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Review Article

Improving photosynthesis through the enhancement of Rubisco carboxylation capacity

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Rising human population, along with the reduction in arable land and the impacts of global change, sets out the need for continuously improving agricultural resource use efficiency and crop yield (CY). Bioengineering approaches for photosynthesis optimization have largely demonstrated the potential for enhancing CY. This review is focused on the improvement of Rubisco functioning, which catalyzes the rate-limiting step of CO_2 fixation required for plant growth, but also catalyzes the ribulose-bisphosphate oxygenation initiating the carbon and energy wasteful photorespiration pathway. Rubisco carboxylation capacity can be enhanced by engineering the Rubisco large and/or small subunit genes to improve its catalytic traits, or by engineering the mechanisms that provide enhanced Rubisco expression, activation and/or elevated [CO_2] around the active sites to favor carboxylation over oxygenation. Recent advances have been made in the expression, assembly and activation of foreign (either natural or mutant) faster and/or more CO_2-specific Rubisco versions. Some components of CO_2 concentrating mechanisms (CCMs) from bacteria, algae and C_4 plants has been successfully expressed in tobacco and rice. Still, none of the transformed plant lines expressing foreign Rubisco versions and/or simplified CCM components were able to grow faster than wild type plants under present atmospheric [CO_2] and optimum conditions. However, the results obtained up to date suggest that it might be achievable in the near future. In addition, photosynthetic and yield improvements have already been observed when manipulating Rubisco quantity and activation degree in crops. Therefore, engineering Rubisco carboxylation capacity continues being a promising target for the improvement in photosynthesis and yield.

Introduction

Crop yield (CY) must be double by 2050 to meet the food and bioenergy demands of a rising world population [1,2]. The accelerated impacts of global change, which includes rising global temperatures and more extreme events, together with a reduction in arable land and water availability are major threats for plant production [3,4]. Therefore, current efforts are focused on increasing CY per unit area to enhance resource use efficiency and avoid expanding agricultural land use at the expenses of natural habitats [5].

The CY depends on the efficiency of light energy interception, the efficiency of light energy conversion into biomass (photosynthesis) and the way carbon is partitioned within the plant into harvested organs. In the last decades, crop breeding has extraordinarily increased yields via processes that optimized carbon partitioning and light energy interception, but the efficiency of photosynthesis remained unaltered [6]. Actually, the efficiency of photosynthesis is the only factor that is far from being at its maximum potential, and therefore remains a target of improvement towards the optimization of CY [7–11]. In this sense, about 3–4% of intercepted photosynthetically active radiation is converted in new biomass at optimum conditions in the field, which represents approximately one-third of the potential maximum energy conversion efficiency that can be attained theoretically [12].

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Increased CY has been widely observed in plants grown under CO_2 enrichment conditions showing enhancement of photosynthetic CO_2 assimilation [13,14], indicating that there is the place for substantial improvements in CY through optimization of photosynthesis. In addition, cross-scale modeling, validated using data from diverse field experiments, also evidenced the potential for CY improvement through engineering photosynthesis [9,15].

The photosynthetic process consists of a series of biochemical reactions that use light energy to fix CO_2 into sugars. First, light energy is harvested by chlorophylls and accessory pigments and transformed in excited electrons that are involved in a sequence of redox reactions (transport of electrons) leading to the synthesis of chemical energy (ATP) and reducing power ($NADPH_2$). Second, the synthetized ATP and $NADPH_2$ are consumed in the Calvin–Benson cycle to reduce CO_2 to trioses phosphates in the chloroplast stroma, that are finally exported out of the chloroplast and used as carbon skeletons for building new biomolecules and to cover the energetic requirements of the plant.

One of the most critical and regulated steps of the photosynthetic process is the CO_2 fixation catalyzed by the enzyme Ribulose-bisphosphate carboxylase oxygenase (Rubisco), consisting of the addition of CO_2 to Ribulose-1,5-biphosphate (RuBP) to produce two molecules of 3-phosphoglycerate (3-PGA). This enzyme possesses a slow carboxylation turnover rate (k^c_{cat}) and a relatively low affinity for CO_2 (i.e. relatively high K_c, which is the Michaelis–Menten constant for CO_2 in the absence of O_2). Moreover, Rubisco not only catalyzes the addition of CO_2 to RuBP (carboxylation), but also the addition of O_2 (oxygenation), producing one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG). The latter will be metabolized through photorespiration, a metabolic pathway that will recover only part of the carbon contained in 2-PG molecules, provoking a net loss of fixed carbon with an extra energy investment [16], and therefore, decreasing the efficiency of photosynthesis. These 'inefficient' catalytic properties explain the large amounts of Rubisco found in C_3 plants (20–50% of leaf soluble proteins [17]), and the consequent demand for a substantial N investment.

