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Energetic considerations for engineering novel biochemistries in photosynthetic organisms

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Humans have been harnessing biology to make valuable compounds for generations. From beer and biofuels to pharmaceuticals, biology provides an efficient alternative to industrial processes. With the continuing advancement of molecular tools to genetically modify organisms, biotechnology is poised to solve urgent global problems related to environment, increasing population, and public health. However, the light dependent reactions of photosynthesis are constrained to produce a fixed stoichiometry of ATP and reducing equivalents that may not match the newly introduced synthetic metabolism, leading to inefficiency or damage. While photosynthetic organisms have evolved several ways to modify the ATP/NADPH output from their thylakoid electron transport chain, it is unknown if the native energy balancing mechanisms grant enough flexibility to match the demands of the synthetic metabolism. In this review we discuss the role of photosynthesis in the biotech industry, and the energetic considerations of using photosynthesis to power synthetic biology.

KEYWORDS

photosynthesis, chloroplast bioenergetics, synthetic biology, chloroplast biotechnology, bioenergetics

Introduction

Plants and algae are attractive platforms for bioengineering pathways for valuable metabolic products because they use freely available solar energy to sustainably power synthetic biology. To make best use of this energy, the localization of engineered synthetic metabolism should be carefully considered relative to where the metabolic precursors are

Abbreviations: ATP, adenosine triphosphate; *cyt bf*, cytochrome *bf* complex; CO₂, carbon dioxide; CBB, Calvin, Benson, Bassham cycle; CF_o, membrane fraction of ATP synthase; CF₁, soluble fraction of ATP synthase; ETC, electron transport chain; FBPase, fructose 1,6 biphosphatase; NADPH, nicotinamide adenine dinucleotide phosphate; O₂, oxygen; O₂⁻ superoxide; PCON, photosynthetic control; PG, 2-phosphoglycolate; PGA, 3-phosphoglycerate; PQ, plastoquinone; PQH₂, plastoquinol; PR, photorespiration; PSI, photosystem I; PSII, photosystem II; q_E, exciton quenching; ROS, reactive oxygen species; rubisco, ribulose 1,5 biphosphate carboxylase/oxygenase; RUBP, ribulose 1,5 biphosphate; Δ*p*, protonmotive force; Δ*p*H, fraction of Δ*p* stored as pH; Δψ, membrane potential since the light reactions.

produced. In the cytosol and other non-energetic subcellular compartments, the metabolic precursors ATP and reducing equivalents (e.g., NADPH and ferredoxin) required for synthetic metabolism must be supplied by other metabolism or transported from the energetic compartments (i.e., mitochondria or chloroplast). For synthetic metabolism with large demands of ATP and/or reducing equivalents, localization to the chloroplast allows the synthetic metabolism direct access to ATP and reducing equivalents generated by the light dependent reactions in the chloroplast. Traditional bioengineering approaches usually focus on engineering carbon flux but seldom think about the types of energy fluxes required to power synthetic metabolism. In point of fact, one should not consider the light reactions as an independent supplier of ATP or NADPH because their production is intrinsically linked. The light dependent reactions in the thylakoid electron transport chain are constrained to produce a fixed stoichiometry of ATP and reducing equivalents that may not match the newly introduced synthetic metabolism, leading to inefficiency or damage. While photosynthetic organisms have evolved several ways to modify the ATP/NADPH output from their thylakoid electron transport chain, it is unknown if the native energy balancing mechanisms grant enough flexibility to match the demands of the synthetic metabolism. In other words, is native photosynthetic energy production able to support maximal yields of bioproduction from engineered photosynthetic organisms, or will photosynthesis itself need to be re-engineered?

In this review, we discuss bioengineering from an energetics perspective and where this may present a hurdle for using photosynthetic organisms for producing bioproducts of interest.

The chloroplast as a factory

The chloroplast sets photosynthetic eukaryotes apart from other organisms for bioengineering. The chloroplast is the solar cell of the plant, utilizing light energy to generate ATP and reducing power for use in downstream metabolism. This makes these organisms attractive as a bioengineering platform for synthetic metabolism for valuable products because growth and yield is sustained by light; a cheap, abundantly available natural resource. Additionally, the chloroplast itself is the site of many metabolic precursors that can be harnessed for synthetic metabolism (e.g., ATP, reducing equivalents, sugar phosphates, fatty acids, isoprenoids, and amino acids). Enzymes in a desired synthetic pathway that utilize these precursors may be expressed in the nucleus of eukaryotes and targeted to the chloroplast, or integrated into the chloroplast genome, with both approaches having their place in bioengineering.

