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ENGINEERING ENHANCED PHOTOSYNTHESIS

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by

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Contents

1. Abstract

Photosynthesis drives all agricultural productivity, yet its efficiency in major crops remains strikingly low. Fewer than five percent of incoming solar photons are stored as biomass, largely due to evolutionary trade-offs that favour survival over productivity. With population growth and climate change intensifying pressure on food systems, targeted redesign of photosynthesis has emerged as a promising strategy to unlock further yield gains.

This monograph synthesizes advances across multiple fronts of photosynthetic engineering. In the light reactions, approaches aim to extend spectral use, balance canopy light distribution, accelerate recovery from photoprotective states, and relieve bottlenecks in electron flow. In the carbon reactions, efforts focus on optimizing Calvin–Benson cycle enzymes, stabilizing and enhancing Rubisco and its activase, introducing carbon-concentrating mechanisms from algae and cyanobacteria, and designing synthetic photorespiratory routes. Complementary strategies include improving gas diffusion, reinforcing source–sink relationships, stacking multiple traits, reshaping canopy structure, and exploring bio-nanomaterials.

Evidence from greenhouse and field trials confirms that these modifications can raise photosynthetic efficiency and, in some cases, crop yield. Yet results are often species-specific, and most single-trait interventions only shift the bottleneck elsewhere. The discussion therefore highlights integration as the central challenge: combining complementary traits in multigene frameworks, supported by modelling and omics, to deliver consistent improvements.

Ultimately, photosynthetic engineering is best conceived as a systems-level redesign that unites molecular, physiological, and architectural innovations. If realized, such integration could underpin a new era of agricultural productivity, enabling crops that are not only higher yielding but also resilient under climate stress.

2. Introduction

Photosynthesis is the foundation of life, capturing sunlight and producing the biomass that sustains ecosystems and agriculture. Yet while the Green Revolution doubled or tripled crop yields through improved varieties, fertilizer, and irrigation (Evenson & Gollin, 2003), gains in staples such as wheat, rice, and maize are now slowing (Ray *et al.*, 2013). With the global population projected to exceed nine billion by 2050 and climate stress increasing, new levers are needed. Traditional agronomy and breeding are nearing their limits, making photosynthesis and its engineering central to future yield improvements (Long *et al.*, 2015).

At its core, photosynthesis involves the conversion of light energy into chemical energy, followed by carbon fixation. Photons are captured by pigments in the antenna complexes of photosystem II (PSII), where excitation energy drives charge separation. To avoid damage when excess light is absorbed, plants dissipate excitation energy as heat—rather than using it for photochemistry—through a crucial but slow-recovering mechanism called non-photochemical quenching (NPQ). From PSII, the resulting electrons flow via plastoquinone through the cytochrome b_6f complex, which contributes to the formation of a proton gradient across the thylakoid membrane, before being transferred by plastocyanin (PC) to photosystem I (PSI). The proton motive force (pmf) then drives ATP synthase to produce ATP, while a second excitation at PSI enables the reduction of $NADP^+$ to NADPH. These energy carriers (ATP, NADPH) power the Calvin–Benson–Bassham (CBB) cycle in the chloroplast stroma, where Rubisco catalyses the fixation of CO_2 into organic molecules. Yet Rubisco is slow and catalyses an alternative oxygenation reaction that generates 2-phosphoglycolate (2PG), which is recycled via energetically costly photorespiration (Sharkey, 2020). The products of carbon fixation—triose phosphates—are allocated to sucrose, starch, and biomass, with overall efficiency further constrained by CO_2 diffusion through stomata and mesophyll, and sink strength—the capacity of plants to utilize and store assimilated carbon.

Despite driving all plant productivity,

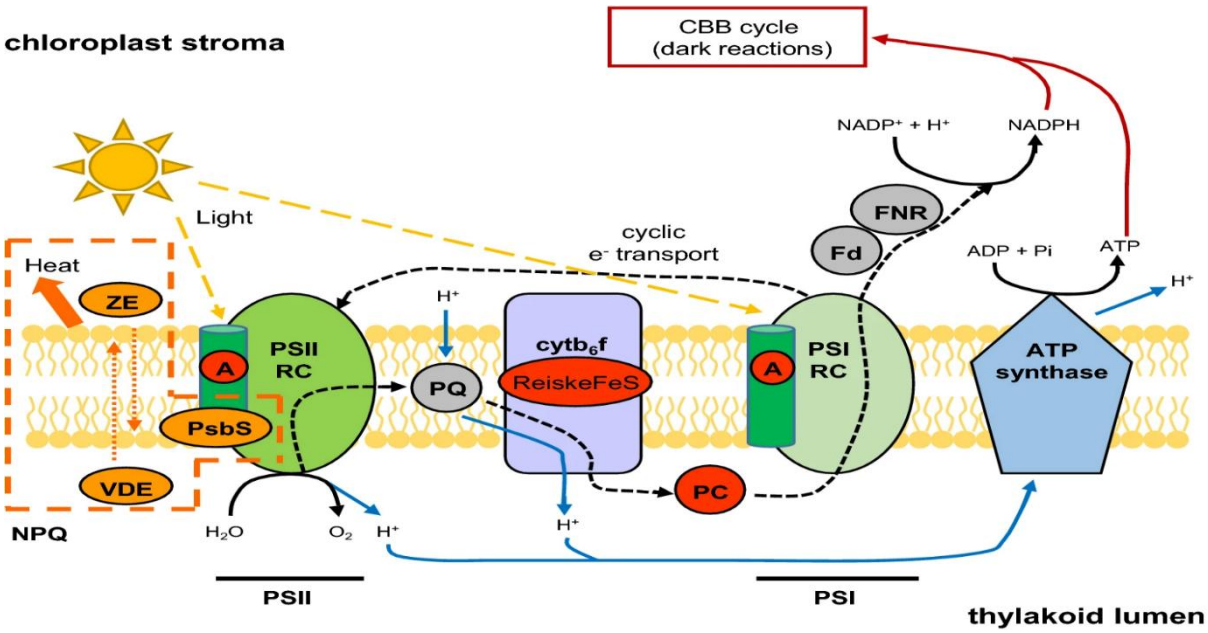

Figure 1 : General diagram of photosynthetic light reactions and non-photochemical quenching in C_3 plants. Blue lines denote proton movement, black discontinuous lines denote movement of electrons, red lines denote movement to the CBB cycle. NPQ is boxed in orange. Components that have been modulated for improvement of photosynthetic efficiency are shown in red and orange. Image and description taken from Singer et al. (2019).

photosynthesis is remarkably inefficient, with less than 5% of incoming solar energy ultimately converted and stored as biomass (Zhu *et al.*, 2010). The (in)efficiency of photosynthesis stems from structural and physiological constraints spanning throughout the process that limit the ability and rate at which plants can utilize sunlight. Such limitations create persistent bottlenecks in energy conversion and carbon assimilation, leading to productivity losses and restricting yield potential. These bottlenecks reflect how photosynthesis evolved for survival, not agricultural productivity—favouring robustness over efficiency. While these strategies ensured adaptability in diverse environments, they impose significant constraints on the photosynthetic capacity and productivity of crops in agricultural environments such as large monocultures. However, it is important to note that, in nature, plants do not *need* to produce more than they require themselves—perhaps partly explaining why photosynthetic efficiency has not been further optimized by evolution. By contrast, in today's societal conditions, we humans *need* plants to produce more than they require—perhaps explaining why we face these bottlenecks. The central challenge is therefore to identify ways of overcoming these inherent limitations, by engineering photosynthesis into a more efficient and resilient system and achieve consistent gains under current and future conditions.

This monograph reviews current progress in photosynthesis engineering, emphasizing how such modifications could improve crop productivity. It examines strategies aiming to optimize light capture and energy conversion (Section 3), enhance carbon assimilation (Section 4), and integrate emerging and whole-plant approaches (Section 5). However, the greatest potential lies in combining complementary traits through coordinated, multigene, and systems-level redesign, as models suggest that alleviating multiple bottlenecks simultaneously could raise efficiency by 30–60% (Zhu et al., 2010). This work highlights successes, trade-offs, and remaining challenges, and argues that such integrated redesign efforts may provide the foundation not only for raising yields, but also to build resilient, sustainable agriculture for the future.

3. Optimizing The Light Reactions

3.1 Broadening the Absorption Spectrum

Sunlight is the primary energy source for photosynthesis. However, pigments in the light-harvesting antenna complexes (LHCs) of most photosynthetic organisms absorb only between 400 and 700 nm, a range called photosynthetically active radiation (PAR), which represents ~43% of all solar energy reaching Earth's surface (Zhu *et al.*, 2010). By themselves, the main pigments—chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids—absorb strongly in the blue and red regions of PAR but only weakly in far-red light (FRL), setting an effective upper PAR limit near 700 nm. As a result, most far-red (FR) photons are excluded from efficient use and conversion, unless they synergistically interact with shorter-wavelength photons (Zhen *et al.*, 2022). This constraint reduces light-use efficiency, particularly in dense canopies where upper leaves intercept visible light, leaving lower leaves with mostly FR radiation and reduced photosynthetic output (Mirkovic *et al.*, 2017; Slattery & Ort, 2021). Expanding absorption into FR could therefore enhance productivity; indeed, Blankenship and Chen (2013) estimated that a 50 nm extension would increase available energy by ~19%, providing rationale for further research.

In this context, several approaches are being pursued. For example, far-red absorption can be extended by engineering binding sites of naturally occurring "red-form" Chl *a* variants, which are FR-shifted due to coupling between multiple Chl *a* molecules (Croce *et al.*, 2024), or by integrating bacteriochlorophyll-containing PSI-like reaction centres from anoxygenic phototrophs that absorb up to ~1100 nm (Blankenship *et al.*, 2011). However, the most promising strategy to spectrum broadening involves the use and manipulation of alternative forms of chlorophyll—named chlorophyll *d* (Chl *d*) and chlorophyll *f* (Chl *f*)—produced by certain cyanobacteria, which can efficiently absorb FR light, thereby enabling the use of photons beyond 700 nm to drive photosynthesis. Their discovery overturned the long-held view that such low-energy photons were insufficient to sustain oxygenic photosynthesis, as efficient water oxidation was thought impossible under FR light (Croce *et al.*, 2024), and provided the first clear demonstration that photosynthesis can operate under far-red light (Miyashita *et al.*, 1996; Chen *et al.*, 2010).