Previous studies suggested that increases in Rubisco carboxylation capacity can considerably enhance photosynthesis [4,18], which is supported by the significant enhancement of photosynthetic CO_2 assimilation frequently observed in plants grown under CO_2 enrichment conditions [13,14], as mentioned above. This review will focus on recent bioengineering approaches to improve photosynthesis by enhancing Rubisco CO_2 fixation capacity, therefore, increasing the synthesis of carbohydrates required for plant growth and yield. Manipulations to enhance Rubisco carboxylation capacity includes improvements in Rubisco kinetic traits, the increase in $[CO_2]$ around Rubisco to limit its oxygenation through the introduction of CO_2 concentrating mechanisms (CCMs), and the modification of Rubisco expression and degree of activation (see Figure 1).

Improving Rubisco kinetic traits

Rubisco catalyzes the main entrance of inorganic carbon into the biosphere, therefore it sustains most trophic webs on Earth. Due to its essential role in carbon assimilation and 'inefficient' catalytic properties, Rubisco performance has been and continues to be one of the main targets to be engineered for improving photosynthesis to increase plant biomass productivity and yield [10,18–21]. Ideally, Rubiscos with a combination of elevated k^c_{cat}, high carboxylation efficiency (k^c_{cat}/K_c), and elevated specificity factor of CO_2 over O_2 ($S_{c/o}$) are the most desirable to be engineered in crops [22], replacing their native Rubiscos.

The improvement of Rubisco kinetic traits entails the exploration of more efficient naturally occurring versions of Rubisco, and the understanding of the molecular causes of variability in the catalytic traits, allowing the design of improved Rubiscos in the laboratory [23]. In either case, the improved (either natural or artificial) Rubisco versions must be successfully transplanted into crops (see Figure 1A), which is a complex challenge that still requires in many cases a better understanding of chloroplast gene regulatory pathways as well as Rubisco folding and assembly, although significant advances in plastid transformation has been achieved [20,24,25].

Screening natural diversity

Rubisco is present in all domains of life (Archaea, Bacteria and Eukarya) with different forms (I, II, II/III, III). All Rubisco types are composed of dimers of two large subunits (L_2) to produce functional active sites, while form I Rubisco also includes small subunits in a L_8S_8 stoichiometry [26]. Form I Rubisco can be classified in the 'green type' (form IA and IB) and 'red type' (form IC and ID) lineages. The only types found in eukaryotes are form IB in green algae and plants, and form ID in non-green algae, except for peridinin-containing dinoflagellates, that possess form II. Form II Rubisco is also found in photoautotrophic or chemoautotrophic

