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EXPERT VIEW

Synthetic biology approaches for improving photosynthesis

Armin Kubis and Arren Bar-Even*,

Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476 Potsdam-Golm, Germany

* Correspondence: bar-even@mpimp-golm.mpg.de

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Abstract

The phenomenal increase in agricultural yields that we have witnessed in the last century has slowed down as we approach the limits of selective breeding and optimization of cultivation techniques. To support the yield increase required to feed an ever-growing population, we will have to identify new ways to boost the efficiency with which plants convert light into biomass. This challenge could potentially be tackled using state-of-the-art synthetic biology techniques to rewrite plant carbon fixation. In this review, we use recent studies to discuss and demonstrate different approaches for enhancing carbon fixation, including engineering Rubisco for higher activity, specificity, and activation; changing the expression level of enzymes within the Calvin cycle to avoid kinetic bottlenecks; introducing carbon-concentrating mechanisms such as inorganic carbon transporters, carboxysomes, and C_4 metabolism; and rewiring photorespiration towards more energetically efficient routes or pathways that do not release CO_2. We conclude by noting the importance of prioritizing and combining different approaches towards continuous and sustainable increase of plant productivities.

Keywords: carbon fixation, Calvin cycle, Rubisco, C_4 metabolism, carbon-concentrating mechanisms, photorespiration.

Introduction

Selective breeding and optimization of cultivation techniques have historically driven increases in agricultural output. In the last century, these efforts have adopted a more scientific approach with the development of the Haber–Bosch process (Haber and Le Rossignol, 1909; Sutton *et al.*, 2008, and, later, the 'green revolution' (Khush, 2001). Since 1961, global rice and wheat yields increased by 150% and 210%, respectively (FAO, 2018). However, we have recently started to witness stagnation in growth improvement of major crops such as rice in China (Peng *et al.*, 2009) or wheat in the USA (Ray *et al.*, 2012). This presents a major problem, as further yield increases are sorely needed to feed the human population, especially considering the global shift towards meat-dependent diets, use of arable lands to feed bio-refineries, deleterious effects of climate change, and continuous erosion of agricultural land (Godfray *et al.*, 2010; Tilman *et al.*, 2011).

Agricultural yield can be modeled as a product of three factors (Monteith and Moss, 1977; Fletcher *et al.*, 2011): (i) efficiency of intercepting light; (ii) efficiency of converting intercepted light into biomass; and (iii) the harvest index (i.e. the fraction of biomass that is captured in the harvested part). In the past, improved yields have largely been achieved by increasing the light capture efficiency and the harvest index; however, these two factors now appear to be approaching their practical limits (Long *et al.*, 2006). Therefore, the efficiency with which plants convert light to biomass has become the prime focus for further improvement (Long *et al.*, 2006). This efficiency is determined by two main processes, the light-dependent reactions, in which photoenergy is used for the generation of the cellular redox and energy carriers NADPH and ATP, and the light-independent reactions, which use these carriers to

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fix CO_2 and reduce it to organic carbon. The efficiency of both processes is unlikely to be improved by a classic selective breeding approach—as demonstrated by a recent study exploring 80 years of soybean breeding (Koester *et al.*, 2016)—but could be potentially increased by dedicated engineering (Zhu *et al.*, 2010). The focus of this review is the use of synthetic biology tools for boosting the efficiency and rate of carbon fixation (Box 1). Rather than discuss the technical aspects of synthetic biology in plants—for which we refer the readers to other reviews (DePaoli *et al.*, 2014; Liu and Stewart, 2015; Boehm and Bock, 2018; Piatek *et al.*, 2018;Vazquez-Vilar *et al.*, 2018)—we emphasize conceptual strategies to boost carbon

Box 1. Key developments in synthetic biology approaches for improving photosynthesis

- **Assembly of Rubisco-containing carboxysomes in tobacco chloroplasts**

  Assembly of a simplified α-carboxysome in tobacco chloroplasts by replacing native Rubisco with large and small subunits of Rubisco from cyanobacteria and two key structural subunits. The introduction of carboxysomes to plant chloroplasts is a key step towards establishing a full biophysical carbon-concentrating mechanism in higher plants (Long *et al.*, 2018).