Chl *d* was first reported in 1943 as a minor pigment in red macroalgae (Manning & Strain, 1943) but long dismissed as an extraction artifact. Its natural role was later confirmed in *Acaryochloris marina*, where it accounts for ~80% of pigments, with only trace amounts of Chl *a* (Miyashita *et al.*, 1996). In 2010, Chl *f* was discovered in *Halomicronema hongdechloris* (Chen *et al.*, 2010) and shown to be synthesized in response to FR light via Far-Red Light Photoacclimation (FaRLiP) (Gan *et al.*, 2014b). FaRLiP is an acclimation response that allows cyanobacteria to maintain oxygenic photosynthesis under FR light, by switching from exclusive Chl *a* production to the additional synthesis of Chl *d* and Chl *f* (Gan *et al.*, 2014b). To enable the use of these FR chlorophylls, this response also involves extensive remodelling of the photosynthetic machinery, with most core subunits of both photosystems (PSI, PSII) and the phycobilisome (PBS) replaced by FRL-specific paralogues of these canonical subunits, encoded in a dedicated multi-gene cluster (Gan *et al.*, 2014a; Gan & Bryant, 2015). Structurally, Chl *d* and Chl *f*

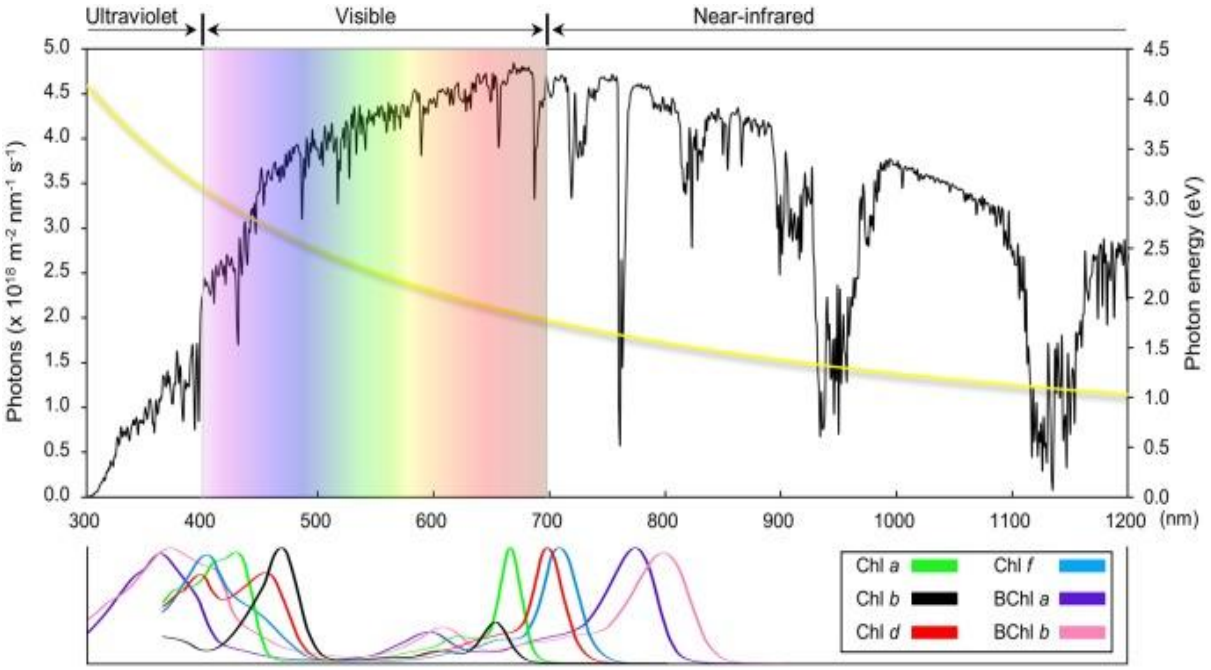

Figure 2 : Solar spectrum and absorption profiles of chlorophyll and bacteriochlorophyll pigments. The photon flux spectrum from 300 to 1200 nm of sunlight is plotted in black. Photon energy at each wavelength is plotted in yellow. Image taken from Cardona et al (2018).

differ from Chl *a* by a single formyl group substitution, suggesting that conversion from the red- to FR-absorbing forms could be achieved by a single enzyme—similar to how chlorophyll *a* oxygenase (CAO) produces Chl *b* from Chl *a* (Tanaka & Tanaka, 2011; Croce *et al.*, 2024). While the enzyme for Chl *d* synthesis is unknown, the light-dependent Chl *f* synthase (*chlF*) was identified within the FaRLiP cluster as a divergent "super-rogue" paralog of *psbA*, which normally encodes the D1 subunit of PSII (Ho *et al.*, 2016). Indeed, *chlF* converts Chl *a* into Chl *f* and is both necessary and sufficient for its synthesis: null mutants lacked Chl *f* and its characteristic FR absorption and long-wavelength fluorescence, while introduction of *chlF* into non-FaRLiP strains induced Chl *f* production and its integration into PSI (Ho *et al.*, 2016; Shen *et al.*, 2019a). Alongside *chlF*, the FaRLiP cluster encodes paralogous pigment-binding proteins of PSI, PSII, and PBS, as well as regulatory components including the photoreceptor RfpA (a phytochrome) and regulators RfpB and RfpC, which together form the RfpABC cascade controlling FaRLiP expression (Gan *et al.*, 2014a; Zhao *et al.*, 2015). To ensure functional pigment–protein complexes under far-red light, these FRL-specific pigment-binding proteins exhibit enhanced affinity for Chl *f* (Gisriel *et al.*, 2021), a trait that may hinder efforts to introduce these novel chlorophylls into non-FR-adapted organisms such as crops. However, Tros *et al*. (2020) showed that canonical PSI proteins can also accommodate Chl *f*, demonstrating compatibility with native pigment-binding sites and highlighting the potential for cross-species transferability. Subsequent work on light-harvesting complexes (LHCs) of higher plants has yielded encouraging results. Through *in vitro* reconstitution experiments using recombinant LHC proteins, researchers demonstrated that, despite structural differences from those of cyanobacteria, plant LHCs can successfully integrate both FR-absorbing chlorophylls with added benefits (Elias *et al.*, 2021, 2024). For example, introducing Chl *d* or Chl *f* into antenna proteins such as the PSI-specific Lhca4 extended absorption into the far-red without impairing structural integrity, energy transfer, or excited-state dynamics, with Chl *d* producing the most extreme red shift (>750 nm) yet observed in a plant LHC, aided by specific pigment–protein interactions (Elias *et al.*, 2024). These results confirm that cyanobacterial chlorophylls *d* and *f* can broaden the photosynthetically active region, highlighting the inherent flexibility of plant antenna systems and the feasibility of engineering crops to exploit far-red light for gains in productivity under low-light conditions.

While this strategy shows promise, practical deployment in crops will require fine control of pigment abundance, positioning, and compatibility to prevent efficiency trade-offs, such as the formation of local energy traps that slow excitation transfer when only a few FR-absorbing chlorophylls are inserted into Chl *a* complexes (Slattery & Ort, 2021; Croce *et al.*, 2024). If successfully implemented, spectrum broadening could enhance photosynthesis by increasing photon availability and improving light-use efficiency, particularly in shaded canopies and low-light conditions.

3.2 Antenna Size and Chlorophyll Content Reduction

In natural ecosystems, plants overinvest in chlorophyll and light-harvesting complexes (LHCs) to outcompete lower and neighbouring vegetation by monopolizing sunlight and shading rivals (Freschet *et al.*, 2011). While adaptive in mixed-species environments, this becomes counterproductive in dense monocultures,

where C_3 photosynthesis saturates at ~25% of full sunlight (Croce *et al.*, 2024). Upper-canopy leaves absorb more light than they can use, leaving lower leaves light-limited and reliant on respiration, which reduces overall canopy photosynthetic efficiency and increases the need for non-photochemical quenching (NPQ) (Slattery & Ort, 2021). Reducing chlorophyll content and antenna size can therefore improve light distribution, enhance lower-canopy photosynthesis, and decrease wasteful NPQ (Kirst *et al.*, 2017).

The idea of reducing antenna size to improve canopy light-use efficiency was first validated in dense cultures of microalgae and cyanobacteria. In *Chlamydomonas reinhardtii*, chlorophyll-deficient mutants *truncated light antennae 1* (*tla1*), possessing smaller yet functional photosystem antennae, exhibited higher photosynthetic efficiency than the wild type under high light (Polle *et al.*, 2003). Subsequent TLA mutants showed similar outcomes and carried lesions in genes of the chloroplast signal recognition particle (cpSRP) pathway, such as *cpSRP43* and *cpSRP54*, which mediate delivery and insertion of LHC proteins into thylakoid membranes (Kirst *et al.*, 2012a,b; Jeong *et al.*, 2017). Disruption of this pathway reduces LHC abundance and antenna size, making it a promising candidate for enhancing photosynthetic productivity (Kirst & Melis, 2014). Translation of the TLA concept to higher plants has yielded promising results. In *Nicotiana tabacum*, downregulation of cpSRP43 produced a light-green phenotype with smaller antennae and reduced chlorophyll, which under high-density cultivation accumulated ~25% more biomass and required higher light intensity to saturate photosynthesis(Kirst *et al.*, 2018). In barley (*Hordeum vulgare*), the pale-green *hus1* mutant, carrying a premature stop codon in *HvcpSRP43*, had ~50% less chlorophyll, reduced carotenoids, and a higher Chl *a*/*b* ratio—consistent with smaller antenna size. Despite these changes, *hus1* lines maintained photosynthetic capacity and photoprotection, yielding biomass and grain similar to wild type (Rotasperti *et al.*, 2022). Pale-green rice (*Oryza sativa*) lines with reduced chlorophyll also showed faster growth and up to a 40% increase in leaf photosynthetic rates while sustaining or improving grain yield under field conditions (Gu *et al.*, 2017). By contrast, chlorophyll-deficient soybean (*Glycine max*) mutants were less successful: across field and controlled studies, pale-green lines showed no consistent improvements in canopy photosynthesis or light-use efficiency, and often reduced yields or biomass compared with wild type (Slattery *et al.*, 2017; Sakowska *et al.*, 2018; Genesio *et al.*, 2020). In the industrially relevant microalga *Chlorella sorokiniana*, truncated antenna mutants accumulated ~30–68% more biomass in dense suspensions and showed higher photon-use efficiency under high light (Cazzaniga *et al.*, 2014), hinting at the scalability of the TLA approach for algal production. These mixed findings highlight the strong influence of species-specific canopy architecture, physiology, and environmental interactions on the success of truncated light antennae strategies. Reducing antenna size can also be achieved by targeting chlorophyll biosynthesis. One strategy focuses on chlorophyllide *a* oxygenase (CAO), which converts Chl *a* to Chl *b*. Partial suppression lowers Chl *b* levels and reduces peripheral antenna size, improving light distribution in dense canopies. In *Chlamydomonas reinhardtii*, CAO-deficient mutants showed increased photosynthetic rates under strong light (Perrine *et al.*, 2012). Since full CAO knockout is detrimental, moderate downregulation is considered optimal. A related strategy targets the rice *YGL1* gene, encoding chlorophyll synthase, which catalyses the final step in Chl *a*

biosynthesis. Suppression of *YGL1* reduces total chlorophyll and antenna size, enhancing light penetration to lower leaves, as shown in the pale-green *ygl* lines, which improved light-use efficiency (Wang *et al.*, 2022; Li *et al.*, 2023). Antenna size can also be truncated by modifying pigment–protein complexes that capture and transfer light energy—namely, the PSII major LHCII, the minor antennas (CP24, CP26, CP29), and the PSI LHCI. Downregulation or knockout of specific LHC subunits decreases chlorophyll-binding capacity and limits excess absorption. However, most *Arabidopsis thaliana* mutants lacking minor antennas show impaired energy transfer, photoprotection, and PSII stability, leading to reduced photosynthesis and growth (Slattery & Ort, 2021; Leister, 2023). In contrast, selective reduction of certain major LHCII subunits lowered chlorophyll content without compromising photosynthetic or photoprotective function (Bielczynski *et al.*, 2020). Thus, precise tuning of LHC composition may be more effective than broad antenna removal.