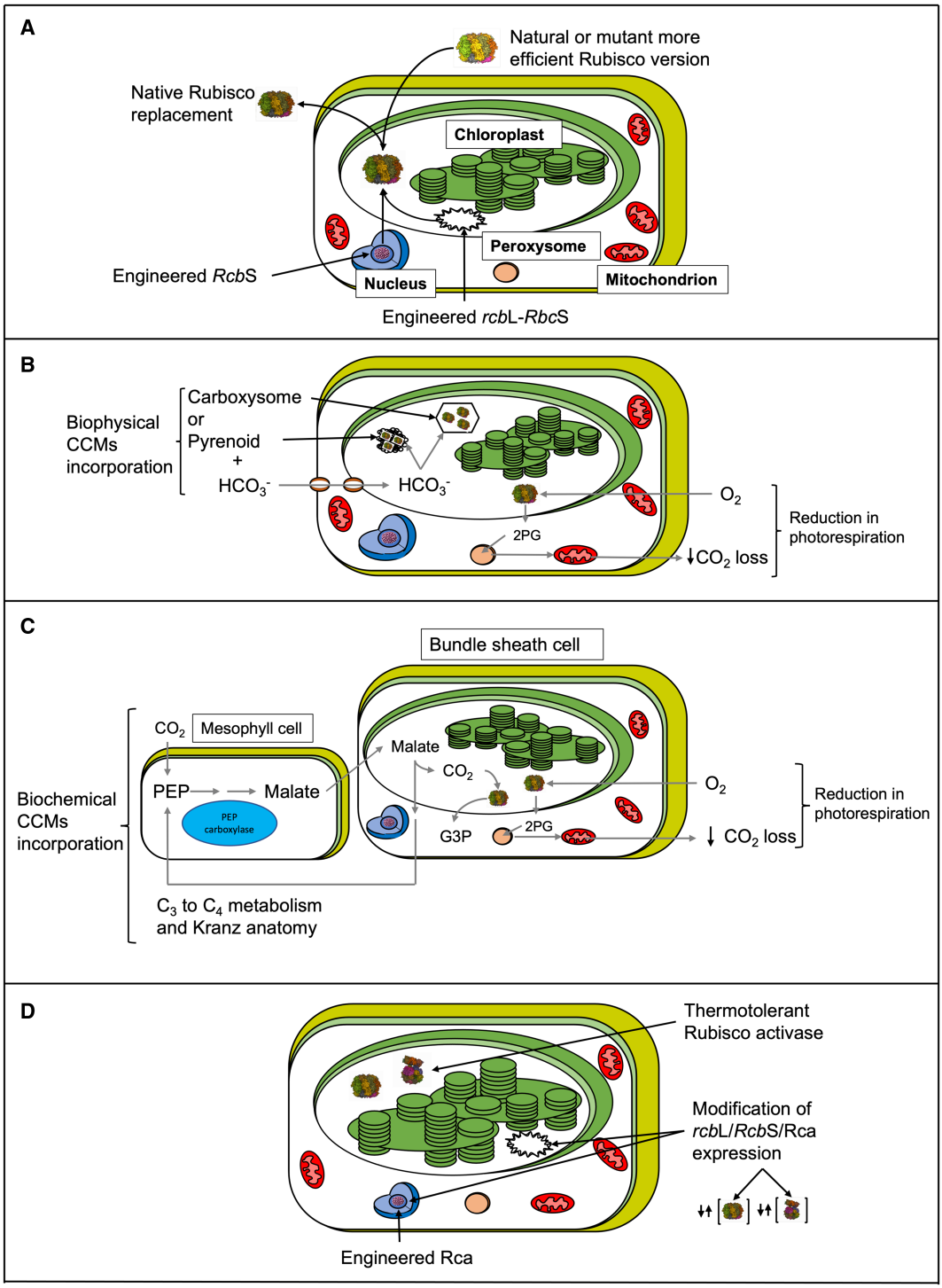

Figure 1. Potential targets to increase Rubisco carboxylation capacity in crops.

Which includes improvements in Rubisco kinetic traits through the replacement of native *rbc*L and/or *Rbc*S by natural or mutant more efficient Rubisco versions (**A**); the introduction of biophysical (**B**) or biochemical (**C**) CO_2 concentrating mechanisms (CCMs) to optimize Rubisco carboxylation and limit its oxygenation, therefore reducing photorespiration; and the modification of Rubisco/Rubisco activase expression and replacement of native Rubisco activases by more thermotolerant versions (**D**). PEP: phosphoenolpyruvate; G3P: glyceraldehyde-3-phosphate; 2PG: 2-phosphoglycolate; Rca: nucleus gene coding Rubisco activase; *rcb*L: plastome-encoded Rubisco large subunit gene; *Rcb*S: nucleus-encoded Rubisco small subunit genes.

Biochemical Society Transactions (2021) 49 2007–2019
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proteobacteria, whereas form III and II/III are found in Archaea and Bacteria, and are usually not involved in autotrophic carbon assimilation pathways [26–28]. Despite possessing 19 conserved amino acids residues essential for catalysis [29], Rubiscos from different forms and phylogenetic groups showed strong differences in their catalytic traits, as a result of different selective pressures during evolution [27].

The characterization of Rubisco kinetic traits from a broader number of phylogenetically distant species in the last decades (reviewed by Iñiguez et al. [27]) has increased the possibility to find more efficient Rubisco versions in Nature to be engineered in crops for potential CO_2 assimilation improvement.