- **Design and *in vitro* realization of carbon-conserving photorespiration**

  A systematic search and analysis of synthetic photorespiration bypass routes that do not release CO_2 reveals that these can enhance the carbon fixation rate under all relevant physiological conditions. Two enzymes were engineered jointly to enable the reduction of glycolate to glycolaldehyde. The combination of these evolved enzymes with existing enzymes supported the *in vitro* recycling of glycolate to RuBP without the loss of CO_2, indicating the feasibility of carbon-conserving photorespiration (Trudeau *et al.*, 2018).

- **The synthetic malyl-CoA–glycerate pathway supports photosynthesis**

  An *in vivo* demonstration of a synthetic pathway that can support photosynthesis in two ways. First, it can produce acetyl-CoA from C_3 sugars without releasing CO_2. It can also assimilate photorespiratory glycolate without loss of carbon (Yu *et al.*, 2018).

- **Carbon fixation via a novel pathway *in vitro***

  An *in vitro* reconstruction of a synthetic carbon-fixing pathway, the CETCH cycle, based on highly efficient reductive carboxylation. The pathway, utilizing 17 enzymes that originate from nine organisms, was optimized by a combination of enzyme engineering and metabolic proofreading (Schwander *et al.*, 2016).

- **Overexpressing the H-protein of the glycine cleavage system increases biomass yield in glasshouse- and field-grown transgenic tobacco plants**

  Increased biomass upon overexpression of a limiting photorespiratory protein in tobacco grown in field conditions. This indicates that optimization of expression levels within native carbon fixation-related pathways could be harnessed to increase productivity, and that photorespiration could be improved even without the need for synthetic pathways (López-Calcagno *et al.*, 2019).

- **The road to C_4 photosynthesis: evolution of a complex trait via intermediary states**

  A case for engineering C_3–C_4 intermediate metabolism as a way to increase photosynthetic efficiency and set the stage towards future realization of complete C_4 metabolism. This study suggests that a detailed and mechanistic understanding of C_3–C_4 intermediates could provide valuable guidance for experimental designs aiming to boost carbon fixation (Schlüter and Weber, 2016).

- **Evolving *Methanococcoides burtonii* archaeal Rubisco for improved photosynthesis and plant growth**

  A demonstration of the use of directed laboratory evolution to improve the kinetic properties of Rubisco from an archaeal origin. The improved Rubisco variant was introduced to tobacco chloroplasts and demonstrated to increase photosynthesis. Such protein engineering strategies could be used to address the kinetic limitations of key enzymes, thus supporting higher metabolic fluxes and boosting productivities (Wilson *et al.*, 2016).

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fixation. In particular, we discuss efforts aiming to improve carboxylation by Rubisco, optimize expression levels of enzymes within the Calvin cycle, introduce carbon-concentrating mechanisms (CCMs), and rewire photorespiration. We claim that multiple complementary strategies are paving the way towards substantial yield increases that are not feasible using conservative selective breeding techniques.

Engineering Rubisco

Rubisco, the key enzyme of the Calvin cycle, is probably the most abundant protein in the biosphere (Ellis, 1979; Raven, 2013), and is responsible for assimilating the vast majority of inorganic carbon (Raven, 2009). The enzyme catalyzes the condensation of ribulose 1,5-bisphosphate (RuBP) with CO_2 to give two molecules of glycerate 3-phosphate (G3P). Despite its key biochemical role, Rubisco is considerably slower than most enzymes in central metabolism (Bar-Even *et al.*, 2011). Moreover, Rubisco is not completely specific to CO_2 and also accepts O_2, leading to the formation of 2-phosphoglycolate (2PG) that needs to be reassimilated. In the C_3 model plant *Arabidopsis thaliana*, the carboxylation to oxygenation ratio was measured to be as low as 2.3:1 under high light conditions (Ma *et al.*, 2014). Suppressing oxygenation reactions by cultivating plants at elevated CO_2 concentrations has repeatedly been shown to increase productivity. For example, a meta-analysis of 70 studies showed that rice yields increased by 23% when CO_2 concentrations were raised to 627 ppm (Ainsworth, 2008). These results indicate that engineering Rubisco for higher CO_2 specificity could substantially boost yield.