There are tradeoffs that must be considered when choosing to express transgenes from either the nucleus or the chloroplast. Most nuclear transformation techniques result in random transgene integration and a variety of transgene expression levels. Chloroplast transformation, however, allows for directed integration of transgenes and every chloroplast transformant is genetically identical, resulting in reproducible expression (Bock, 2001; Bock, 2015; Ahmad et al., 2016). Nuclear transformation may lead to undesirable transgene silencing in later generations (De Wilde et al., 2000). While there are approaches to avoid silencing and increase transgene expression in the nucleus, chloroplast transgenes are not subject to silencing,

resulting in continued high expression generations later. Therefore, chloroplast transformation may be an approach to avoid the silencing problem completely if the transgene product is ultimately desired in the chloroplast (Bock, 2001).

There are vastly more species with transformable nuclei than chloroplasts, but the number of valuable species amenable to chloroplast transformation is constantly increasing, and established protocols exist for many model organisms and economically important crops (e.g., soybean) (Boynton et al., 1988; Svab and Maliga, 1993; Ahmad et al., 2016; Nimmo et al., 2019; Kaushal et al., 2020; Ruf et al., 2021). Additionally, the molecular toolbox for control of chloroplast transgene expression is continually growing, offering synthetic biologists a variety of options to fine-tune expression levels and timing (Bock, 2022). These molecular transformation tools allow us to utilize the chloroplast as a molecular factory, using the raw materials of the chloroplast to supply the production of valuable molecules. However, synthetic metabolism will be in competition for energy and carbon flux within the native chloroplast metabolism. Strategies for optimizing efficiency and yield of our synthetic metabolism will need to account for the demands of the chloroplast's native metabolism, and how those demands are currently supplied.

The native chloroplast energy demand

Natively, the largest sinks for the ATP and reducing equivalents in the chloroplast are the Calvin-Benson-Bassham (CBB) cycle and photorespiration (Noctor and Foyer, 1998; Walker et al., 2020). The CBB cycle begins when ribulose 1,5 bisphosphate carboxylase/oxygenase (rubisco) catalyzes reaction with CO₂ (carboxylation reaction) and ribulose 1,5 bisphosphate (RuBP), forming two 3-carbon molecules of 3-phosphoglycerate (PGA). PGA is converted to 1,3-bisphosphoglycerate and then to glyceraldehyde-3-phosphate, a portion of which is used in the construction of glucose and other sugars with the remainder feeding back into the regeneration of RuBP, consuming ATP and NADPH in a stoichiometry of 3:2. Alternatively, when rubisco catalyzes reaction with O₂ (oxygenase reaction), one molecule of PGA and one molecule of 2-phosphoglycolate (PG) are formed (Ogren, 1984; Foyer et al., 2009). The PG is inhibitory and must be detoxified in a series of enzymatic reactions that occur across three organelles in a process termed photorespiration (PR) which loses CO₂ in the process. Energetically, the whole-cell demand ratio for ATP : NADPH for photorespiration is 1.75 (Edwards and Walker, 2003; Walker et al., 2020). Regardless of the assumptions used for determining the whole-cell energy demand of photorespiration, the conversion of glycerate to PGA requires an ATP within the chloroplast. This increased consumption in the chloroplast means that in the absence of ATP or NADPH transport, photorespiration increases the relative ATP demand within the chloroplast above that of the CBB cycle.

Other pathways in the chloroplast have higher relative reductant demands than the CBB cycle and PR. Both synthesis of fatty acids and desaturation of lipids require reducing equivalents in the form of NADPH, making the demand for reducing equivalents from lipid biosynthesis larger than that of the CBB cycle (Ohlrogge and Browse, 1995). Additionally, the methylerythritol 4-phosphate portion of the

isoprenoid biosynthesis pathway in the chloroplast also has a larger demand for reducing equivalents relative to ATP (Zhao et al., 2013). While under normal conditions these metabolic fluxes are much smaller than that of the CBB cycle and PR, metabolic engineering with the goal of increasing flux through these pathways would increase the relative reductant demand within the chloroplast.

The chloroplast energy supply

Since the light reactions of photosynthesis provide all the energy for native and synthetic metabolism in autotrophs, it is essential to

understand how light energy is converted to usable chemical energy and what constrains production of this chemical energy. It is also important to understand the constraints to this energy production for optimal bioengineering of reliant synthetic metabolism.