The few analyzed red algae have shown to possess the highest $S_{c/o}$ and carboxylation efficiency (k^c_{cat}/K_c) ever reported [30–32], and models reveal a potential benefit for CO_2 assimilation rate and plant growth of up to 30% [4] at ambient conditions, if crops native Rubiscos are replaced by these more efficient Rubisco versions [20]. Nevertheless, green-type and red-type Rubiscos strongly differ in their folding, assembly and regulation requirements [33,34], which has still prevented form ID Rubiscos to be expressed in transplantomic plant lines [32,35]. Specifically, this is due to the inability of plant ancillary chaperones to recognize foreign form ID Rubisco subunits. A substantial recent advance has been the successful expression and activation of a red-type Rubisco from the proteobacterium *Rhodobacter sphaeroides* (form IC) in transplantomic tobacco lines [25]. This plant lines expressed a chloroplastic operon containing the large (*rbc*L) and small subunit (*Rbc*S) genes from *R. sphaeroides* and included a nuclear transformation with the metabolic repair protein CbbX, an AAA+ protein (ATPase associated with various cellular activities) which act as a red-type Rubisco activase [25,33]. Although these transplantomic Rubisco lines need to be grown under elevated CO_2 conditions due to the low affinity for CO_2 of *R. sphaeroides* Rubisco, it will serve as a platform to integrate potential catalysis-enhancing structural elements from Form ID Rubisco by phylogenetic grafting. Efforts in this direction are sustained on the structural similarity between form IC and ID Rubisco, which has already allowed the successful assembly of hybrid *R. sphaeroides* large subunit and form ID small subunit in tobacco chloroplast [25], even though the produced hybrid Rubiscos were catalytically incompetent.

The highest carboxylation turnover rates (k^c_{cat}) so far reported have recently been found in proteobacteria possessing form II Rubiscos, with a maximum of 22 s^{-1} obtained for *Gallionella* sp. (more than 6-fold faster than the mean for plant Rubiscos [36]). However, in order to support elevated photosynthetic rates under environmental conditions, the *Gallionella* Rubisco variant would require operating along with CCMs within the transplantomic plant (see the section below), due to its low affinity and low specificity for CO_2 ($K_c = 276$ μM; $S_{c/o} = 10$).

It has been previously suggested that plant Rubisco catalytic traits are already optimized and cannot be improved, since the active site chemistry constrains the enzyme's evolution [37,38], meaning that the canonical trade-offs between k^c_{cat} and K_c and between k^c_{cat} and $S_{c/o}$ would impede to find faster and more CO_2-specific Rubisco versions. Still, recent studies suggested that Rubisco carboxylation kinetics are not so constrained [27,39–41], and so it might be possible to find improved Rubisco versions in Nature, with enhanced k^c_{cat}, $S_{c/o}$ and k^c_{cat}/K_c than crop Rubiscos. The systematic survey of kinetic traits by mining metagenomic data to search for natural undiscovered Rubiscos [36] might become a useful tool to find faster and more CO_2-specific variants for crop photosynthesis improvement programs.

For improving crop photosynthesis, the temperature response of Rubisco catalytic traits must be also considered. A recent study [23] revealed a large natural variability in the thermal responses of Rubisco kinetic traits in higher plant species, related to their photosynthetic type and adaptation to the species thermal environment, and the authors identified some variants with the potential to improve Rubisco-limited CO_2 gross assimilation rate in crops under future climatic conditions.

Selecting improved Rubisco mutant versions through directed evolution

Another interesting bioengineering tool for finding improved Rubisco versions consists of the application of screening systems to study the artificial evolution of Rubisco. The development of directed protein evolution approaches to search for Rubisco versions with improved catalytic properties from a mutant library comprising sufficient genetic diversity can reveal novel fitness solutions that would otherwise be unexplored during natural evolution [42].

Through directed evolution approaches using Rubisco dependent *Escherichia coli* (RDE) selection, catalytic improvements have been observed in form I, II and III Rubisco mutants during the last years. Relative to form I Rubiscos, a cyanobacterial *Synechocystis* PCC6803 Rubisco mutant with 3-fold improvements in carboxylation efficiency was obtained by Duraõ et al. [43], that improved photosynthesis rates by more than 50% relative to

the wild type (WT), when re-integrated into the cyanobacterium carboxysome. Wilson et al. [44] identified a cyanobacterial *Thermosynechococcus elongatus* Rubisco mutant with improved k^c_{cat} by 28%, enhanced carboxylation efficiency under ambient [O_2] (k^c_{cat}/K^{air}_c) by 43%, and improved $S_{c/o}$ by 6%, relative to WT, although the Rubisco mutant expression in tobacco transplantomic lines was unsuccessful. Recently, Zhou and Whitney [45] identified an *R. sphaeroides* Rubisco mutant with improved k^c_{cat} and k^c_{cat}/K^{air}_c by 27% and 17%, respectively, relative to WT, even though the mutations led to a 40% lower capacity for Rubisco accumulation in the host *E. coli*. Form III from the archaea *Methanococcoides burtonii* has also been subjected to directed evolution. The selected mutants from this non-photosynthetic Rubisco showed 2 and 3-fold higher k^c_{cat} and k^c_{cat}/K^{air}_c, respectively, relative to WT Rubisco, along with a 15% increase in $S_{c/o}$ [46]. When the selected Rubisco mutants were transformed in tobacco, a strong increase in photosynthesis and growth relative to tobacco controls producing WT *M. burtonii* Rubisco was also observed. Therefore, Wilson et al. [46] revealed that carboxylation kinetics from archaeal Rubiscos could be easily enhanced by single aminoacid changes due to its evolutionary specialization in an alternative non-photosynthetic metabolic role, and advantage can be taken from the extremely thermotolerance of some archaeal Rubiscos [47,48] and its elevated affinity for RuBP [46]. Improvements in form II carboxylation kinetics by directed evolution has been limited, although mutant versions of the proteobacterial *Rhodospirillum rubrum* Rubisco with increased thermotolerance and biogenesis capacity have been identified [49].