Approaches to improve Rubisco catalysis by random or site-directed mutagenesis have generally failed to yield substantial kinetic enhancements (Somerville and Ogren, 1982; Spreitzer *et al.*, 2005; Whitney *et al.*, 2011; Wilson *et al.*, 2016). Comparisons between Rubisco variants from a range of different organisms have revealed a trade-off between CO_2 specificity and carboxylation velocity (Tcherkez *et al.*, 2006; Savir *et al.*, 2010; Galmés *et al.*, 2014), although several recent studies challenge this finding (Young *et al.*, 2016; Cummins *et al.*, 2018). Considering this trade-off, it actually seems that most Rubisco variants are well adapted to their intracellular environment. Still, as ambient CO_2 concentrations are changing at a rate faster than plants can adapt to them, it was suggested that replacing plant Rubisco with another variant could boost carbon fixation by up to 25% (Zhu *et al.*, 2004; Orr *et al.*, 2016). Substituting one Rubisco variant with another is undoubtedly a challenging task, but has already been demonstrated using homodimeric Rubisco from the α-proteobacterium *Rhodospirillum rubrum* (Whitney and Andrews, 2001) and, more recently, using a fast hexadecameric Rubisco from *Synechococcus elongatus* (Lin *et al.*, 2014; Occhialini *et al.*, 2016). Co-expression of supporting chaperones, including the appropriate accumulation factors, can assist in producing an active Rubisco recombinantly, and can further facilitate efforts to enhance the kinetics of this key enzyme via mutagenesis (Aigner *et al.*, 2017).

Carbon fixation via Rubisco can potentially be improved by means other than direct engineering of its catalytic parameters. The addition of a CO_2 molecule to an active site lysine, namely carbamylation, is a prerequisite for Rubisco activity (Lorimer and Miziorko, 1980), but can be hindered by the premature binding of RuBP or other sugar phosphates (Portis, 2003; Parry *et al.*, 2008). The catalytic chaperone Rubisco activase (Rca) removes the sugar phosphate inhibitors from an inactive uncarbamylated enzyme or an inhibited carbamylated Rubisco (Portis, 2003). As the thermal instability of Rca was shown to constrain carbon fixation under moderate heat stress (Salvucci *et al.*, 2004), it has become an attractive target for engineering towards enhanced photosynthesis. For example, by increasing the thermostability of Rca in *A. thaliana*, improved photosynthesis and growth rate were demonstrated under a moderate heat stress (Kurek *et al.*, 2007; Kumar *et al.*, 2009). Similarly, overexpression of maize Rca in rice led to a higher activation state of Rubisco in low light and a faster response of photosynthesis when light intensities increased (Yamori *et al.*, 2012).

Optimizing expression of Calvin cycle enzymes

G3P produced by Rubisco needs to be metabolized by nine enzymes of the Calvin cycle to regenerate RuBP. This regeneration process, whose rate has to match that of Rubisco, is known to limit the carbon fixation rate under certain conditions. Computational models have suggested that the natural distribution of enzymes within the Calvin cycle is not optimal and could limit photosynthesis (Zhu *et al.*, 2007). Specifically, it was predicted that higher levels of sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphate aldolase, as well as enzymes linked to sink capacity, could support higher productivity.

Unsurprising, under elevated CO_2 concentrations, the rate of Rubisco becomes less limiting and carbon fixation is mostly constrained by RuBP regeneration. For example, studies of *Nicotiana tabacum* at 930 ppm CO_2 showed that reducing Rubisco levels by 30–50% did not inhibit growth (Masle *et al.*, 1993). Similar results were obtained in rice plants in which Rubisco levels were reduced by 65% at 1000 ppm CO_2. On the other hand, overexpression of sedoheptulose-1,7-bisphosphatase in *N. tabacum* at 585 ppm CO_2 resulted in a higher carbon fixation rate (Rosenthal *et al.*, 2011). Similarly, at 700 ppm, increased levels of fructose-1,6-bisphosphate aldolase in *N. tabacum* led to increased biomass (Uematsu *et al.*, 2012).

Even at ambient CO_2 concentration, overexpression of limiting enzymes of the Calvin cycle was shown to boost carbon fixation. In *N. tabacum*, overexpression of sedoheptulose-1,7-bisphosphatase (Lefebvre *et al.*, 2005) and fructose-1,6-bisphosphatase (Miyagawa *et al.*, 2001) increased photosynthetic rates and biomass. Similarly, the co-overexpression of sedoheptulose-1,7-bisphosphatase and fructose-1,6-phosphate aldolase enhanced photosynthesis and yield (Simkin *et al.*, 2015).