The light reactions of photosynthesis use light excitation to drive a series of redox reactions along an electron transport chain (ETC) to shuttle protons and electrons across the thylakoid membrane generating ATP and reducing power for downstream metabolism [Figure 1, (Hill and Bendall, 1960; Arnon, 1971; Mitchell, 1975; Blankenship, 2002; Mitchell, 2011)]. The coupling of these proton and electron circuits determine the ATP/NADPH produced from the system. When light hits the photosystems, the excited reaction centers

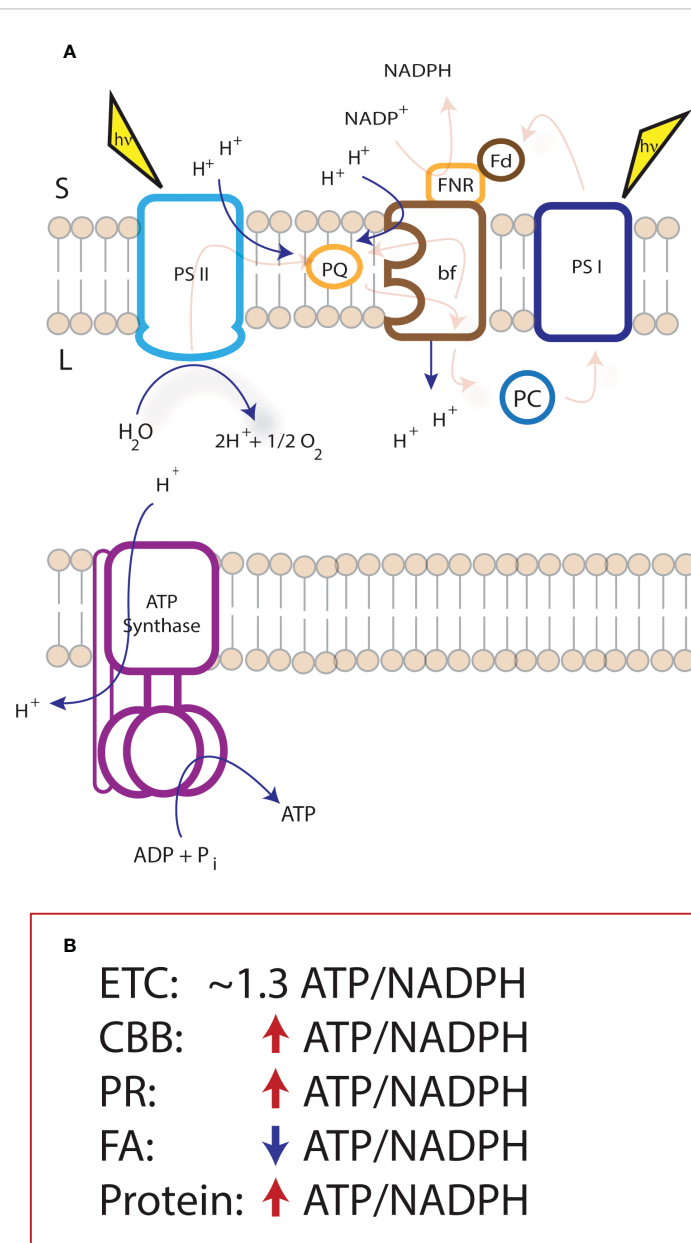


FIGURE 1 Supply and demand of NADPH and ATP within the chloroplast. The proton to electron stoichiometry of proton coupled electron transfer of the light reactions of photosynthesis (as pictured in panel A) is fixed at $3H^+/e^-$ and, together with the c-ring structure of the ATP synthase, constrains the electron transport chain production to ~1.3 ATP/NADPH. The light reactions supply multiple metabolic sinks within the chloroplast that do not match the ATP/NADPH resulting in an ATP deficit or surplus. (B) indicates pathways of interest for metabolic engineering, and their difference in demand (within the chloroplast) from the ATP/NADPH production of the electron transport chain. CBB, Calvin-Benson-Bassham cycle; PR, photorespiration; FA, Fatty acid biosynthesis; Protein, Protein synthesis.

lose an electron. At photosystem II (PSII), electron loss at P680 supplies the oxidative force ($P680^+$) to release electrons from water, reducing $P680^+$ and releasing protons into the lumen. Electron transfer falls downhill in energy to plastoquinone (PQ), the reduction of which leads to the uptake of protons from the stroma. These protons are released into the lumen when plastoquinol (PQH_2) is oxidized at the cytochrome *bf* (cyt *bf*) complex. Electron transfer continues through plastocyanin to reduce the oxidized photosystem I (PSI) reaction center, $P700^+$. When light energy oxidizes P700, the electron travels through PSI to the soluble electron carrier ferredoxin which can provide reducing power to metabolism, redox regulation, or be used to reduce $NADP^+$ to NADPH. NADPH is used as a two-electron donor to a wide variety of metabolism in the chloroplast as discussed above.