Despite all the kinetics improvements previously described for bacterial form I, form II, and archaeal form III mutant Rubisco versions relative to WT enzymes, their carboxylation efficiencies and $S_{c/o}$ are still too low to improve photosynthesis in crop chloroplasts at the current atmospheric [CO_2]. This is the main problem that has prevented from obtaining transformed plants with improved carboxylation capacity yet. Nevertheless, continued directed evolution of bacterial or archaeal Rubiscos confers promising avenues for potential enhancement of leaf photosynthesis and plant growth. In addition, recombinantly expressed form II and form III Rubiscos, that are structurally simpler than form I, are often successfully assembled and activated in model hosts like *E. coli* or *Nicotiana tabacum* [36,46]. The main limitations for developing directed evolution approaches in eukaryotic form I Rubiscos (L_8S_8) was related to the fact that their folding and assembly depend on the co-expression of specific ancillary chaperones [50]. Recently, functional plant Rubisco from *Arabidopsis thaliana* was produced in *E. coli* through the co-expression of five chloroplast chaperones, including Cpn60/Cpn20, Rubisco accumulation factors 1 (raf1) and 2 (raf2), *Rbc*X, and bundle-sheath defective-2 (BSD2) [51,52]. The developed *E. coli* system has already been successfully adapted for the expression of recombinant tobacco Rubiscos [53]. This important advance will allow the selection of improved Rubisco mutants of eukaryotic form I Rubiscos in future studies by directed evolution approaches, with much higher $S_{c/o}$ and/or carboxylation efficiencies than native crop Rubiscos.

Engineering Rubisco large or small subunit

Viable transplantomic plant lines expressing functional foreign form I Rubiscos have been carried out by chloroplast transformation, using the foreign *rbc*L–*Rbc*S operon that replaced native *rbc*L gene [24,25,54] (Figure 1A), along with specific assembly factors involved in Rubisco biogenesis. However, chloroplast transformation technology has been only available in tobacco, not in other important crops such as wheat, rice or cotton [20,55], but recent advances in plastid transformation technology for the model plant *A. thaliana* [56] has opened the door for other recalcitrant crop species.

Rubisco large subunit contains the essential amino acids for active site conformation [57], and some amino acids from the Rubisco large subunit have been identified, through site-directed mutagenesis, as catalytic switches for improving its carboxylation capacity [58,59]. Still, recent studies have clearly demonstrated that Rubisco small subunit can also be an important determinant of kinetic traits [53,54,60–64]. Since nuclear transformation is already optimized in many species, the nuclear-encoded *Rbc*S multigene family is becoming an emergent target for crop photosynthetic enhancement (Figure 1A).

Overexpression of C_4-plant *Rbc*S in rice increased Rubisco k^c_{cat} and K_c by 30–50% and decreased $S_{c/o}$ by 5–15% [60,63], partially reflecting the catalytic properties of the C_4-plant enzyme. Moreover, when the native *Rbc*S multigene family was knocked out and completely replaced by *Sorghum* C_4 *Rbc*S in rice, the produced hybrid Rubisco showed increased k^c_{cat} and K_c by 80–90% and reduced $S_{c/o}$ by 15% relative to WT rice Rubisco [64], almost matching the *Sorghum* C_4 Rubisco kinetics.

Overexpression of an unusual rice *Rbc*S isoform, that is only expressed in non-photosynthetic cells, also significantly enhanced k^c_{cat} and K_c, and reduced $S_{c/o}$, relative to native leaf rice Rubisco [61]. Similarly, an *Rbc*S

Biochemical Society Transactions (2021) 49 2007–2019
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isoform expressed in trichomes, which belongs to a different phylogenetic cluster than *Rbc*S genes expressed in mesophyll cells, increased k^c_{cat} and K_c in tobacco [53,62] and potato [54]. *Arabidopsis Rbc*S mutants expressing algal *Chlamydomonas reinhardtii* small subunit also differed in Rubisco catalysis (reduced k^c_{cat} and $S_{c/o}$) from WT plants, which was also reflected in significant changes in photosynthesis and growth [65]. These results demonstrate the importance of *Rbc*S on Rubisco kinetics that can be exploited for the improvement of crop photosynthesis.