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Establishing carbon-concentrating mechanisms

To mitigate the problem of oxygenation, and further enable the use of faster (and less specific) Rubisco, multiple organisms have developed CCMs to concentrate CO_2 at the site of Rubisco. As C_3 plants lack CCMs, it was proposed to introduce them to increase photosynthetic efficiency. Two main approaches are actively pursued: (i) introduction of biophysical CCMs from cyanobacteria and green algae (Long *et al.*, 2016; Rae *et al.*, 2017); and (ii) introduction of C_4 anatomy and metabolism (Hibberd *et al.*, 2008; Schuler *et al.*, 2016).

Biophysical CCMs are found in cyanobacteria (Kupriyanova *et al.*, 2013) and in green algae such as *Chlamydomonas reinhardtii* (Mackinder, 2018). In such CCMs, bicarbonate is actively transported into the cytosol in which carbonic anhydrase is lacking. From there, bicarbonate is further transported into specialized compartments packed with Rubisco—carboxysomes in cyanobacteria and pyrenoids in green algae—where it is dehydrated to CO_2 by carbonic anhydrase. It is thought that both carboxysomes and pyrenoids present a diffusion barrier for CO_2 and O_2, keeping the former molecule in and the latter molecule out, and thus enhancing carboxylation and suppressing oxygenation (Mangan *et al.*, 2016).

Establishing biophysical CCM in plants is a challenging task that first requires the expression and correct localization of inorganic carbon transporters. It was suggested that the transporters themselves could increase the carbon fixation rate albeit to a limited extent (McGrath and Long, 2014; Yin and Struik, 2017). Indeed, overexpression of the putative inorganic carbon transporter from cyanobacteria, ictB, in *A. thaliana*, tobacco, rice, and soybean was reported to increase the photosynthetic rate and biomass (Lieman-Hurwitz *et al.*, 2003, 2005; Yang *et al.*, 2008; Simkin *et al.*, 2015; Hay *et al.*, 2017). In contrast, expression of other transporters from cyanobacteria or *C. reinhardtii* did not increase yield or improve growth, despite correct localization within the plant cells (Atkinson *et al.*, 2016; Rolland *et al.*, 2016; Uehara *et al.*, 2016). Optimizing transporter activity is therefore still an open challenge that needs to be resolved before commencing with the next step: assembly of Rubisco-containing compartments. The establishment of these sophisticated structures would enable a further increase in CO_2 concentration at the site of Rubisco and could therefore substantially enhance carbon fixation. Recently, simplified carboxysome structures were introduced into the chloroplasts of *N. tabacum* (Long *et al.*, 2018). Yet, these are expected to enhance photosynthesis only after combination with functional inorganic carbon transporters (McGrath and Long, 2014).

Engineering C_4 metabolism

As an alternative to biophysical CCMs, ongoing research is dedicated to introducing C_4 metabolism into C_3 plants (Schuler *et al.*, 2016). C_4 metabolism utilizes the most efficient carbon fixation enzyme, phosphoenolpyruvate (PEP) carboxylase, to capture inorganic carbon temporarily, which is then transported to the vicinity of Rubisco (Jenkins *et al.*, 1989). Specifically, PEP carboxylase in the mesophyll cells 'borrows' PEP and converts it to oxaloacetate, which is further metabolized to malate or aspartate. These C_4 acids are transported to the bundle sheath cells and decarboxylated to release CO_2 next to Rubisco, which is mainly localized in these cells. Pyruvate, the product of this decarboxylation, is then transported back to the mesophyll cells to regenerate PEP. Hence, the entire C_4 cycle, which depends on a special anatomy termed 'Kranz anatomy' (mesophyll cells surrounding bundle sheath cells), can be regarded as a sophisticated CO_2 pump that results in an ~10 times higher concentration of inorganic carbon in the vicinity of Rubisco (Jenkins *et al.*, 1989).