The proton-coupled electron transfer reactions at PSII and cyt *bf* lead to proton accumulation in the thylakoid lumen, generating an electrochemical proton gradient used for ATP synthesis at the ATP synthase. At PSII, water splitting results in the release of one proton into the lumen per electron entering the electron transport chain. When PQH_2 is oxidized at the cyt *bf* complex, electrons enter a bifurcated pathway, one of which is eventually passed to PSI, and another is passed to another site on cyt *bf* to half reduce another PQ [i.e., the Q-cycle, (Mitchell, 1975)]. Therefore, for each electron passed from cyt *bf* to PSI, two protons are deposited into the lumen. Across the membrane, the difference in charge imposed by this proton gradient comprises a membrane potential ($\Delta\psi$) and the difference in pH generates a pH gradient (ΔpH) (Cruz et al., 2001). Together, these comprise the protonmotive force (Δp) for ATP synthesis *via* the ATP synthase (Nicholls and Ferguson, 2002; Kramer et al., 2003). The chloroplast ATP synthase is comprised of two complexes, the CF_o in the membrane, and the CF_1 in the stroma (Hahn et al., 2018). The CF_o contains a 14-subunit c-ring and a proton half channel, releasing 14 protons per one full turn (Seelert et al., 2003). This turn moves the g-subunit, which in turn drives the conformational change in the CF_1 domain, comprised of 3 heterodimers, and responsible for ATP synthesis. Therefore, 14 protons are required to synthesize 3 ATP, and $NADP^+$ reduction to NADPH requires 2 electrons from the electron transport chain.

From the H^+/e^- at PSII and cyt *bf*, there is a total stoichiometry of the ETC of $3H^+/e^-$, which results in an ATP/NADPH of ~ 1.3 , in a perfectly coupled system. This means that, the largest sinks for ATP and NADPH in the chloroplast (i.e., CBB cycle and photorespiration) are running at an ATP deficit. The ATP/NADPH production stoichiometry is flexible to a certain extent by the activation of, or leak of electrons into, alternative electron transfer pathways [discussed in depth elsewhere, e.g., (Kramer and Evans, 2011; Strand et al., 2016; Alric and Johnson, 2017; Walker et al., 2020)]. The most studied alternative pathways lead to an increase in the relative production of ATP from the ETC. This is likely due to the largest sinks in the chloroplast imposing an ATP deficit on the ETC (discussed below), however, with the introduction of synthetic biology into the chloroplast, we must consider the impact of new or altered metabolic sinks on chloroplast energetics.

Consequences of energetic imbalance

To optimize efficiency and avoid damage, the chloroplast must balance the supply and demand of ATP and reducing equivalents.

Energy demand from native metabolism in the chloroplast does not match the calculated output of the light reactions, therefore it is likely that throughout the organism's life cycle it experiences periods of ATP deficits or surplus. What problem do these conditions pose for the plant?

When ATP demand relative to NADPH exceeds the supply of the light reactions (ATP deficit, Figure 2A), sinks for reducing power may be closed as they do not have ATP as substrate to turnover acceptors. For example, if ATP is limiting to the CBB cycle, 1,3-bisphosphoglycerate is not produced from 3-phosphoglycerate. Since 1,3-bisphosphoglycerate is an electron acceptor, $NADP^+$ is not being regenerated for reduction by PSI. This shifts the $NADPH/NADP^+$ to a more reduced state, lowering the redox potential of the acceptor side of PSI. A more reduced acceptor side of PSI is dangerous because it will increase the likelihood of electrons being passed to oxygen. Reduction of oxygen generates superoxide (O_2^-), a reactive oxygen species that can damage protein and lipid, and must be detoxified [i.e., through the water-water cycle, (Asada, 2000)]. This detoxification itself diverts electrons from NADPH, and activates another electron sink (i.e., cyclic electron flow) to increase ATP/NADPH production (Casano et al., 2001; Strand et al., 2015).