Introducing CO_2 concentrating mechanisms in C_3 plants

Atmospheric CO_2 gas has to diffuse through several leaf barriers to Rubisco's active site in C_3 plants. Among these resistances, leaf mesophyll conductance (g_m) is the main limitation for photosynthesis in many plant species. Leaf anatomy is the most determining component of g_m, and, since it is encoded by multiple genes, it is too hard to directly improve g_m (but see [66] for increased CO_2 conductance by overexpression of aquaporins).

Some photosynthetic organisms have developed CCMs, which increase [CO_2] around Rubisco during steady-state photosynthesis, promoting a nearly saturated carboxylation activity and a strong reduction in the oxygenation activity of Rubisco. There is a wide variability in CCMs that have evolved in some plants, algae and bacteria, ranging from biochemical processes involving a first carbon fixation into a C_4 molecule before the definitive Rubisco-mediated carbon fixation (C_4 and crassulacean acid metabolism plants), to biophysical processes based on active transporters of dissolved inorganic carbon (HCO_3 and/or CO_2) and/or proton pumps contributing to the creation of acid zones, coupled with carbonic anhydrases that accelerate the interconversion between HCO_3^- and CO_2 [67]. Efficient CCMs in cyanobacteria, some proteobacteria and many eukaryotic algae include the packaging of Rubisco along with carbonic anhydrase into non-membranous compartments (i.e. carboxysomes in bacteria and pyrenoids in algae [68,69]). Rubisco kinetics co-evolved with CCMs, leading to higher k^c_{cat} and K_c values along with a relaxation in $S_{c/o}$ and carboxylation efficiency promoted by the high CO_2 environment around Rubisco [27]. Therefore, organisms with CCMs possess potentially higher nitrogen efficiencies (and higher water use efficiencies in the case of terrestrial plants) relative to their counterparts without CCMs, since the former can fix the same amount of CO_2 with less Rubisco protein, which supposes an important reduction in N investment (and reduced time for stomatal aperture in the case of terrestrial plants [70]). Models predict that the introduction of CCMs in C_3 plants (Figure 1B,C) could significantly increase the net CO_2 uptake, producing >50% enhancement in CY [15].

Biophysical CCMs: carboxysomes and pyrenoids expression in chloroplasts

Cyanobacteria and some proteobacteria possess bicarbonate transporters in the plasma membrane that allow bicarbonate accumulation in the cytosol. Carboxysomes encapsulate Rubisco within a polyhedral protein shell that allows the transport of HCO_3^- and RuBP inside it but restrict the efflux of CO_2 out of the microcompartment, maintaining an internal elevated [CO_2]. There are two different types of carboxysomes, α-carboxysomes (associated to form IA Rubisco) and β-carboxysomes (associated to form IB Rubisco), with similar physiological functioning despite intrinsic structural differences [71]. Recent advances have been made in the expression and assembly of carboxysomes within heterologous hosts (Figure 1B). Functional proteobacterial α-carboxysome has been assembled in *E. coli* by expressing the carboxysome operon [72]. Recently, simplified cyanobacterial α-carboxysomes have been reconstituted in tobacco chloroplasts with a minimum set of genes (*rbc*L and *Rbc*S, together with two key α-carboxysome structural proteins) which were able to encapsulate Rubisco and to allow autotrophic plant growth [24]. Regarding β-carboxysomes, Lin et al. [73] and Orr et al. [74] observed functional macromolecular complexes within tobacco chloroplast stroma by co-expressing cyanobacterial Rubisco with an internal carboxysomal protein (CcmM35), which represent an early step in the biogenesis of this type of carboxysomes. In addition, Fang et al. [75] was able to produce functional β-carboxysome-like structures in *E. coli* using 12 genes from *Synechococcus elongatus*. However, none of the transformed tobacco lines expressing simplified carboxysome-like structures were able to grow equal or faster than WT plants. This result was expected, since no pumping and accumulation mechanisms for inorganic carbon inside the chloroplast was still introduced in the transplantomic plants, and only Rubisco encapsulation is not enough for a functional CCM operation. Directions towards fully functional bacterial CCM expression in C_3 chloroplasts go through targeting functional bicarbonate transporter proteins into the chloroplast membranes (Figure 1B), a matter that is still underway [76–78].