Engineering C_4 photosynthesis in C_3 plants has been outlined as a stepwise process (Schuler *et al.*, 2016) that includes alteration of plant tissue anatomy, establishment of bundle sheath morphology, as well as ensuring a cell type-specific enzyme expression. Although challenging, engineering a C_3 plant to have C_4 metabolism seems to be a feasible goal as it is known to have emerged independently at least 66 times in different phylogentic backgrounds (Sage *et al.*, 2012). Importantly, C_3 plants already harbor the main enzymes of C_4 metabolism, such as PEP carboxylase (Aubry *et al.*, 2011), and are known to shuttle carbon from the vasculature into the surrounding cells in a way similar to C_4 plants (Hibberd and Quick, 2002; Brown *et al.*, 2010). This provides a solid basis to replicate the emergence of C_4 metabolism by direct engineering.

Nevertheless, despite international efforts, a synthetic C_4 plant has yet to be reported. Following Richard Feynman's famous quote 'What I cannot create, I do not understand', it seems that incomplete understanding of C_4 metabolism hampers its engineering. Specifically, the metabolic shuttling of intermediates between mesophyll and bundle sheath cells and the factors necessary to create Kranz anatomy are still not fully clear and need to be elucidated (Schuler *et al.*, 2016).

It might not be necessary to establish a complete C_4 metabolism in order to improve carbon fixation. It was recently suggested that engineering a C_3–C_4 intermediate metabolism could enhance productivity (Schlüter and Weber, 2016). For example, in C_3–C_4 intermediate type I plants, photorespiratory glycine is transported from the mesophyll cells to the bundle sheath cells for decarboxylation. In the bundle sheath cells, the mitochondria are closely associated with the chloroplast, thereby enhancing re-assimilation of released CO_2 by nearby Rubisco (Monson and Edwards, 1984; Rawsthorne *et al.*, 1988). Establishing this intermediary metabolism within C_3 plants, besides being useful on its own to boost carbon fixation, would provide a milestone towards further engineering of complete C_4 metabolism.

An interesting alternative engineering target is crassulacean acid metabolism (CAM). While C_4 metabolism increases CO_2 concentration in the vicinity of Rubisco via spatial organization, CAM accomplishes the same goal via temporal decoupling. Specifically, inorganic carbon is temporarily fixed by the highly efficient PEP carboxylase during the night, when the stomata are open and CO_2 can freely enter the cell. Malate, the indirect product of the carboxylation, is stored within the vacuole. During the day, when the stomata are closed, malate is decarboxylated, releasing CO_2 and maintaining its

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high concentration for subsequent fixation by Rubisco and the Calvin cycle. Besides increasing CO_2 concentration in the vicinity of Rubisco, CAM reduces water evaporation and increase water use efficiency by 20–80% (Borland *et al.*, 2009), making CAM plants highly suitable for arid climates. Similarly to C_4 metabolism, CAM has arisen multiple times in a taxonomically diverse range of plants, indicating that its necessary components exist in C_3 plants which could potentially be engineered towards this unique carbon metabolism (DePaoli *et al.*, 2014). Furthermore, the existence of C_3–CAM intermediate species and plants that switch between both metabolic modes further supports the potential of engineering C_3 metabolism towards CAM (Borland *et al.*, 2011). Such engineering would require precise control of the activity of key enzymes (e.g. PEP carboxylase, malic enzyme, and Rubisco), stomatal conductance, and intracellular transport (e.g. to and from the vacuole) (Borland *et al.*, 2014; DePaoli *et al.*, 2014; Yang *et al.*, 2015).

Rewiring photorespiration

2PG, the product of Rubisco's oxygenation activity, is recycled to the Calvin cycle in a process termed photorespiration. This rather long pathway requires the shuttling of metabolites across multiple organelles and is considered inefficient as it dissipates energy by releasing ammonia and using oxygen as an electron acceptor. Moreover, photorespiration releases one CO_2 molecule in the recycling of two 2PG molecules and hence directly counteracts carbon fixation by the Calvin cycle. The inefficiencies associated with the recycling of 2PG cannot be prevented by simply blocking photorespiration, as this pathway plays an essential role in plant metabolism (Somerville and Ogren, 1979) and reduction of its flux was shown to affect photosynthesis negatively (Servaites and Ogren, 1977; Wingler *et al.*, 1997; Heineke *et al.*, 2001). One explanation for this lies in the inhibitory effects exerted by several photorespiratory intermediates. For example, 2PG was shown to inhibit triosephosphate isomerase and sedoheptulose-1,7-bisphosphate phosphatase (Anderson, 1971; Flügel *et al.*, 2017), glyoxylate impairs Rubisco activation (Chastain and Ogren, 1989; Campbell and Ogren, 1990; Hausler *et al.*, 1996; Savir *et al.*, 2010), and glycine interferes with Mg^{2+} availability (Eisenhut *et al.*, 2007). Based on these observations, it was suggested that increased photorespiratory flux could prevent the accumulation of inhibitory intermediates and enhance photosynthesis; indeed, this was demonstrated upon overexpression of components of the glycine cleavage system in *A. thaliana* (Timm *et al.*, 2012, 2015) and in *N. tabacum* (López-Calcagno *et al.*, 2019).