3.3 Accelerating NPQ Kinetics and Recovery

Non-photochemical quenching (NPQ) is a ubiquitous photoprotective mechanism in oxygenic photosynthetic organisms, dissipating excess absorbed light as heat to prevent PSII overexcitation and the formation of damaging reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) (Pinnola & Bassi, 2018). It consists of several components acting on different timescales, from the rapid qE to the slower qZ, qH, and qI (Leister, 2023). While essential for protection, NPQ relaxes slowly after high-to-low light transitions, leading to continued energy dissipation instead of photochemistry (Hu *et al.*, 2023). This keeps PSII below optimal efficiency and constrains CO_2 fixation (Li *et al.*, 2023), with modelling suggesting up to ~30% losses in carbon assimilation under fluctuating light, such as in dense crop canopies (Zhu *et al.*, 2004). Accelerating NPQ recovery is therefore a key engineering target for improving light-use efficiency and crop productivity.

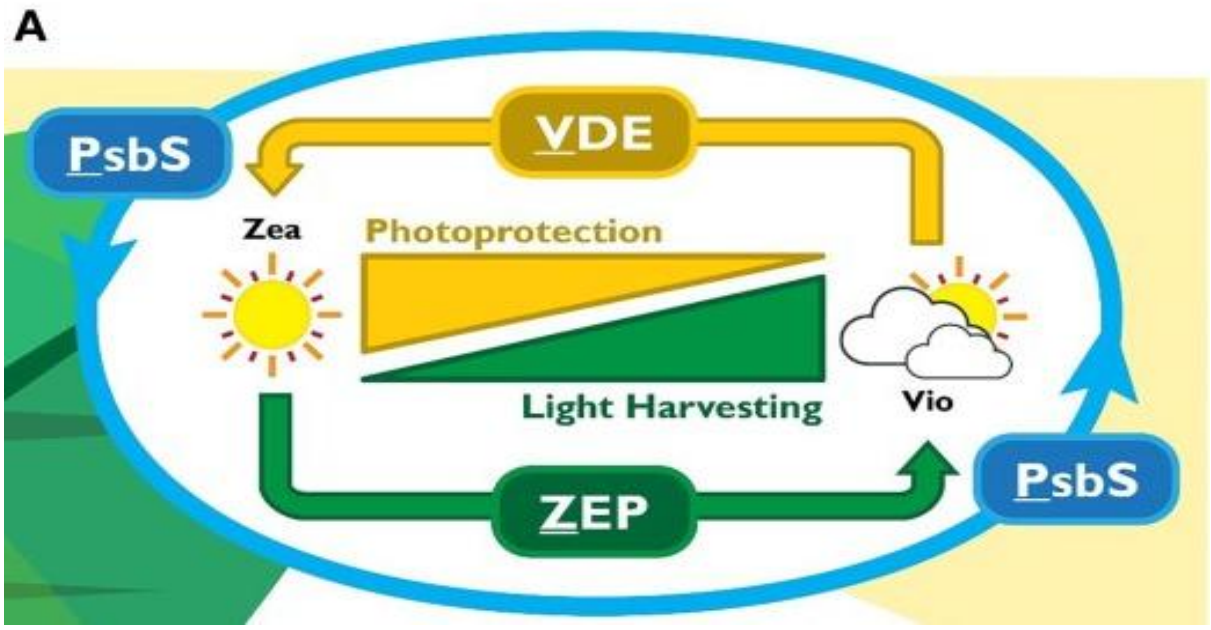

Figure 3 : Diagram of the VPZ mechanism. Image adapted from Croce et al. (2024).

The most validated and scalable strategy for accelerating NPQ recovery—known as the VPZ approach—involves simultaneous overexpression of three proteins of energy-dependent quenching (qE), the fastest NPQ component, which activates and relaxes within seconds to minutes. qE operates through a trans-thylakoid pH gradient (ΔpH), which regulates quenching via PsbS and the xanthophyll zeaxanthin (Zea), a carotenoid that dissipates excess excitation energy (Bassi & Dall'Osto, 2021). The targeted proteins are: violaxanthin de-epoxidase (VDE), which produces Zea; PsbS, which under low luminal pH promotes qE formation and quenching; and zeaxanthin epoxidase (ZEP), which reconverts Zea to violaxanthin (Ruban, 2016). Increasing their abundance accelerates both induction and relaxation of NPQ during light fluctuations, enabling plants to switch more rapidly between photoprotection and efficient light use, thereby reducing photodamage and wasted energy. The VPZ approach has shown notable success in some crops. In tobacco, overexpression accelerated NPQ relaxation and increased biomass by ~15% in replicated field trials and up to 20% in glasshouse experiments (Kromdijk *et al.*, 2016). In transgenic soybean, faster NPQ relaxation improved CO_2 assimilation, linear electron transport, and raised elite seed yield by ~20% in small-scale field trials (De Souza *et al.*, 2022). Although NPQ optimization is widely regarded as a promising and validated strategy with significant agronomic potential, results have been inconsistent. In fact, in *Arabidopsis thaliana* (Garcia-Molina & Leister, 2020) and *Solanum tuberosum* (potato) (Lehretz *et al.*, 2022), VPZ overexpression did accelerate NPQ dynamics but unexpectedly reduced biomass and yield under greenhouse and fluctuating light conditions. Thus, while promising, evidence indicates that this approach is not always applicable, likely due to species-specific responses to altering VPZ proteins or the need for a highly precise balancing of expression (Leister, 2023).

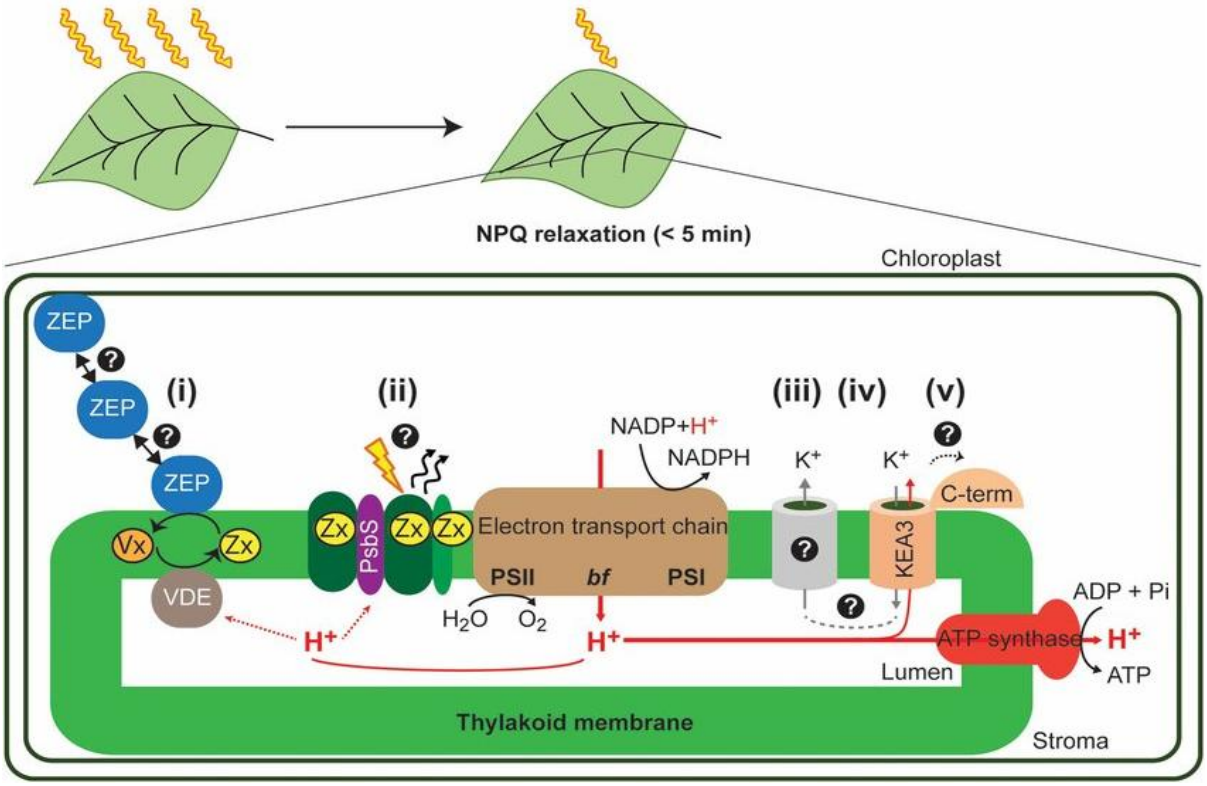

Figure 4 : Summary of chloroplast processes involved in NPQ relaxation. The figure summarizes known components involved in rapid NPQ relaxation. Image taken from Kaiser et al. (2019).

Other NPQ-targeting strategies focus on manipulating light-harvesting complex stress-related proteins (LHCSR) in microalgae such as *Chlamydomonas reinhardtii*, where NPQ induction—especially qE—depends largely on LHCSR1 and LHCSR3 (Perozeni *et al.*, 2020). A mutant lacking both proteins (*npq4lhcsr1*) showed no rapidly inducible and reversible NPQ and a marked increase in photosynthetic rates when grown under intense light, which led to enhanced singlet oxygen production and subsequent photodamage (Cantrell & Peers, 2017). In sinusoidal light cycles, while the mutant showed consistent total daytime carbon accumulation relative to the wild type, its overall daily growth was significantly reduced due to fewer cell divisions occurring at night. Cantrell and Peers (2017) suggest that, because the mutant experienced low photoinhibition of PSII and maintained daytime carbon gain, the growth penalty likely comes from the metabolic cost of repairing photodamage during the day in the absence of qE, diverting resources that would otherwise support cell division at night. Similarly, mutants deficient in both LHCSR1 and LHCSR3 exhibited better photosynthetic efficiency and a shorter NPQ lag phase, resulting in faster initial growth rates following slow light intensity transitions. However, under continuous and fast intermitting light conditions, mutants suffered greater photodamage and no significant differences in growth kinetics or final biomass were observed (Barera *et al.*, 2021). These results suggest that removing LHCSR can enhance instantaneous photosynthesis under high light but does not reliably improve overall biomass productivity in more natural and dynamic lighting conditions.

3.4 Enhancing Electron Transport Chain Efficiency (ETC)

The cytochrome b_6f (Cyt b_6f) complex plays a central role in both linear (LET) and cyclic (CET) electron transport by oxidizing plastoquinol, transferring electrons to the copper-containing plastocyanin (PC), and contributing to proton translocation across the thylakoid membrane to generate the proton motive force (pmf) (Tikhonov, 2014). Plastoquinol oxidation is a relatively slow step, making Cyt b_6f a major regulatory bottleneck in both C_3 and C_4 photosynthesis (Price *et al.*, 1995, 1998; Yamori *et al.*, 2011).

