: location and incorporation of key components. Plant Biotechnology Journal **14**, 1302–1315
79. Wong S-C, Cowan IR, Farquhar GD (1985) Leaf conductance in relation to rate of CO_2 assimilation. I. Influence of nitrogen nutrition, phosphorus nutrition, ontogeny, photon flux density, and ambient partial pressure of CO_2. Plant Physiol. **78**, 821-825
80. von Caemmerer S, Evans JR (1991) Determination of the average partial pressure of CO2 in chloroplasts from leaves of several C3 plants. Aust. J. Plant Physiol. **18**, 287-305
81. Hawkesford MJ, Araus J-L, Park R, Calderini D, Miralles D, Shen T, Zhang J, Parry MAJ. (2013) Prospects of doubling wheat yields. Food and Energy Security 2, 34-48Ishikawa C Hatanaka T Misoo S Miyake C Fukayama H. (2011) Functional incorporation of sorghum small subunit

increases the catalytic turnover rate of Rubisco in transgenic rice. Plant Physiology **156**, 1603–1611.
82. Flexas J. (2016) Genetic improvement of leaf photosynthesis and intrinsic water use efficiency in C_3 plants: why so much little success? Plant Science **251**, 155-161
83. Parry MAJ, Flexas J, Medrano H. (2005) Prospects for crop production under drought. Annals of Applied Biology **147**, 211-278
84. Flexas J, Barbour MM, Brendel O, Cabrera HM, Carriquí M, Díaz-Espejo A, Douthe C, Dreyer E, Ferrio JP, Gago J, Gallé A, Galmés J, Kodama N, Medrano H, Niinemets Ü, Peguero-Pina JJ, Pou A, Ribas-Carbó M, Tomás M, Tosens T, Warren CR. (2012) Mesophyll diffusion conductance to CO2: an unappreciated central player in photosynthesis. Plant Science **193–194**, 70-84.
85. Jones HD. (2015) Challenging regulations: Managing risks in crop biotechnology. Food Energy Security **4**, 87–91
86. Zhang, Y-J, Sack L, Cao, K-F, Wei X-M, Li N. (2017) Speed versus endurance tradeoff in plants: Leaves with higher photosynthetic rates show stronger seasonal declines. Scientific Reports **7**, 42085

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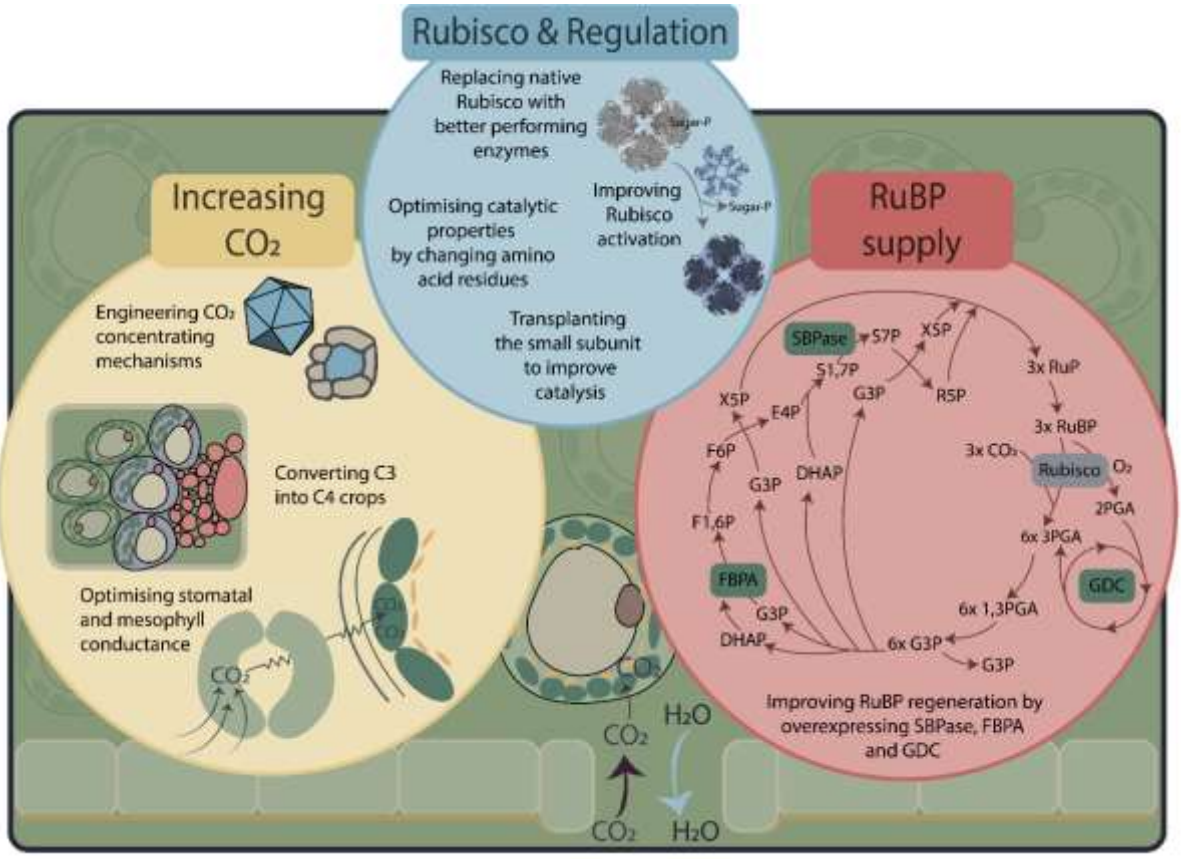

Figure 1. Strategies to improve carbon fixation based on three key processes: increasing CO2 fixation, Rubisco regulation, and RuBP supply. In the illustration of the Kranz anatomy of C4 photosynthesis, green represents the mesophyll cells and purple the bundle-sheath cells. In the Calvin-Benson-Bassham (CBB) cycle, only the enzymes mentioned in the text have been included for simplicity. Enzymes: SBPase, sedoheptulose-1,7-bisphosphatase; FBPA fructose-1,6-bisphosphate aldolase; GDC, glycine decarboxylase. Metabolites: RuBP, ribulose-1,5-bisphosphate; 3PGA, 3-phosphoglycerate; 2PGA, 2-phosphoglycolate; 1,3PGA, 1,3-bisphosphoglycerate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; F1,6P, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; X5P, xylulose-5-phosphate; E4P, erythrose-4-phosphate; S1,7P, sedoheptulose-1,7-bisphosphate; S7P, sedoheptulose-7-phosphate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate. Rubisco and Rubisco activase structures were adapted from http://www.rcsb.org/pdb