Most of the knowledge about eukaryotic algal CCMs comes from the model species *C. reinhardtii*. The CCMs in this species is based on HCO_3^- transport across the periplasmic, chloroplast and thylakoid

membranes. Then, HCO_3^- is converted to CO_2 by carbonic anhydrase at the acidic pH of the thylakoid lumen, and finally CO_2 diffuses to Rubisco active sites in the pyrenoid matrix, since *Chlamydomonas* pyrenoid is connected by cylindrical pyrenoid tubules with thylakoids [79]. Although pyrenoids do not have a protein shell restricting gas diffusion, starch granules form a sheath around pyrenoid matrix [79] that probably limits CO_2 leakage. The multiple repeat linker-protein, EPYC1, is associated with Rubisco small subunits during aggregation within the *Chlamydomonas* pyrenoid [80]. EPYC1 was successfully expressed and localized in higher plant chloroplasts [81], and recently, hybrid Rubisco composed of *Arabidopsis* large subunit and *Chlamydomonas* small subunit has been shown to aggregate with EPYC1 into a proto-pyrenoid, with similar liquid-like properties, in *Arabidopsis* chloroplasts [82]. Novel research is now focused on the binding mechanism between Rubisco small subunit and EPYC1, as well as with other key proteins, that will allow the engineering of minimal sequence changes into native crop Rubiscos to be able to reconstitute a functional pyrenoid in C_3 plant chloroplasts [83] (Figure 1B). Future research steps in this field must focus on the mostly unexplored introduction of eukaryotic HCO_3^- transport mechanisms in the periplasmic, chloroplast and thylakoid membranes, in order to enhance plant Rubisco carboxylation capacity.

Biochemical CCMs: transforming C_3 in C_4 plants

The CCMs of C_4 plants with Kranz anatomy consist of a first non-definitive carbon fixation, in which CO_2 is fixed in the mesophyll cells by phosphoenolpyruvate carboxylase (PEPc), producing C_4 acids. The resulting C_4 acids are then transported to and decarboxylated in the bundle-sheath cells, where the liberated CO_2 is fixed by Rubisco [84].

Since C_4 plants possess enhanced energy conversion efficiencies, higher nitrogen and water use efficiencies, and higher yields than C_3 plants, long-standing efforts has been done during the last two decades to transfer C_4 photosynthesis to C_3 crops (see the C_4 rice project; https://c4rice.com/ [84,85]). The most important genes required for the C_4 pathway have been successfully introduced into rice [86], and evidences for *in vivo* incorporation of CO_2 into C_4 acids was revealed, but still without subsequent decarboxylation [53,87], presumably due to low enzyme levels or poor enzymatic regulation in the rice transgenic lines.

Engineering the C_4 pathway into a C_3 plants not only requires the introduction of the biochemical pathway, but also the implementation of Kranz anatomical traits (Figure 1C). Recently, some progress has been made by obtaining a 'proto-Kranz' anatomy with induced photosynthetic development of vascular sheath cells through constitutive expression of maize transcriptional regulator of cellular differentiation [88], which resulted in increased photosynthetic efficiency and yield (by 30–40%) under excessive or fluctuating light conditions [89], but not under optimal light conditions. Although a full Kranz two cell-type C_4 mechanism has still not being implemented in rice, the results obtained up to date suggest that it may be achievable in the future [85].

Altering expression and activation of Rubisco

Changes in the expression of Rubisco large and/or small subunits altering Rubisco content have also been shown to enhance photosynthesis and yield in both C_3 and C_4 crops (Figure 1D). Transgenic rice with increased Rubisco concentration (by 30%) exhibited ∼15% enhanced biomass production in lab experiments [90] and up to 30% increased yields with improved N-use efficiency for increasing biomass production when receiving sufficient N fertilization in field experiments [91]. This has been the first study reporting enhanced CO_2 assimilation and yield by increasing Rubisco content, and therefore, Rubisco activity, in a C_3 species. Previously, models have predicted that an increase in Rubisco activity at ambient [CO_2] would benefit CO_2 assimilation in C_4 species but not in C_3 species, where it would only benefit CO_2 assimilation at lower CO_2 concentrations [9,92]. In the C_4 species, *Zea mays*, overexpression of Rubisco large and/or small subunits, together with the Rubisco assembly chaperone RAF1, resulted in a >30% increase in Rubisco content, which was translated in improvements in CO_2 assimilation and growth [93,94].