A key strategy to relieve this bottleneck has been overexpression of the Rieske FeS (PetC) protein, a critical subunit for Cyt b_6f assembly and function (Singer *et al.*, 2019). In *Arabidopsis thaliana*, overexpressing the tobacco *petC* gene increased other Cyt b_6f core subunits (PetA, PetB) and photosystem proteins, enhancing electron transport, CO_2 assimilation under saturating light, and biomass and seed yield (Simkin *et al.*, 2017b). In the C_4 model plant *Setaria viridis*, Rieske FeS overexpression increased Cyt b_6f content in both mesophyll and bundle sheath cells, enhancing linear electron transport (LET) capacity. Under high light and non-limiting CO_2, such transgenic lines exhibited higher photosynthesis and CO_2 assimilation rates, supported by a greater proton motive force (pmf), indicating that Cyt b_6f can be a key control point for photosynthetic efficiency in C_4 plants (Ermakova *et al.*, 2019). In the C_3 crop *Nicotiana tabacum*, Rieske FeS overexpression also increased Cyt b_6f levels, but produced only transient gains in electron transport following light shifts, linked to faster proton gradient formation and quicker qE induction, with no consistent steady-state improvements in

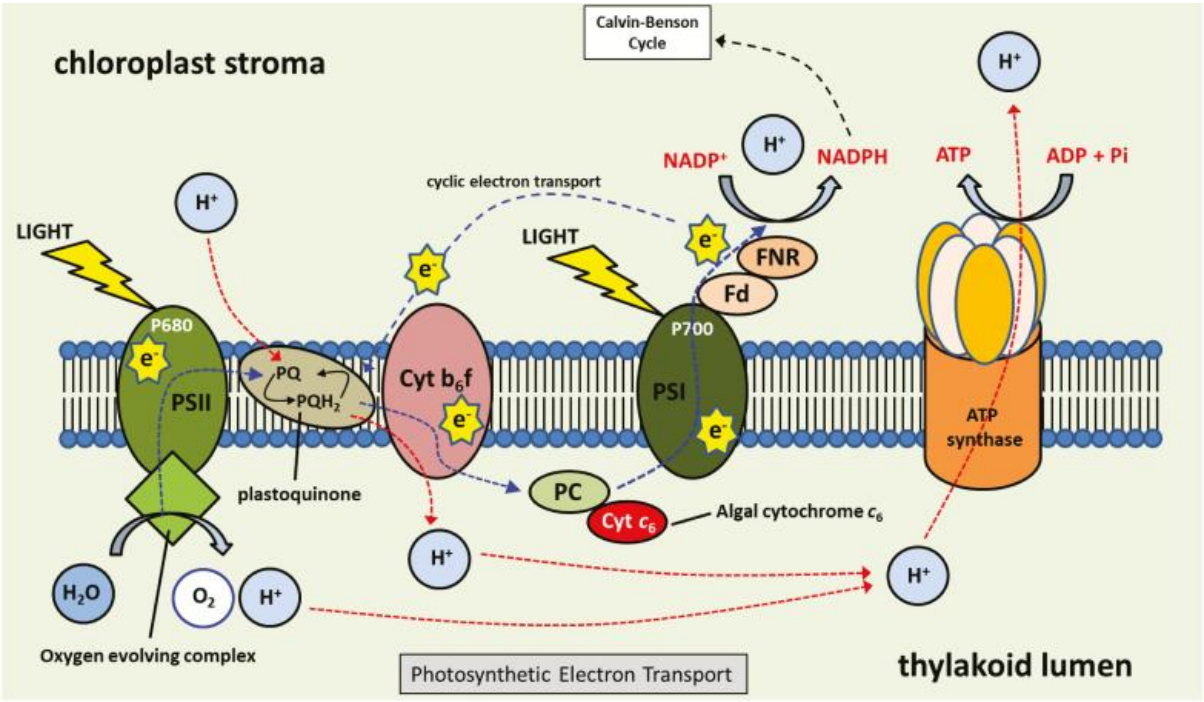

Figure 5 : Diagram of the photosynthetic Electron Transport Chain. Ferredoxin (Fd), ferredoxin-NADP reductase (FNR), cytochrome b_6f complex (Cyt b_6f), plastocyanin (PC), cytochrome c_6 (Cyt c_6). Image taken from Simkin et al. (2019).

CO_2 assimilation (Heyno *et al.*, 2022). This suggests that Cyt b_6f is not the sole limitation to electron transport in tobacco under saturating conditions. More recently, introducing the *BdpetC* gene from *Brachypodium distachyon* into sorghum increased Rieske FeS abundance by ~40%. Although steady-state electron transport and CO_2 assimilation rates were unchanged under high light/CO_2, transgenic plants showed higher biomass and grain yield under glasshouse conditions, attributed to faster photosynthetic induction, more dynamic NPQ responses, and improved performance under fluctuating light (Ermakova *et al.*, 2023). The contrasting outcomes in C_3 and C_4 species highlight the need for species-specific strategies, especially as breeding may have already optimized Cyt b_6f content in crops (Croce *et al.*, 2024). Overall, Rieske FeS overexpression enhances photosynthesis and productivity mainly by accelerating induction and improving light-use efficiency, rather than steady-state performance. This is because the cytochrome b_6f complex does not limit steady-state electron transport under saturating light and CO_2, where downstream processes define the maximum capacity of photosynthesis, rendering increased Cyt b_6f levels through Rieske FeS overexpression ineffective. In contrast, during dark-to-light transitions, elevated Cyt b_6f levels enhance proton gradient formation, enabling faster activation of electron transport and CO_2 fixation. As a result, photosynthetic induction is accelerated and light-use efficiency improved under fluctuating conditions, even though steady-state capacity remains unchanged.

Beyond alleviating the Cyt b_6f bottleneck itself, several strategies target the downstream transfer of electrons to PSI. One approach is the heterologous expression of cytochrome c_6 (Cyt c_6), a soluble heme-containing carrier naturally present in cyanobacteria and green algae. In its native context, Cyt c_6 replaces plastocyanin (PC) under copper-deficient conditions, maintaining electron flow between Cyt b_6f and PSI (Merchant & Bogorad, 1987). Transgenic expression of

algal Cyt c_6 in *Arabidopsis thaliana* and *Nicotiana tabacum* improved photosynthetic performance. In *Arabidopsis*, which lacks a functional Cyt c_6 homolog (Molina-Heredia *et al.*, 2003), algal Cyt c_6 efficiently substituted for PC *in vivo*, transferring electrons to PSI more rapidly than the native carrier, thereby increasing ATP and NADPH production, chlorophyll and starch content, CO_2 assimilation, and biomass accumulation (Chida *et al.*, 2007). In tobacco, similar enhancements were observed, including higher photosynthetic rates, water-use efficiency, and growth, particularly under low light, where electron transport is limiting (Yadav *et al.*, 2018). When combined with Calvin–Benson cycle optimizations, Cyt c_6 further boosted photosynthesis (see Section 5.3), underscoring its promise in multigene engineering strategies. A complementary approach enhances plastocyanin (PC), the only soluble copper-containing carrier, which transfers electrons from Cyt b_6f to PSI. PC is considered rate-limiting because its abundance depends on copper availability (Zhang *et al.*, 2014). The conserved microRNA408 (*miR408*) regulates copper homeostasis by promoting delivery to the thylakoid lumen, thereby supporting PC synthesis (Trindade *et al.*, 2010). Overexpression of *miR408* increased PC abundance in *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Oryza sativa*, while in rice it also upregulated genes encoding upstream ETC components (Zhang *et al.*, 2017; Song *et al.*, 2018; Pan *et al.*, 2018). These changes raised photosynthetic rates, light saturation points, and linear electron flow between PSII and PSI. More importantly, lines overexpressing *miR408* showed significant increases in biomass and seed size, with rice exhibiting higher grain yield under field conditions (Pan *et al.*, 2018), highlighting its agronomic relevance. Other mobile electron carriers have also been engineered. Cyanobacterial flavodoxin can partially substitute for ferredoxin, conferring stress tolerance, while overexpressing endogenous plastocyanin or ferredoxin has been shown to enhance growth in higher plants. Similarly, introducing cyanobacterial flavodiiron proteins improves PSI protection and maintains linear electron transport under fluctuating light (Leister, 2023).

Together, these strategies demonstrate that optimizing electron delivery from Cyt b_6f to PSI—whether by introducing algal carriers, enhancing PC availability, or modulating other electron transport proteins—offers a complementary route to boosting photosynthetic efficiency and crop productivity. Yet, these gains can only be fully realized if matched by downstream capacity in the CBB cycle and carbon metabolism, highlighting the need to integrate ETC optimization with improvements in carbon fixation (see Section 4).

4. Optimizing the Carbon Reactions

The Calvin–Benson–Bassham (CBB) cycle is the central pathway for carbon fixation in C_3 and C_4 plants, where ribulose-1,5-bisphosphate (RuBP) serves as the CO_2 acceptor and substrate for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme that catalyses the initial carboxylation step, but also initiates the costly photorespiratory pathway via its oxygenase activity. Rubisco's activity is maintained by Rubisco activase (Rca), a catalytic chaperone protein that facilitates carbamylation by removing inhibitory sugar phosphates. Whether through enhancing enzyme activity, optimising Rubisco or its regulatory proteins, or introducing photorespiratory bypasses and carbon-concentrating mechanisms (CCMs), improving the CBB cycle and its components is widely recognised as a

crucial and promising strategy for boosting photosynthesis and crop productivity, with numerous studies reporting substantial gains in carbon fixation, biomass, and yield (Batista-Silva *et al.*, 2020). Within this framework, recent efforts have increasingly focused on multigene stacking to avoid shifting bottlenecks and to achieve balanced enhancement across the cycle (Simkin *et al.*, 2015).

a complementary strategy to boost carbon assimilation and crop productivity (Kubis & Bar-Even, 2019; Simkin *et al.*, 2019).