While photorespiration cannot be avoided, it might be possible to replace the natural pathway with more efficient alternatives. The first bypass suggested in this regard was inspired by cyanobacterial photorespiration (Eisenhut *et al.*, 2006), where glyoxylate is condensed and reduced to directly generate the key photorespiratory intermediate glycerate. This pathway was implemented in *A. thaliana* (Kebeish *et al.*, 2007) and later in *Camelina sativa* (Dalal *et al.*, 2015) using glycolate dehydrogenase, glyoxylate carboxyligase, and tartronic semialdehyde reductase from *Escherichia coli*. In both cases, this metabolic bypass, dissipating less energy and shifting CO_2 release from the mitochondria to the chloroplast, was shown to increase photosynthesis and biomass.

However, it was shown that expression of only the first enzyme of the pathway, glycolate dehydrogenase, suffices to enhance photosynthesis. Supporting this, chloroplastic expression of glycolate dehydrogenase in *Solanum tuberosum* induced a 2.3-fold increase in tuber yield (Nölke *et al.*, 2014). This suggests that the benefits of the glycerate pathway might not stem from more efficient recycling of 2PG but rather from oxidation of glycolate to glyoxylate. Indeed, incubation with glyoxylate was shown to increase carbon fixation—potentially due to suppression of Rubisco oxygenation—in both tobacco leaf disks (Oliver and Zelitch, 1977) and soybean mesophyll cells (Oliver, 1980).

Another photorespiratory bypass, which was reported to increase biomass and photosynthesis, involves the complete oxidation of 2PG to CO_2 via a catabolic pathway that consists of glycolate dehydrogenase, malate synthase, malic enzyme, and pyruvate dehydrogenase (Maier *et al.*, 2012). A recent study showed that a variant of this bypass can increase the productivity of tobacco plants in the field by >40% (South *et al.*, 2019). However, the mechanism that underlies the beneficial effects of the pathway remains vague as a theoretical model predicts a negative effect when 2PG is completely oxidized (Xin *et al.*, 2015).

Carbon-conserving photorespiration

As the main problem associated with photorespiration is (arguably) the release of CO_2, bypasses that do not lead to the loss of carbon could dramatically boost carbon fixation. Several synthetic carbon-conserving bypasses have been suggested. In the *de novo* 2PG salvage pathway (Ort *et al.*, 2015), 2PG was suggested to be reduced to 2-phosphoglycolaldehyde, which is subsequently condensed with dihydroxyacetone phosphate to give xylulose bisphosphate. This intermediate is then dephosphorylated to xylulose 5-phosphate, a Calvin cycle metabolite. The main challenges of this proposed bypass is the reversibility of most of its reactions (resulting in a low driving force), the low concentration of 2PG, and the inhibitory effect of xylulose bisphosphate (Yokota, 1991; Zhu and Jensen, 1991; Parry *et al.*, 2008).

Recently, a systematic analysis identified multiple synthetic routes that can bypass photorespiration without the release of CO_2. Several of these pathways involve the reduction of glycolate (the concentration of which is considerably higher than that of 2PG) to glycolaldehyde, which then undergoes an aldol condensation with a phosphosugar from the Calvin cycle to generate a longer chain phosphosugar that is reintegrated into the Calvin cycle (Bar-Even, 2018; Trudeau *et al.*, 2018). A computational model indicated that these pathways can boost photosynthesis under all physiologically relevant irradiation and intracellular CO_2 levels.