Not only the content, but also the degree of activation of Rubisco is crucial for enhancing *in vivo* CO_2 assimilation (Figure 1D). The active site of Rubisco is prone to deactivation by tight-binding of inhibitory sugar-phosphates, which are released by the ATP-dependent chaperone Rubisco activase [19]. It has been shown that the alteration in Rubisco content might also affect Rubisco activase activity [93], as well as overexpression of Rubisco activase can produce a reduction in Rubisco content [95,96], revealing the intrinsic connection between Rubisco and Rubisco activase expression and functioning.

The *in vivo* degree of activation of Rubisco under optimum conditions in illuminated crops is about 80–90% but it decreases at higher temperatures due to the thermolability of plant Rubisco activase [97], producing a

Biochemical Society Transactions (2021) **49** 2007–2019
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decrease in crop CO_2 assimilation by heat stress. There have been substantial improvements in the understanding of the regulation of *in vivo* CO_2 assimilation under heat stress in plant species from different thermal environments [98,99]. Increased Rubisco activase content has been associated with increased grain yield in the C_4 species, *Z. mays* [100]. The replacement of native Rubisco activase by, or overexpression of, a more thermostable isoform has led to significant yield improvement under heat stress in *Arabidopsis* and rice [101,102]; while mutational variants of Rubisco activase including a conserved sequence from heat-adapted species, improved wheat Rubisco activase thermostability [103] (Figure 1D). Therefore, Rubisco activase has been identified as an important target for improving photosynthesis and crop productivity in near-future climate change scenarios [102].

Conclusions and future prospects

Substantial progress has been made regarding the expression and metabolic regulation of more efficient and CO_2-specific Rubisco versions in crops, and major steps toward implementing CCMs in C_3 crops have been achieved, including the assembly of bacterial carboxysomes in tobacco chloroplast and the establishment of proto-Kranz anatomy in rice. Photosynthetic and yield improvements have already been observed when manipulating Rubisco quantity and activation degree. Still, engineering Rubisco is a complex challenge that requires a better understanding of chloroplast gene regulatory pathways, Rubisco catalysis and biogenesis [20].

In addition to the improvements in Rubisco carboxylation capacity discussed in this review, it is likely that for improving CY to meet the future food and bioenergy demands, it would be necessary to engineer a large number of different traits targeting photosynthesis. This might include improvements in electron transport capacity [104,105] manipulations of Calvin Cycle enzymes other than Rubisco, such as sedoheptulose-1,7-bisphosphatase [106–108], and engineering more efficient photorespiratory pathways to reduce the loss of fixed carbon [109–111]. The previously mentioned bioengineering approaches have already demonstrated an increase in plant biomass production and yield in both glasshouse and field experiments, and have been reviewed in detail by Simkin et al. [8], Nowicka et al. [112] and Timm and Hagemann [113].

Indeed, cross-scale modeling revealed that other photosynthetic components such as light energy capture efficiencies must be manipulated simultaneously to Rubisco activity to obtain the greatest improvements in C_3 and C_4 CYs [9].

Finally, alternative (natural or synthetic) carbon assimilation pathways substituting for the Calvin Cycle are other promising targets for photosynthetic improvement [114,115].

A combination of the mentioned strategies may hopefully contribute to improving agricultural productivity in future climate change scenarios to meet the food and bioenergy demand of a growing human population.

Perspectives

- Rubisco catalyzes the rate-limiting step of CO_2 fixation, but also initiate the carbon wasteful photorespiratory pathway, therefore, bioengineering its carboxylation capacity is a promising target for the improvement in crop photosynthesis and yield.
- Recent advances in expressing more efficient foreign Rubisco versions and some CCMs components (e.g. carboxysomes) in crops have been achieved, as well as modifications in the level of expression and activation of crop native Rubisco.
- A better understanding of Rubisco folding and assembly, and photosynthetic gene regulatory pathways, is needed to obtain the greatest improvements in crop photosynthesis, although the results obtained up to date suggest that it might be achievable in the near future.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

C.Í. wrote most parts of the manuscript with support and contributions from J.G. and P.A.-N.. All authors provided critical feedback and helped shape the research and manuscript to its final version.

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Abbreviations

2-PG, 2-phosphoglycolate; 3-PGA, 3-phosphoglycerate; BSD2, bundle-sheath defective-2; CCMs, CO_2 concentrating mechanisms; CY, crop yield; RDE, Rubisco dependent *E. coli*; RuBP, Ribulose-1,5-biphosphate; WT, wild type.

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