Sedoheptulose-1,7-bisphosphatase (SBPase) catalyses a key step in RuBP regeneration, and its overexpression consistently enhances photosynthesis, biomass, and grain yield in several species, including tobacco, wheat, and tomato, across diverse growth conditions (Lefebvre *et al.*, 2005; Rosenthal *et al.*, 2011; Ding *et*

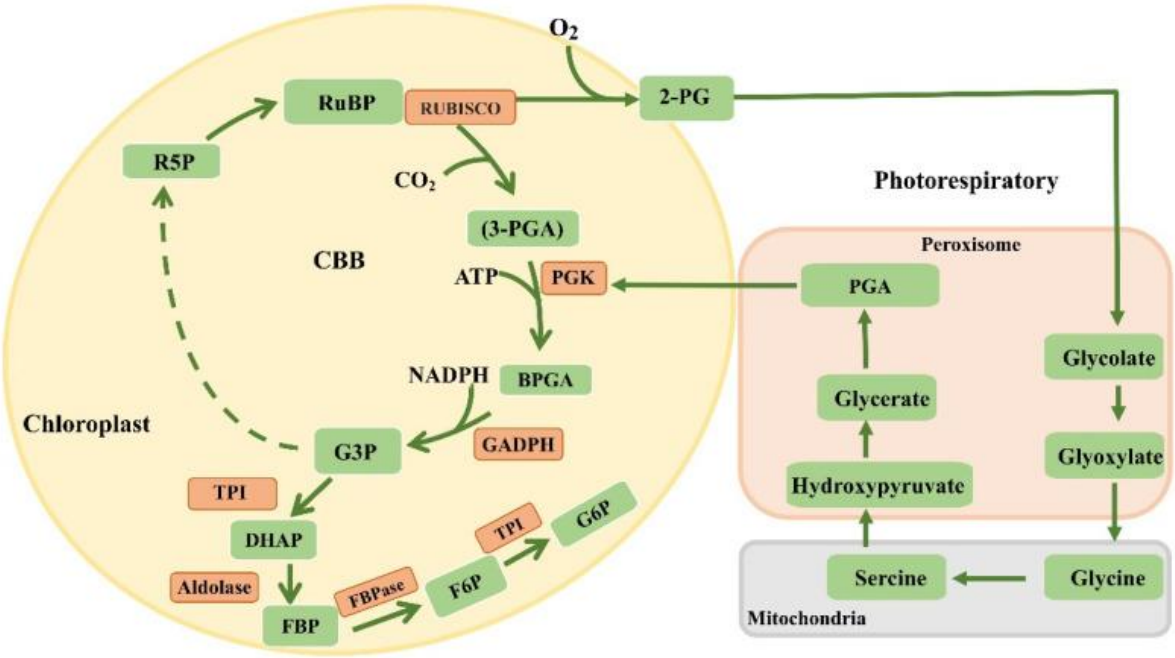

Figure 6 : Carbon metabolism cycle diagram: the Calvin–Benson–Bassham (CBB) cycle and photorespiratory pathway. Image and description taken from Li et al. (2023)

4.1 Improving Calvin Cycle Enzyme Activity (CBB Cycle)

While Rubisco has long been considered the primary bottleneck to photosynthetic efficiency, modelling shows that other CBB cycle enzymes can also impose limitations, as their natural distribution was suggested to be suboptimal (Zhu *et al.*, 2007). Both experimental and computational analyses have revealed additional control points, particularly within the RuBP regeneration phase (Orr *et al.*, 2017; Bar-Even, 2018). This has led to growing interest in improving the activity of several CBB cycle enzymes as

al., 2016; Driever *et al.*, 2017). SBPase overexpression has also been associated with increased tolerance to abiotic stresses such as chilling, salinity, and high temperature (Batista-Silva *et al.*, 2020). However, as further detailed in Section 5.3, the largest gains are typically achieved when SBPase is co-overexpressed with other enzymes involved in RuBP regeneration, such as fructose-1,6-bisphosphate aldolase (FBPA) and fructose-1,6-bisphosphatase (FBPase), which have shown only moderate benefits when overexpressed individually (Uematsu *et al.*, 2012). Similarly, phosphoribulokinase (PRK), which catalyses the final step in

RuBP regeneration, and the potentially limiting transketolase (TK) are frequently included in multigene constructs and co-expressed with SBPase (Croce *et al.*, 2024). Other Calvin–Benson cycle genes have also been targeted, for example through simultaneous upregulation of FBA1, RCA1, FBP5, and PGK1 via elevated expression of the BZR1 transcription factor, which enhanced photosynthetic capacity (Yin *et al.*, 2022). Introduction of a bifunctional cyanobacterial SBPase/FBPase—a single enzyme catalysing two reactions—improved photosynthesis in several crops and algae (Hu *et al.*, 2023; Croce *et al.*, 2024), especially in soybean where it enhanced CO_2 assimilation and growth while sustaining yields even under heat and elevated CO_2 (Köhler *et al.*, 2017). Improvements in RuBP regeneration can also be achieved through cross-pathway strategies involving non-CBB cycle proteins (see Section 5.3). The benefits of Calvin cycle engineering are influenced by broader physiological factors. Increased protein production raises nitrogen demand, and enhanced carbon assimilation must be matched by sink strength to avoid feedback inhibition (see Section 5.2). Moreover, strategies validated under controlled conditions must also prove effective under fluctuating field environments where light, temperature, and water availability are variable (Batista-Silva *et al.*, 2020).

4.2 Rubisco Activase (Rca) Engineering

Rubisco activase (Rca) is an essential regulator of photosynthesis, enabling the carbamylation of Rubisco by removing inhibitory sugar phosphates (Portis, 2003). Rca's thermolability is a major constraint—moderate heat stress can impair its activity, reducing Rubisco activation and CO_2 fixation (Salvucci & Crafts-Brandner, 2004). Activation also lags during transitions from low to high light, causing up to 20% carbon assimilation losses (Taylor & Long, 2017). Identifying and engineering thermostable, more abundant Rca variants is therefore key to maximizing carbon fixation, particularly under projected warmer conditions (Qu *et al.*, 2023), and this strategy has already delivered significant gains. In *Arabidopsis*, thermostable forms consistently improved photosynthetic rate, growth, and heat tolerance (Kurek *et al.*, 2007; Kumar *et al.*, 2009). Overexpressing maize Rca in rice enhanced Rubisco activation under low light and accelerated induction after light increases (Yamori *et al.*, 2012). These benefits are amplified when Rca is co-overexpressed with Rubisco. In rice, a typical C_3 crop, combined overexpression of native Rubisco with maize Rca—a C_4 species adapted to warmer conditions—improved CO_2 assimilation and increased biomass by up to 26% under both ambient and high temperatures compared with the wild type (Suganami *et al.*, 2021; Qu *et al.*, 2021), mitigating the reduced activation seen in Rubisco-only overexpression lines (Suzuki *et al.*, 2009). In microalgae, Rca overexpression in boosted lipid and biomass production by 40% (Wei *et al.*, 2017), highlighting its wide-range potential. Overall, Rca engineering is a proven method for sustaining Rubisco activation under elevated heat and dynamic light. When integrated with other Calvin cycle enhancements and sink-targeting strategies (see Section 5.2), it represents a key component of multi-gene approaches for climate-resilient photosynthesis.

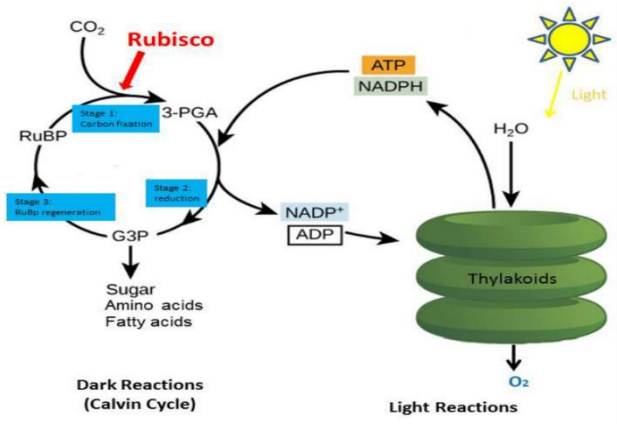

Figure 7 : Diagram of RuBP regeneration. Image taken from https://www.chess.cornell.edu/crystal-structure-type-iii-rubisco-complex-its-product-3-phosphoglycerate

4.3 Rubisco Engineering

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the primary CO_2-fixing enzyme of the Calvin–Benson–Bassham cycle, yet its slow catalytic turnover (*k*cat) and poor CO_2/O_2 discrimination impose major limitations, promoting energetically costly photorespiration. In C_3 crops, Rubisco can constitute up to 50% of soluble leaf protein and a substantial nitrogen investment, making it a prime target for photosynthetic improvement (Parry *et al.*, 2013).

One strategy to enhance Rubisco performance is protein engineering via site-directed mutagenesis or directed evolution. Rubisco-dependent *E. coli* selections have yielded variants with higher turnover or improved folding/assembly, though gains can trade off with CO_2/O_2 discrimination (Parikh *et al.*, 2006; Mueller-Cajar & Whitney, 2008a,b; Wilson *et al.*, 2018). A complementary approach exploits natural diversity through subunit swapping or heterologous expression. Assembling plant small subunits with algal large subunits in *Chlamydomonas* increased CO_2/O_2 specificity while retaining near-normal carbon fixation (Genkov *et al.*, 2010). In crops, interspecific Rubisco hybrids were created by expressing the sorghum small unit in rice raised Rubisco catalytic turnover but did not increase leaf CO_2 assimilation (Ishikawa *et al.*, 2011). More radically, non-native cyanobacterial Rubisco has been installed in tobacco plastids; the enzyme shows higher per-site carboxylation rates, but plants require elevated CO_2 and/or co-expressed assembly factors, underscoring compatibility constraints (Lin *et al.*, 2014b; Occhialini *et al.*, 2016). Collectively, these studies indicate that kinetic enhancement is possible, but benefits depend on balancing turnover with substrate discrimination and on solving assembly/activation in the plant chloroplast. Given these barriers, improving assembly and activation by engineering assembly and chaperone proteins has become a parallel focus. Overexpressing the BSD2 chaperone in maize and tobacco increased Rubisco content and photosynthetic rates (Aigner *et al.*, 2017), while co-expression of Rubisco Assimilation Factor 1 (RAF1) and related factors enabled functional assembly of foreign Rubisco in plants (Salesse-Smith *et al.*, 2018). Overall, no single modification resolves Rubisco's intrinsic limitations, as the most promising route integrates catalytic enhancement, leveraging natural diversity, and optimizing assembly and regulation. Given these limitations, nature has evolved carbon-concentrating mechanisms to overcome Rubisco's inefficiency.

4.4 Introduction of Carbon-Concentrating Mechanisms

Rubisco is catalytically slow and, at its active sites, O_2 competes with CO_2, leading to the oxygenation of RuBP and substantial photorespiration, which can reduce net photosynthetic carbon gain by up to 30% under current atmospheric conditions (Walker *et al.*, 2016). Many photosynthetic organisms have independently evolved carbon-concentrating mechanisms (CCMs) to elevate the concentration of CO_2 at the site of carboxylation, thereby suppressing Rubisco's oxygenase activity and enhancing photosynthetic efficiency (Atkinson *et al.*, 2016). In nature, these mechanisms range from biophysical systems in cyanobacteria and algae (cCCMs, pCCMs) to biochemical pathways in higher plants such as C_4 and crassulacean acid metabolism (CAM) photosynthesis. Modelling predicts that installing a functional CCM into C_3 crops could increase photosynthetic efficiency by 30–60% (McGrath & Long, 2014). Recent and more conservative estimates suggest

that introducing a cyanobacterial CCM in wheat could raise yield by ~8% under current climate conditions (Wu *et al.*, 2023). Although the genetic and structural complexity of these systems makes engineering them into crops challenging, CCM installation remains a high-priority target within broader efforts to optimize the carbon reactions of photosynthesis.

Biophysical CCMs (cCCMs and pCCMs)

Biophysical CCMs are widespread in cyanobacteria and many eukaryotic algae, where they function to elevate CO_2 concentrations at the site of Rubisco carboxylation. This is done by transporting and accumulating inorganic carbon (Ci) in the chloroplast or the cytosol—often via active HCO_3^- uptake—and by localizing carbonic anhydrase (CA) within specialized microcompartments containing Rubisco, called carboxysomes (cyanobacteria) and pyrenoids (algae). Inside these compartments, CA converts HCO_3^- to CO_2 around Rubisco, while a surrounding protein shell (carboxysomes) or a starch sheath (pyrenoids) limit CO_2 leakage. Together, CA localization and diffusion barriers create a high-CO_2, low-O_2 microenvironment that suppresses oxygenation and photorespiration (Badger & Price, 2003; Atkinson *et al.*, 2016).