However, if a large enough ATP sink was introduced into the chloroplast, it is not clear if the self-balancing machinery of the chloroplast is robust enough to meet the new demand without side effects like decreased growth. An example of when the self-balancing machinery fails to meet a new metabolic demand may be seen in a mutant of the chloroplast fructose 1,6 bisphosphatase (FBPase) (Livingston et al., 2010). This mutant seemingly survives disruption to the main metabolic sink in the chloroplast by bypassing the FBPase lesion through the glucose-6-phosphate shunt, which has increased relative ATP demand (Sharkey and Weise, 2016). Despite activation of multiple alternative electron transfer pathways to increase ATP supply from the ETC (Livingston et al., 2010; Strand et al., 2015), these plants grow slower and smaller.

This may, in part, be due to the secondary impact cyclic electron flow around PSI (CEF), an alternative electron transport pathway that is increased in the FBPase knockout which may decrease the efficiency of photosynthesis. CEF increases proton translocation into the lumen relative to electron transfer from PSII. Additional proton translocation can increase the Δp in the lumen, activating pH dependent feedback regulation i.e., downregulation of light harvesting (exciton quenching, q_E) and electron transfer at cyt *bf* (photosynthetic control, PCON). In other words, the responses used to mitigate the ATP deficit may lead to total decrease in output of the light reactions. In an industrial process, this would be an undesired side effect limiting the yield of the engineered pathways.

While the chloroplast has many identified routes of alternative electron transfer to deal with an ATP deficit, much less is known about how an ATP surplus (or, alternatively, a reducing equivalent deficit). For those routes that are proposed, there is little evidence that they contribute substantially to chloroplast energy balancing in the light (Flügge et al., 2011; Strand et al., 2017). The prevalence of pathways to mitigate an ATP deficit rather than mitigate an ATP surplus may be due to selective pressure stemming from native metabolism being poised towards an ATP deficit as discussed above (Walker et al., 2020). However, many proposed synthetic biology pathways result in an ATP surplus such as increased lipid production