The operation of these carbon-conserving bypass routes depends on the conversion of glycolate to glycolaldehyde, but

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this activity is not supported by any known enzyme. To establish this activity, two enzymes were engineered (Trudeau *et al.*, 2018). First, acetyl-CoA synthetase from *E. coli* was engineered to accept glycolate, thus generating glycolyl-CoA. Next, propionyl-CoA reductase from *Rhodopseudomonas palustris* was engineered to accept glycolyl-CoA, reducing it to glycolaldehyde. The cofactor specificity of this latter enzyme was switched, such that it could use NADPH—the photosynthetic electron carrier—as an electron donor. The two engineered enzymes were combined, in a test tube, with fructose 6-phosphate aldolase (condensing glycolaldehyde with glyceraldehyde 3-phosphate to generate arabinose 5-phosphate), arabinose 5-phosphate isomerase, and phosphoribulokinase. Upon addition of glycolate and glyceraldehyde 3-phosphate, NADPH and ATP were consumed and RuBP was found to accumulate (Trudeau *et al.*, 2018), demonstrating the *in vitro* activity of an alternative photorespiration route that does not release CO_2.

It was further proposed to go beyond carbon conservation, and engineer a photorespiration bypass that fixes CO_2 and thus directly supports the activity of the Calvin cycle. One such carbon-positive bypass was inspired by the 3-hydroxypropionate bi-cycle (Shih *et al.*, 2014). Here, glycolate is oxidized to glyoxylate, which is then metabolized and further carboxylated to pyruvate. Towards the implementation of this bypass, six non-native genes from *C. aurantiacus* were expressed in cyanobacteria, but no distinct growth phenotype was evident.

In another study, glycolate was not recycled to the Calvin cycle but instead was metabolized to acetyl-CoA via the synthetic malyl-CoA–glycerate pathway (Yu *et al.*, 2018). This pathway can further be used to generate acetyl-CoA from photosynthetic C_3 sugars via an additional CO_2-fixing step, thereby bypassing CO_2 release by pyruvate dehydrogenase. In cyanobacteria, the pathway facilitated a 2-fold increase in bicarbonate assimilation.

Conclusions

The increasing numbers of studies demonstrating improved photosynthesis and growth by engineering different components of the light-dependent and independent reactions indicates that we are on the right path. Yet, many challenges are ahead of us. Besides the technical difficulties, which we did not discuss here and for which we refer the reader to other reviews (DePaoli *et al.*, 2014; Liu and Stewart, 2015; Boehm and Bock, 2018; Piatek *et al.*, 2018;Vazquez-Vilar *et al.*, 2018), there is one key barrier that is worth elaborating on, which is system complexity. Complex systems are notoriously difficult to engineer as the effect of even small changes can have substantial effects that cannot be easily predicted. While mathematical models can help deal with such complexity, the lack of knowledge regarding many of the involved components commonly hinders accurate prediction. Plant carbon metabolism provides an excellent example of a complex system, the response of which to changes is hard to foretell. Previous attempts to engineer carbon fixation demonstrate this vividly. Perhaps the best example is the engineering of photorespiration bypass routes as described above. While few bypasses were already shown to enhance photosynthesis (most notably in recent field experiments; South *et al.*, 2019), the cause of this effect is probably different from that originally suggested. Unraveling this mystery would require deep understanding of the intricate interplay between all system components, a task which we have yet to fully achieve.

Moreover, while some engineering efforts show only minor benefits in isolation, the key for future improvements lies in the correct combination of multiple strategies. Indeed, first examples of beneficial cumulative effects have been reported (Simkin *et al.*, 2015). It is further clear that not all strategies can be implemented with similar ease. Overexpressing a Calvin cycle enzyme, for example, is considerably easier than rerouting photorespiration via a synthetic pathway that does not release CO_2. It is therefore important to choose targets carefully for the near and medium future and progress in a way that ensures intermediate gains. For example, establishing a C_3–C_4 intermediate metabolism not only provides a solid basis for further engineering of a complete C_4 metabolism, but is also expected to boost carbon fixation by itself. Once we gain the required proficiency in rewiring plant central metabolism, we can aim at even bigger targets, for example replacing Rubisco with a set of enzymes, each responsible for a different catalytic step (Bar-Even, 2018), or replacing the Calvin cycle with a synthetic carbon fixation pathway (Schwander *et al.*, 2016).

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