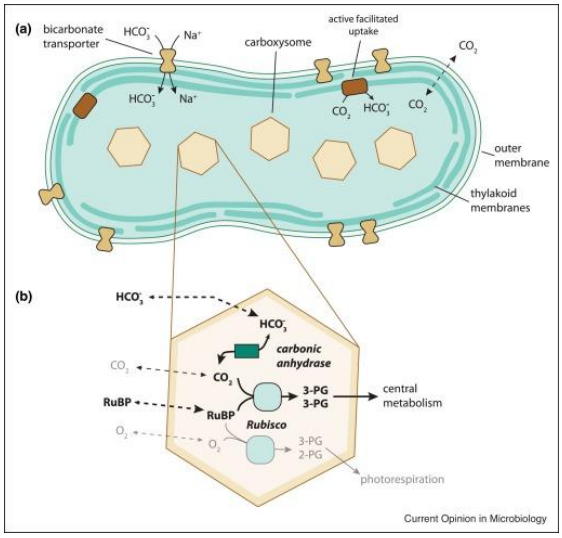

Figure 8 : Diagram of a cyanobacterial carboxysome. Image taken from Borden et al., (2021).

Carboxysomes are small proteinaceous microcompartments found in chloroplast of cyanobacteria and some chemoautotrophs, encapsulating Rubisco along with CA and structural/scaffold proteins such as CcmM, CcmN, and CcmK. The surrounding protein shell limits CO_2 diffusion and leakage, while active bicarbonate transporters accumulate HCO_3^- inside the cytosol. Within the carboxysome, CA dehydrates HCO_3^- around Rubisco, rising local CO_2 concentrations by up to 1000-fold (Badger & Price, 2003).

Engineering efforts have successfully expressed individual cyanobacterial bicarbonate transporters in C_3 plants. Despite recent uncertainty regarding its transporter function (Johnson, 2022), *ictB* has been targeted to the chloroplast inner envelope in *Arabidopsis*, rice, and soybean, with its expression leading to increased photosynthesis and yield (Lieman-Hurwitz *et al.*, 2003; Gong *et al.*, 2015; Hay *et al.*, 2017). Aiming to introduce cCCMs into crops, more advanced work has achieved partial assembly of carboxysomes in tobacco chloroplasts (Lin *et al.*, 2014a), for example by replacing native Rubisco subunits with cyanobacterial Form-1A Rubisco subunits and co-expressing essential shell and scaffold proteins (Long *et al.*, 2018). The additional inclusion of cyanobacterial Rubisco activase components (CbbQ, CbbO) further enhanced CO_2 fixation in synthetic carboxysomes (Chen *et al.*, 2022). However, full functionality under ambient CO_2 has not yet been demonstrated, in part due to the need to remove or suppress stromal CA to allow HCO_3^- accumulation inside the carboxysome (Price *et al.*, 2011) and introduce active bicarbonate transporters in the chloroplast membrane to increase HCO_3^- concentrations in the stroma around the carboxysome (Price *et al.*, 2013).

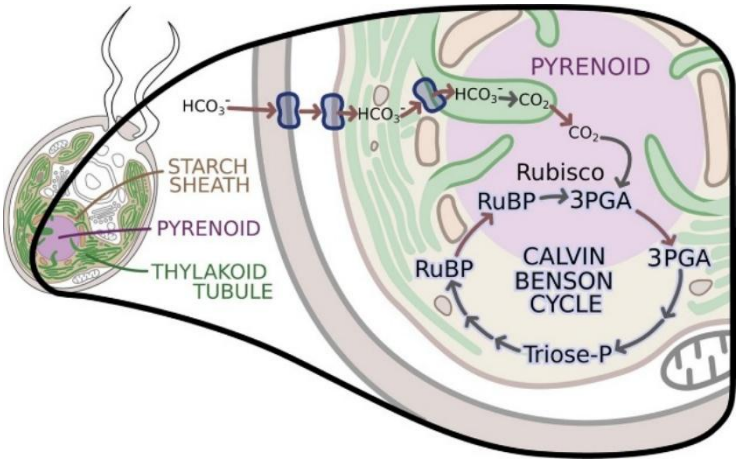

Figure 9 : Diagram of an algal pyrenoid. Image taken from https://www.energy.gov/sites/default/files/2023-04/beto-09-project-peer-review-algae-a-apr-2023-reardon%20.pdf

In many green algae, Rubisco is concentrated into a pyrenoid, a liquid-like phase-separated condensate (or matrix) organized by the linker protein EPYC1 (Essential Pyrenoid Component 1), that is enveloped in a starch sheath and traversed by thylakoid tubules. Localized in the thylakoid lumen, CAH3—a *C. reinhardtii*-specific isoform of CA—dehydrates HCO_3^-, and with the surrounding starch sheath limiting back-diffusion to the stroma, the resulting CO_2 diffuses into the pyrenoid matrix, therefore elevating concentrations near Rubisco (Mackinder, 2018; He *et al.*, 2020, 2023). While the enrichment achieved by pCCMs—around 40-fold—is typically lower than cCCMs, it is nonetheless highly effective in suppressing photorespiration, as modelling suggests that their introduction into C_3 chloroplasts could triple CO_2 assimilation rates (Fei *et al.*, 2022). Progress towards implementing pCCMs in higher plants has been promising. The combined expression of EPYC1 and of a plant-algal Rubisco hybrid successfully induced the condensation of Rubisco into proto-pyrenoid structures (Meyer *et al.*, 2020; Atkinson *et al.*, 2020). This suggests that phase separation can be reproduced in vascular plants, potentially avoiding the need for active bicarbonate pumps or removing stromal CA, which may make pCCMs more compatible with C_3 plant physiology. However, full functional reconstitution requires additional components, including CAH3 targeting, thylakoid tubule integration, and an effective CO_2 diffusion barrier. These remain significant engineering challenges, and it is unclear whether pyrenoid-based systems in terrestrial crops could achieve the same CO_2 concentrations as their aquatic counterparts.

Biochemical CCMs (C_4 and CAM)

Biochemical CCMs operate by spatially or temporally separating the initial fixation of inorganic carbon from Rubisco carboxylation. This separation, combined with the action of phosphoenolpyruvate carboxylase (PEPC) and decarboxylating enzymes, results in a localized increase in CO_2 concentration at the site of Rubisco, thereby suppressing photorespiration (Sage *et al.*, 2012). Unlike biophysical CCMs, which rely on intracellular microcompartments, biochemical CCMs require extensive changes to plant metabolism and, in some cases, leaf anatomy.

C_4 photosynthesis is characterized by the Kranz anatomy (Sedelnikova *et al.*, 2018), a dual-cell arrangement in which atmospheric CO_2 is initially fixed in mesophyll cells by PEPC into four-carbon (C_4) acids (e.g., malate, aspartate). These C_4 acids are transported to bundle sheath cells, where they are decarboxylated, depending on the plant subtype, by either NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), or phosphoenolpyruvate carboxykinase (PEPCK) to release CO_2 around Rubisco. This mechanism can elevate CO_2 concentrations at Rubisco up to 10-fold relative to ambient levels, greatly reducing oxygenation (von Caemmerer & Furbank, 2016). Engineering C_4 traits into C_3 crops is a long-standing goal exemplified by the C4Rice Project (https://c4rice.com/).

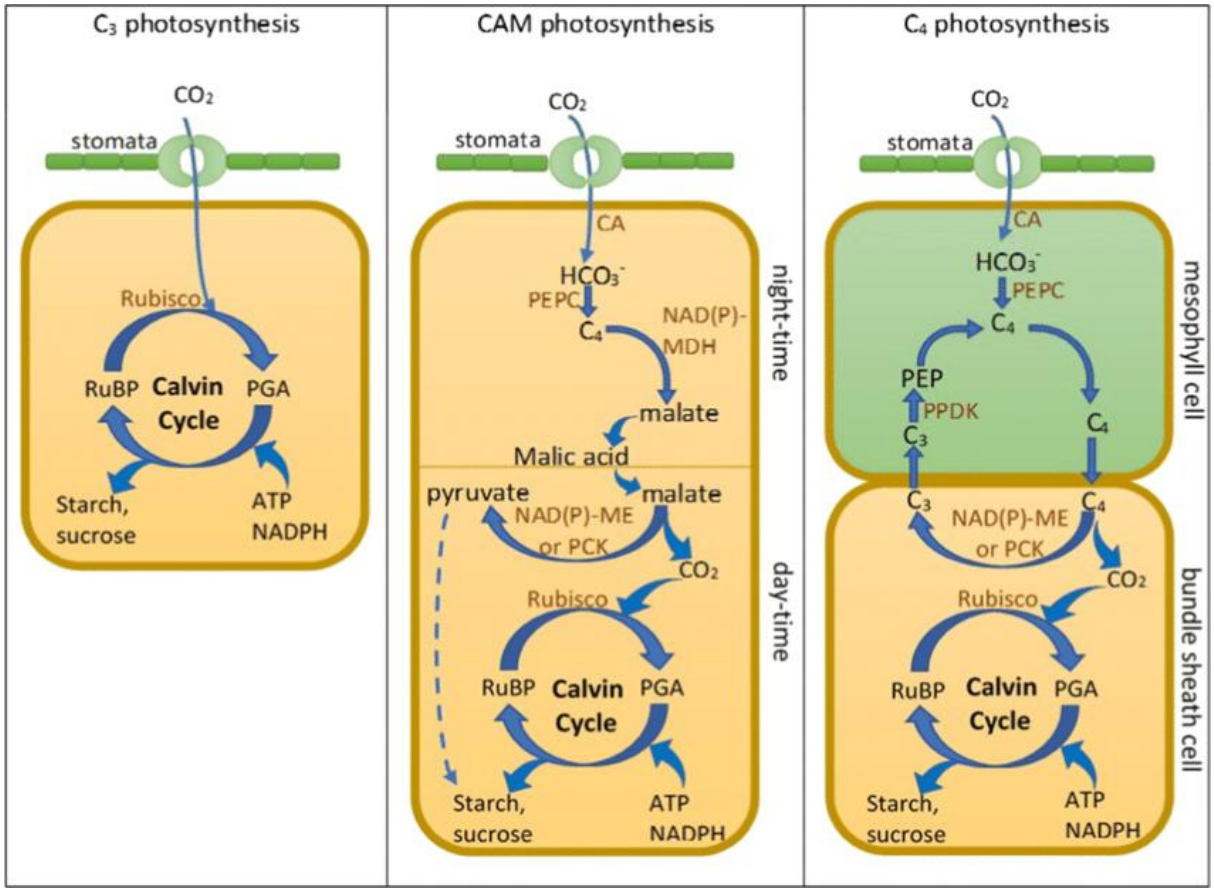

Figure 10 : C3, C4 and CAM photosynthesis. Image taken from Dehigaspitiya et al. (2019).