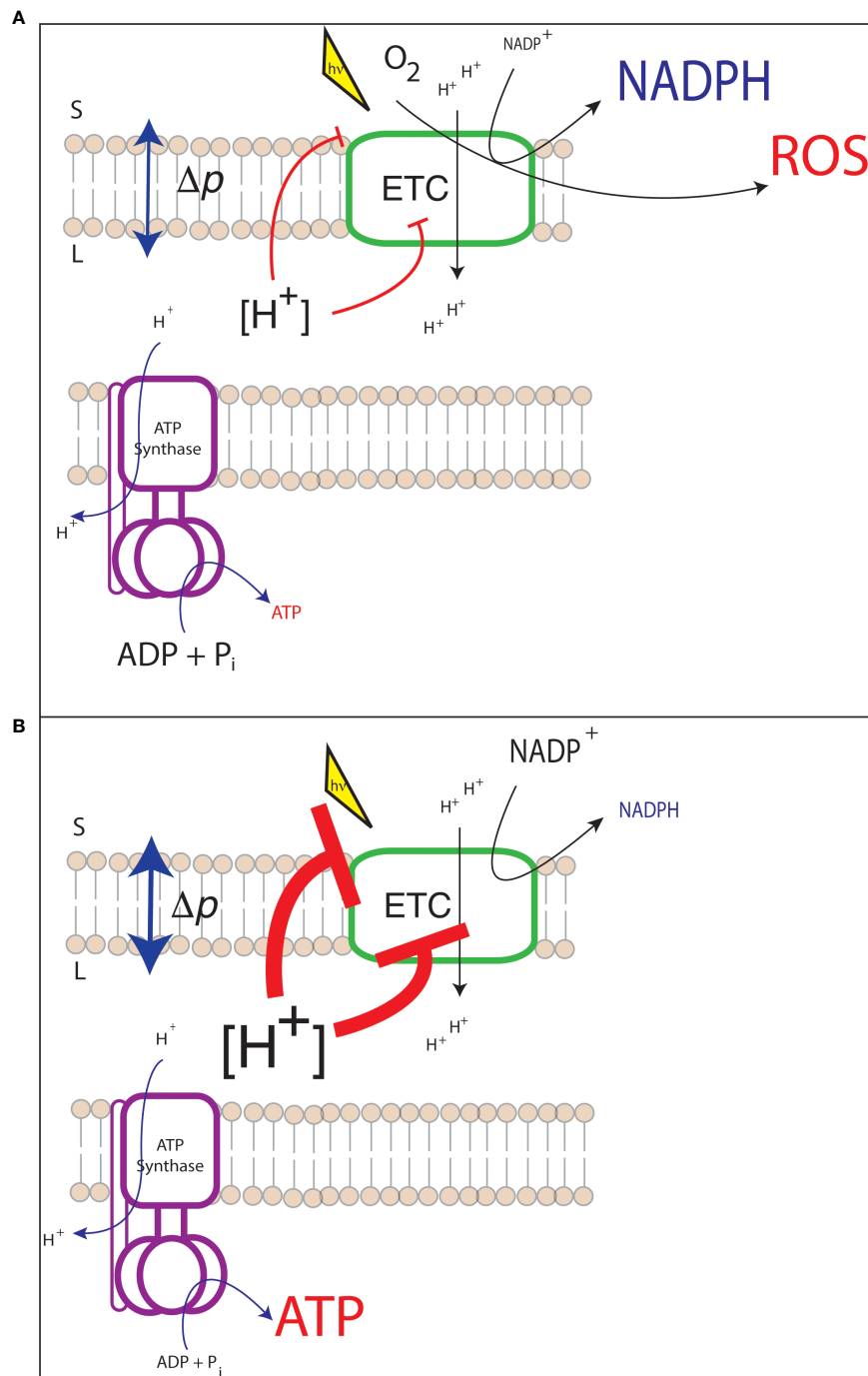


FIGURE 2

Possible consequences of energetic mismatch between the electron transport chain (ETC) and downstream metabolism. (A) An ATP deficit may result in the closure of the acceptor side of PSI and the generation of harmful reactive oxygen species (ROS); (B) An ATP surplus may lead to substrate limitation of ATP synthase and the downregulation of light harvesting and electron transfer resulting in lower photosynthetic efficiency.

in microalgae and photorespiratory bypasses in plants (Miller et al., 2020).

Under an ATP surplus condition the threat of damage to the chloroplast is not a primary concern, instead the efficiency of the light reactions will decrease. This inefficiency would occur through a shift in the $[ATP]/[ADP] [P_i]$ pool which may lead to an increase in Δp required to drive ATP synthesis. All else being equal, increased Δp leads to an activation of q_E and PCON (Figure 2B), decreasing light harvesting and electron transfer rates. In the case of a synthetic pathway with an

increased electron demand, activation of these photoprotective processes would lead to decreased yield of the synthetic pathway. This response to increased electron demand may be a limiting factor for any synthetic pathway with high reductant demand, such as increased lipid production.

The conditions described above are an important consideration for bioengineering pathways relying on output from the light reactions of photosynthesis. This is because final product yield may ultimately be limited by the light reactions if new metabolism exceeds the innate

energetic flexibility of the chloroplast, even if the engineered metabolism yields the expected product to some degree. To increase product yield, it may be enticing to modify the regulatory processes that are activated in response to energetic imbalance. However, feedback regulation that limits light utilization and electron transfer (i.e., q_E and PCON) are important to protect the photosystems from photodamage and in the case of PCON, it is *essential* for plant survival under fluctuating conditions (Müller et al., 2001; Suorsa et al., 2012). This is an important point: energetic limitations can't simply be rectified by eliminating feedback regulation, and instead may require additional engineering to optimize our desired yield.

Case studies in chloroplast energetics

Since the invention of plant transformation, there have been many successful applications of bioengineering metabolism to produce valuable metabolites, either by the introduction of synthetic metabolism or altering the flux through metabolic pathways to favor a specific molecule [reviewed in detail in (O'Neill and Kelly, 2017)]. While there have been many successes without energetic considerations, it may be worthwhile to consider the ATP and NADPH requirements when yield from synthetic pathways is lower than expected. Here we offer examples of synthetic metabolism that, if successfully implemented into a photosynthetic organism would drastically alter the energetic demands in the chloroplast and may need additional engineering to ensure maximal yield of the desired product.