Progress has been made in co-expressing multiple C_4 enzymes—PEPC, pyruvate phosphate dikinase (PPDK), NADP-ME, NADP-malate dehydrogenase (MDH), and CA—with correct subcellular localization in rice (Ermakova *et al.*, 2020). However, while modelling predicts potential yield gains of 20%, the anatomical modifications required to establish Kranz anatomy, along with appropriate cell-specific expression of C_4 transporters and regulators remain a major obstacle (Schuler *et al.*, 2016; Wang *et al.*, 2021). Moreover, C_4's advantage diminishes under elevated atmospheric CO_2, making its benefits context-dependent (Singer *et al.*, 2019). C_3–C_4 intermediates are plant species whose photosynthetic metabolism and anatomy show features of both C_3 and C_4 pathways, representing evolutionary "transition states" between the two photosynthetic types. These achieve a moderate CO_2 concentrating effect through a "glycine shuttle," in which photorespiratory glycine produced in mesophyll cells is metabolized in bundle sheath cells. This process releases CO_2 close to Rubisco, increasing carboxylation efficiency without the full metabolic and anatomical complexity of C_4 photosynthesis (Kubis & Bar-Even, 2019). Modelling suggests that engineered C_3–C_4 intermediates could provide yield benefits in a wider range of climates than C_4 plants (Bellasio & Farquhar, 2019). Crassulacean acid metabolism (CAM) is a temporal CCM in which CO_2 uptake occurs at night through PEPC, producing malate that is stored in the vacuole. During the day, stomata close to conserve water, and malate is decarboxylated by NADP-ME, NAD-ME, or PEPCK, releasing CO_2 for the CBB cycle, which increases water-use efficiency (WUE) by 20–80%, making CAM photosynthesis highly advantageous in arid environments (Borland *et al.*, 2009, 2014; Kubis & Bar-Even, 2019). Engineering CAM

into C_3 crops is challenging, as it requires precise circadian regulation of PEPC, malate transporters, and decarboxylases, as well as modifications to vacuolar capacity. However, the existence of C_3–CAM intermediates suggests that partial CAM traits could be introduced incrementally (Borland *et al.*, 2011).

CCMs—whether biophysical or biochemical—offer substantial potential for improving photosynthetic efficiency and yield in C_3 crops. Biophysical systems could, in principle, increase CO_2 concentrations and may integrate with existing chloroplast architecture, though they require precise assembly of complex protein structures. Biochemical CCMs (C_4, CAM) are proven in nature but necessitate large-scale anatomical and regulatory changes. Hybrid strategies, such as combining bicarbonate pumps with pyrenoid formation or engineering C_3–C_4 intermediates, may offer more immediate yield gains. Given the multi-gene complexity and physiological integration required, a stepwise engineering approach, with extensive field testing, is considered essential for the successful deployment of CCMs in agriculture.

4.5 Rewiring/Redesigning Photorespiration

Photorespiration imposes a major cost in C_3 plants because Rubisco frequently fixes O_2 instead of CO_2, generating 2-phosphoglycolate (2-PG), a toxic metabolite that must be recycled via an energetically expensive, multi-organelle pathway. This process can reduce net photosynthetic output by up to 50% under current atmospheric conditions (South *et al.*, 2019). Consequently, synthetic bypasses that process glycolate more efficiently have become a major focus of photosynthetic engineering, with models suggesting that more efficient recycling could raise gross photosynthesis by 12–55% (Peterhansel & Maurino, 2011; Walker *et al.*, 2016).

Current bypass designs fall into three functional classes : (i) CO_2-releasing routes, shorten recycling but release CO_2, resulting in net carbon loss (e.g., GDH and GOC pathways); (ii) carbon-conserving routes, which recover glycolate without net carbon loss (e.g., synthetic glycolaldehyde bypass); and (iii) synthetic carbon-positive cycles (e.g., CETCH pathway), that could, in principle, fix additional CO_2. One early demonstration of CO_2-releasing routes (i) involved the Escherichia coli glycolate dehydrogenase (GDH) pathway, which enables the direct use of glycolate as a carbon and energy source. Its introduction into Arabidopsis confined glycolate metabolism entirely within the chloroplast, bypassing native recycling and enhancing photosynthetic performance (Kebeish *et al.*, 2007). Similarly, a new chloroplastic photorespiratory bypass—the GOC pathway—was designed in rice by introducing glycolate oxidase, oxalate oxidase, and catalase, which enabled direct glycolate metabolism within the chloroplast, improving photosynthetic efficiency, biomass yield, and nitrogen content under both greenhouse and field conditions (Shen *et al.*, 2019b). Building on these advances, three distinct bypass designs were tested in field-grown tobacco. The most successful combined glycolate dehydrogenase (GDH) with additional enzymes to form a complete chloroplastic glycolate oxidation cycle, together with RNAi downregulation of the chloroplast glycolate transporter Plgg1 to block flux through the native photorespiratory pathway and redirect it into the synthetic bypass. This construct increased biomass by up to ~40% and significantly improved water-use efficiency (South *et al.*, 2019). These studies provided the first clear evidence that synthetic photorespiration

can deliver benefits under agronomic conditions and that it can be redesigned for yield gains in crops. Carbon-conserving photorespiratory bypasses (ii) aim to eliminate CO_2 loss entirely. One approach engineered enzymes to convert glycolate into glycolaldehyde, which was then condensed with Calvin cycle intermediates to regenerate RuBP, consuming NADPH and ATP in the process and leading to RuBP accumulation. While it remains to be tested in plants, this strategy provided proof-of-principle for a CO_2-neutral bypass, offering potential to boost photosynthesis under diverse conditions and illustrating the future and long-term potential of synthetic biology (Trudeau *et al.*, 2018; Kubis & Bar-Even, 2019).

al., 2016). Although photorespiration bypasses are among the most advanced photosynthetic modifications and have been field-validated for yield benefits, their translation to crops will require species-specific optimization. Looking ahead, such bypasses must be integrated with Calvin cycle capacity, Rubisco performance, and sink strength to ensure assimilates are effectively utilized. In this sense, bypass engineering is not a standalone solution but part of a coordinated redesign of carbon metabolism—one that complements enzyme engineering (Section 4.1) and Rubisco optimization (Section 4.3), while being integrated into broader multigene stacking strategies (Section 5.3).

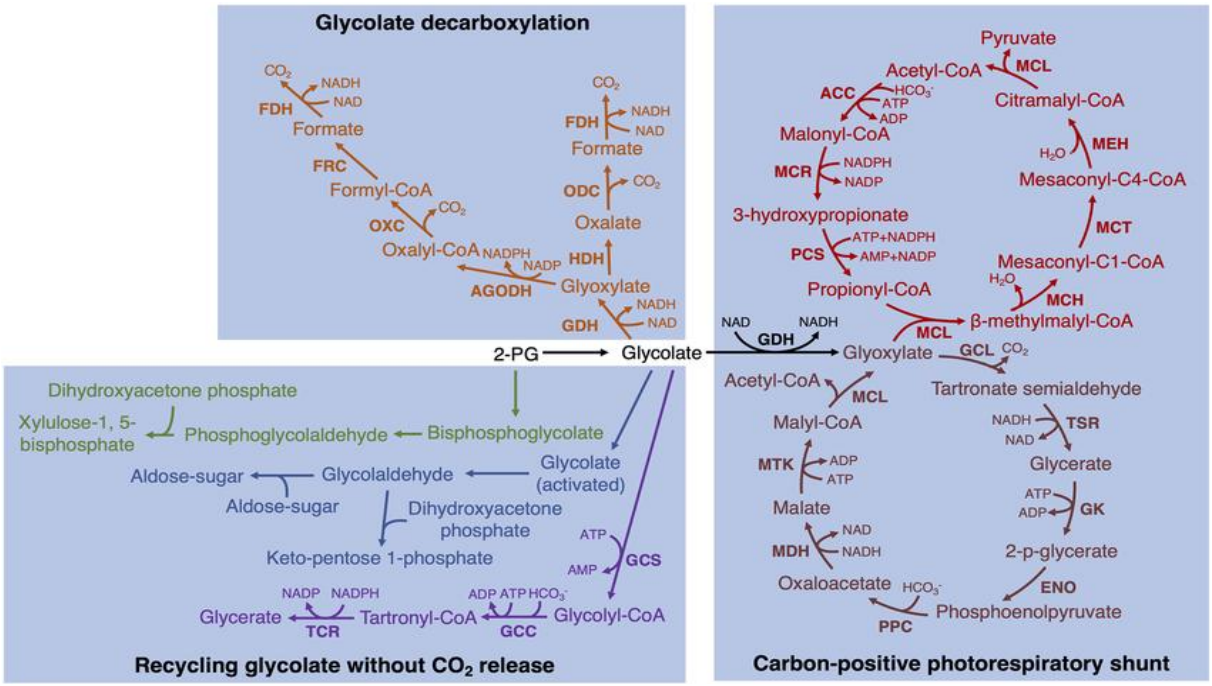

Figure 11 : Diagram of multiple Synthetic Bypasses of photorespiration. Image taken from Wang et al. (2022).

As a longer-term prospect, fully synthetic carbon-positive cycles (iii) have been assembled in vitro. The most advanced, the CETCH pathway, combines 17 enzymes from nine different organisms to catalyse the reductive carboxylation of enoyl-CoA esters, efficiently converting CO_2 into glyoxylate and demonstrating the feasibility of replacing or augmenting the CBB cycle with entirely artificial routes (Schwander *et*

5. Additional Strategies

5.1 Improving Gas Exchange and Water Use

Photosynthetic efficiency in C_3 plants is constrained by CO_2 assimilation and diffusion into the leaf and chloroplasts. This process depends on stomatal conductance (g_s), which regulates CO_2 entry through stomata, and mesophyll conductance (g_m), governing diffusion to the chloroplast stroma. Together they influence both photosynthetic rates and water-use efficiency (WUE), since CO_2 uptake is coupled to transpirational water loss (Lawson & Blatt, 2014). Moreover, stomatal constraints have been shown to be a major limiting factor for photosynthesis under certain stress conditions (Li *et al.*, 2023). Mesophyll conductance (g_m) can be improved by altering both molecular and anatomical traits. For example, overexpression of aquaporins—which facilitate CO_2 transport—such as *NtAQP1* or *OsPIP1;2* has enhanced CO_2 diffusion, photosynthesis, and biomass accumulation, particularly under elevated CO_2 (Aharon *et al.*, 2003; Flexas *et al.*, 2006; Xu *et al.*, 2019). Structural strategies aim to expand the chloroplast surface exposed to intercellular airspaces or to alter cell wall properties, thereby reducing resistance to CO_2 diffusion. Modifying leaf anatomy by increasing the number of mesophyll cells through manipulation of genes such as the rice *NAL1* or *ERECTA* in *Arabidopsis* also improved g_m, resulting in enhanced photosynthesis (Li *et al.*, 2023). Similar results were observed after cell-specific overexpression or down-regulation of certain regulators involved in leaf development (*KRP1*, *RBR1*), which modified cell density and arrangement (Lehmeier *et al.*, 2017). Cell wall composition has also been shown to affect porosity and diffusion capacity, representing future research directions in this field (Ellsworth *et al.*, 2018).