Due to the loss of CO₂ during the detoxification and recycling of PG, photorespiration is thought to be a large inefficiency in C3 plants and is an active area of research for improvement of crop productivity (Fu et al., 2022b). There have been several approaches to decrease the rate of photorespiration, e.g., by introducing a bypass modifying the compartment of CO₂ release (Peterhansel et al., 2010; South et al., 2019), or compartmentalization of rubisco (Lin et al., 2014). However, an alternative approach to decreasing biomass loss due to photorespiration is the complete bypass of rubisco to fix carbon. One approach that has been shown to operate *in vitro* is the so called 'CETCH' cycle (Schwander et al., 2016; Miller et al., 2020), a completely synthetic pathway for CO₂ fixation achieved by combining enzymes from multiple organisms across the tree of life. This approach is interesting due the pathway's increased relative demand in reducing equivalents. The challenges of introducing this cycle into a plant are extensive (e.g., expression, availability of cofactors), but the prospect does introduce an intriguing case study of the need for multiple engineering targets to optimize yield. Since the demand of this cycle for reductant is extremely altered (0.66 ATP/NADPH) from that of native CO₂ fixation (1.5 ATP/NADPH), organisms utilizing the 'CETCH' cycle would certainly require secondary bioengineering to overcome the substantial change in chloroplast energetics for growth to be sustainable.

Another engineering target is in the use of photosynthetic organisms to produce biofuel feedstocks (Blatti et al., 2013). One attractive approach to sustainable biofuel production is the use of lipid rich algae. There has been substantial research effort into both identifying algal species that are suitable for robust lipid production and developing molecular toolkits to allow for their genetic modification (Alishah Aratboni et al., 2019). While there have been some successes in increasing lipid production or shifting lipid production to more valuable lipid species, more effort is needed to

make algae an economically viable biofuel feedstock (Blatti et al., 2013; Alishah Aratboni et al., 2019; Gilmour, 2019; Verma and Kuila, 2020). From an energetic (i.e., ATP : NADPH budget) perspective, increasing relative lipid production has different considerations than increasing overall biomass. The latter may be approached by looking at metabolic bottlenecks, but substantial flux through the fatty acid biosynthetic pathway, with its relatively high NADPH demand, may introduce a bottleneck to the light reactions (Figure 2B).

Due to the targeted nature of synthetic biology, the energetic demands of the synthetic pathway can be determined from its ATP and reductant demands. This presents an opportunity to incorporate the new metabolism into existing models [e.g., (Noctor and Foyer, 1998)] and estimate the impact the synthetic pathways may have on cellular energetics. Several flux models exist quantifying metabolic flux under various environmental conditions (Ma et al., 2014; Wieloch and Sharkey, 2022; Fu et al., 2022a) and may help to gauge the impact introduction of new metabolic sinks may have on energetics relative to native metabolism. This could also serve as a starting point for the design of accessory metabolism to offset any energetic imbalances imposed by the introduced metabolism.

Translation to crops

Broadly, increasing photosynthetic output by targeting inefficiencies has been shown to increase overall plant biomass [e.g. (Driever et al., 2017; South et al., 2019; Chen et al., 2020; Wang et al., 2020)]. In biofuel crops where general biomass is utilized for fermentation, this will likely result in the increased yield of desired product, but most bioengineering approaches are concerned with increasing a specific plant product (e.g., grain, lipids). Due to the complex regulation of carbon partitioning in crops (Yu et al., 2015; Paul, 2021), it is unclear if targeting photosynthetic inefficiencies or energetics will translate to increased grain or fruit production.

Despite these potential issues, engineering approaches that increase CO₂ assimilation have yielded higher overall biomass, including an increase in grain (Driever et al., 2017). Since many breeding successes have been measured by increasing yield while decreasing overall plant size [i.e., increased harvest index (HAY, 1995)], increasing grain yield by increasing overall crop biomass may increase the land use need, offsetting our perceived increases.

Improving photosynthesis for growth and resilience

Despite the uncertainty of downstream partitioning of biomass, photosynthesis researchers have a goal focused on improving the efficiency of photosynthesis (Long et al., 2006; Ort et al., 2015). With increased understanding of the regulation of these processes, we may increase the amount of light energy harnessed into driving CO₂ assimilation. Regardless of the downstream success in biomass partitioning, these increases will be an important building block in our steps to improve crops. There have been several approaches taken to improving the electron transport chain, many of which have resulted in small increases photosynthetic efficiency and/or in downstream carbon assimilation or overall plant biomass.

One large target of improvement is the group of processes activated by the accumulation of the Δp H component of Δp (i.e., q_E and PCON). Even though q_E and PCON are important to protect the thylakoid electron transport chain from damage, they are often cited as points for crop improvement to minimize energy loss and increase electron transfer rates (Murchie and Niyogi, 2011). In the case of an ATP surplus introduced by synthetic biology, these processes may also be a target to increase electron transfer to synthetic electron sinks. This should be undertaken with caution, as it may seem attractive to alter the sensitivity of these photoprotective processes to increase electron transfer, but this approach will also open our organisms to increased risk of photodamage. Under controlled environmental conditions, this may not be problematic, however, if engineered plants are grown in greenhouse or field conditions, they will experience rapidly changing light conditions. Without feedback regulation excess excitation energy can lead to the damage of PSII which must be repaired, leading to the long-term downregulation of light harvesting (Müller et al., 2001). Additionally, under high or rapidly fluctuating light, unregulated transfer of electrons through *cyt bf* can lead to damage of PSI, a potentially lethal situation for the plant (Suorsa et al., 2012).

Despite these concerns, there has been limited success with approaches to increase the flexibility of feedback regulation. Overexpression of *cyt bf* components (the rate limiting step of the electron transport chain) in a C4 plant has led to small increases in electron transfer and CO₂ assimilation (Ermakova et al., 2019). This suggests there is some limitation due to protein content of the *cyt bf* complex, and that subtle increases of electron transfer through *bf* may be a route to improving photosynthetic efficiency. This is especially of interest because overexpression of *cyt bf* would not impact dynamic PCON, maintaining PSI photoprotection. Increased expression of a K⁺/H⁺ antiporter involved in exchanging Δp H for $\Delta \psi$ during fluctuating light changes increased photosynthetic efficiency due to the increased capacity to relax q_E (Armbruster et al., 2016). Overexpression of q_E components, which has been shown to alter the sensitivity of q_E to the Δp H component of Δp (Li et al., 2002), has led to biomass increases in tobacco and soybean (Kromdijk et al., 2016; De Souza et al., 2022). However, in Arabidopsis the results were opposite (Garcia-Molina and Leister, 2020). Interpretation of these conflicting results is complicated, as the xanthophyll cycle component of NPQ is intrinsically linked to abscisic acid biosynthesis, the plant hormone involved in plant water relations (Niyogi et al., 1998).

While the light reactions supply ATP and NADPH for downstream carbon assimilation, except for low light, they do not appear to be the major limitation on bulk photosynthetic yield. There have also been improvements in assimilation and growth increases when the carbon reactions of photosynthesis are targeted, without modification of the light reactions. For example, overexpression of sedoheptulose 1,7 biphosphatase, the rate limiting step in the CBB cycle leads to larger plants (Miyagawa et al., 2001; Driever et al., 2017), suggesting the bulk supply of the light reactions are able to meet increased demand from the carbon side. However, if we think about the system dynamically, there are parts of the day that plants are light limited, such as early morning. Under very low light, q_E and PCON are not active, and suppression of them wouldn't likely offer substantial benefit. However, these processes are activated at intermediate light, where there is room to modify feedback regulation to improve photosynthesis (Murchie and Niyogi, 2011). Rather than modify the response of q_E and PCON directly, one

approach that has potential is the modification of the metabolic sensor of the ETC, the ATP synthase.

The ATP synthase is the central regulator of photosynthesis. It senses downstream metabolic states and controls Dp accumulation. For example, when the CBB cycle is substrate limited, the rate constant of proton efflux decreases, leading to the activation of q_E and PCON, even at low light (Kanazawa and Kramer, 2002). The ATP synthase is post-translationally regulated, the most understood of which is the light to dark inactivation of the redox regulated g-subunit (Kramer et al., 1990; Evron et al., 2000; Wu et al., 2007). However, the ATP synthase has also been proposed to be regulated by phosphorylation and protein-protein interactions, but these are not clearly understood (Evron et al., 2000; Bunney et al., 2001; Reiland et al., 2009). Additionally, it has been demonstrated that up to 50% of the ATP synthase content in tobacco leaves is inactive, and the molecular mechanism of this inactivation is completely unknown (Rott et al., 2011). Clearly, more basic research into the regulation of ATP synthase could lead to strategies to delay the onset of feedback regulation leading to q_E and PCON, and increase the integrated efficiency of the light reactions during the course of the day.

While the energetics of target pathways are not the only engineering consideration, we hope we have emphasized the importance of chloroplast energetics in synthetic biology. While some bioengineering strategies are promising, relatively extreme differences from native chloroplast metabolic demands may require secondary engineering to maximize the yield of the desired product. Finally, bioengineering of plants to fine tune energetic responses may lead to improved growth and resilience to stress conditions. Fine tuning should be considered as a strategy for plant improvement in addition to methods that may increase yield more dramatically.

Author contributions

Both authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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