Stomatal conductance (g_s) has been targeted through density and kinetics. Changes in stomatal density (SD) can be achieved by altering the expression of genes involved in stomatal development and patterning pathways. Overexpression of *EPF1/EPF2* reduces SD, improving drought tolerance and WUE without limiting carbon assimilation (Caine *et al.*, 2019; Dunn *et al.*, 2019). Conversely, increasing SD via *EPFL9/STOMAGEN* enhances CO_2 uptake but reduces WUE (Tanaka *et al.*, 2013). Stomatal kinetics are also an important factor limiting photosynthesis: slow opening reduces assimilation by ~10%, while slow closure wastes up to 50% of transpirational water (McAusland *et al.*, 2016). While stomatal traits have traditionally received greater attention, several reviews emphasize that mesophyll conductance may represent an equally or even more significant limitation in many C_3 crops, particularly under elevated CO_2, making it a critical but often overlooked target for future engineering (Flexas *et al.*, 2012). Optimizing g_s and g_m therefore represents an emerging strategy not only for improving photosynthesis, but also for enhancing resilience to climate stress, since water-use efficiency, CO_2 uptake, and drought tolerance are directly linked to conductance traits.

5.2 Source–Sink Balance and Sink Strengthening

Another critical determinant of productivity is source–sink balance, encompassing both growth sinks (e.g., developing grains, roots, panicles) and storage sinks (e.g., starch or soluble sugars). Even when carbon fixation is enhanced, gains in photosynthetic efficiency can be limited if sink strength is insufficient, leading to carbohydrate accumulation in leaves and feedback inhibition of the CBB cycle (Paul & Foyer,

2001; Smith & Stitt, 2007). Key strategies to strengthen sink capacity involve improving assimilate export or expanding storage capacity by increasing sink size or number. Assimilate export enhancements have been achieved by overexpressing sucrose-phosphate synthase (SPS)—which catalyses the synthesis of sucrose-6-phosphate, an intermediate metabolite in sucrose production and catabolism—or by upregulating phloem loaders, which improved yield and sink strength (Singer et al., 2020). Increasing sink size or number, for instance through cytokinin-mediated modulation of panicle or seed development, has boosted grain number and size (Ashikari *et al.*, 2005). Metabolic engineering of storage capacity has also elevated starch accumulation and seed weight, including overexpression or midification of ADP-glucose pyrophosphorylase (AGPase), which catalyses the rate-limiting step of starch biosynthesis (Smidansky *et al.*, 2002, 2003, 2007).

However, the key point of sink strengthening is integration. As is often the case, the largest benefits arise when source and sink traits are engineered together: pairing photosynthetic improvements with sink-targeted modifications has produced sustained biomass and yield increases in field-grown crops, highlighting the importance of source-sink balance (Chang *et al.*, 2017; Sonnewald & Fernie, 2018).

5.3 Multigene Stacking & Coordinated Multigene Engineering

Generally, single-gene modifications in photosynthesis deliver modest gains and, since control of energy conversion and carbon flux is distributed across multiple components, such interventions often shift the limitations elsewhere. To achieve durable improvements, current strategies are moving toward multigene stacking and coordinated engineering, where multiple complementary targets are modified simultaneously to optimize pathways, accelerate protective responses, integrate new functions, and relieve sequential bottlenecks (Ort *et al.*, 2015; Leister, 2023).

Proof-of-concept studies illustrate this potential. In the light reactions, co-overexpression of three photoprotective proteins using the VPZ approach (see Section 3.3) accelerated NPQ relaxation, leading to increased biomass or seed yield under field conditions (Kromdijk *et al.*, 2016; De Souza *et al.*, 2022). Multigene approaches are often employed in the CBB cycle. Stacking SBPase with FBPA produces remarkable additive effects in tobacco, as their combined overexpression significantly enhanced carbon assimilation and increased biomass by 62% compared to the 34% gain observed when SBPase is expressed alone (Simkin *et al.*, 2017a), while pairing SBPase with cytosolic FBPase has also been shown to improve growth (Li *et al.*, 2022). Despite these successes, the effectiveness of these strategies can vary, as co-overexpression of SBPase and FBPA was found to reduce water use efficiency due to increased stomatal conductance (Simkin *et al.*, 2015). Species-specific outcomes have also been reported, with positive effects in tobacco but limited improvements in rice under optimal conditions when SBPase is co-overexpressed with Rubisco and Rca (Suzuki *et al.*, 2019). Numerous studies confirm the benefits of cross-pathway combinatorial approaches, achieving enhanced RuBP regeneration by introducing proteins outside of the CBB cycle. For example, co-expressing SBPase and FBPA, together with either the photorespiratory enzyme GDC-H or the cyanobacterial protein *ictB*, increased photosynthesis and biomass, even under low light (Simkin *et al.*, 2015, 2017a). Combining CBB cycle and electron transport chain improvements also

yielded positive results, as co-overexpression of SBPase with the algal electron carrier cytochrome c_6 improved yield and water-use efficiency, boosting biomass by ~52% in field-grown tobacco (López-Calcagno *et al.*, 2020). Synthetic photorespiratory bypasses provide another example, requiring multiple enzymes assembled as functional modules, exemplified by the engineering of a complete chloroplastic glycolate oxidation cycle in tobacco that delivered substantial yield gains in field trials (South *et al.*, 2019), or the more advanced CETCH pathway, a 17-enzyme bypass that can convert CO_2 into glyoxylate, highlighting the future potential of such strategies (Schwander *et al.*, 2016). Progress toward installing C_4 photosynthesis in rice also relies on stacking, with the correctly localized co-expression of five core enzymes from a single construct representing a milestone in complex trait transfer (Ermakova *et al.*, 2020). Beyond photosynthesis, stacking root growth regulators (*CKX3* and *AVP1*) produced additive gains in both root and shoot biomass (Vercruyssen *et al.*, 2011).

These successes highlight both the promise and the challenges of multigene engineering. However, outcomes depend on species-specific responses and precise stoichiometric balance, while field variability often limits predictability. Advances in transgene stacking technologies (single-promoter polycistronic constructs, CRISPR multiplexing) and predictive modelling will be crucial for designing effective combinations (Zhu *et al.*, 2010; Ort *et al.*, 2015). Ultimately, multigene and cross-pathway approaches represent the most powerful route to transform photosynthesis, integrating improvements across light capture, electron transport, carbon fixation, and photorespiration into coordinated, high-yielding systems. Multigene engineering acts as a framework to combine isolated strategies, serving as a stepping stone toward whole-plant redesign.

5.4 Whole-Plant Approaches

Beyond single-gene and coordinated multigene modifications, whole-plant strategies integrate molecular gains with canopy architecture, carbon allocation, and long-term breeding or crop design. The “smart canopy” concept emphasizes optimizing leaf angle and morphology to improve light distribution in dense stands. Erect leaves at the top of the canopy allow sunlight to penetrate to lower layers, while more horizontal lower leaves maximize light-capture and absorption. Modelling and field experiments in rice and wheat demonstrate that such architectures increase canopy-wide photosynthesis and yield (Drewry *et al.*, 2014; Ort *et al.*, 2015). In a recent breakthrough, the natural *lac1* mutant in maize was identified, which embodies the smart canopy architecture (Tian *et al.*, 2024). This mutant shows upright upper leaves, less erect mid-canopy leaves, and flat lower leaves, resulting in enhanced photosynthetic capacity and attenuated responses to shade under dense planting. To complement the benefits of leaf and plant architecture modifications, this concept could be combined with other molecular strategies targeting physiological constraints. For example, a “smart canopy” could be strengthened by reducing chlorophyll content (see Section 3.2) in the upper leaves to improve light penetration and reduce NPQ, while also incorporating far-red–absorbing chlorophylls f or d (see Section 3.1) into the shaded lower leaves to enable the use of the remaining FR light and improve light-use efficiency, further enhancing canopy-wide photosynthesis. Finally, de-novo domestication and redomestication leverage genome editing to accelerate crop design. Using genetic tools, wild relatives can be rapidly domesticated to combine photosynthetic or stress-

tolerant traits with agronomic performance, while traditional crops can be re-domesticated to restore lost efficiency traits (Zsögön *et al.*, 2018; Fernie & Yan, 2019). Together, these plant-level strategies complement molecular approaches, underscoring that durable gains will only be achieved through integration across scales.

5.5 Enhancement by Nanomaterials

Nanomaterials represent a novel frontier for improving photosynthesis by extending the usable solar spectrum or by creating bio-nano hybrids. Certain compounds or nanoparticles can convert underutilized regions of the spectrum into photosynthetically active radiation. For example, quantum nanodots, aggregation-induced emission materials and conjugated polymers (CPs) are nanomaterials that absorb UV or green light and emit blue or red light, which can then be used for photosynthesis (Leister, 2023). Indeed, aggregation-induced emission materials conjugated to chloroplasts enhanced ATP production (Bai *et al.*, 2021), while coating chloroplasts with conjugated polymers (CPs) expanded the absorption spectrum (Wang *et al.*, 2017), observations that resulted from or led to enhanced photosynthesis. Other CP-based nanoparticles increased O_2, NADPH, ATP production and growth in algae and Arabidopsis (Zeng *et al.*, 2021; Zhou *et al.*, 2022). Similarly, introduction of carbon quantum dots, which are nanoparticles small enough to enter chloroplasts, also improved photosynthesis in rice by boosting electron transport, ultimately leading to increased growth (Li *et al.*, 2021). Another line of work uses bio-nano hybrids, in which photosystem I (PSI) is coupled with abiotic catalysts or electrodes for photocurrent generation or hydrogen production. PSI–platinum hybrids are robust but costly, while more naturally abundant alternatives are still being explored. Attachment of plasmonic metal nanoparticles has also enhanced PSI absorption, even in the green spectral gap (Leister, 2019). Despite promising lab-scale results, translation to crops is still restrained by challenges of nanoparticle delivery, stability, cost, and potential toxicity to plants or ecosystems. Additionally, unlike genetic interventions that can be stably inherited, nanomaterial enhancements require external application, further limiting the current scalability of these approaches. While still largely experimental, nanomaterials demonstrate the potential to broaden spectral use and create artificial energy-harvesting systems, illustrating how synthetic and abiotic strategies could one day complement genetic engineering, expanding the toolkit for photosynthetic enhancement.

6. Discussion, Perspectives and Conclusion

Photosynthesis remains the fundamental bottleneck for crop productivity. Addressing its limitations is essential if agriculture is to meet the demands of food security and sustainability in a changing climate. Photosynthesis engineering has therefore progressed from single-gene interventions to increasingly complex strategies, spanning photosystem manipulation, NPQ kinetics and electron transport optimization, carbon fixation enhancement, photorespiration bypasses, and whole-plant redesigns. As shown in this monograph, each strategy addresses a specific inefficiency, but their true potential emerges only when they are considered together as parts of a tightly integrated system. Indeed, in almost every case, the approaches reviewed here show that improving one component often merely shifts the limitation elsewhere, thereby

reducing the overall benefit of these modifications. The challenge, therefore, is not simply to identify promising targets, but to integrate them coherently across scales.

particularly in smart canopy architectures (Section 5.4). Yet such adjustments only become significant when matched by stronger electron transport (Section 3.4)

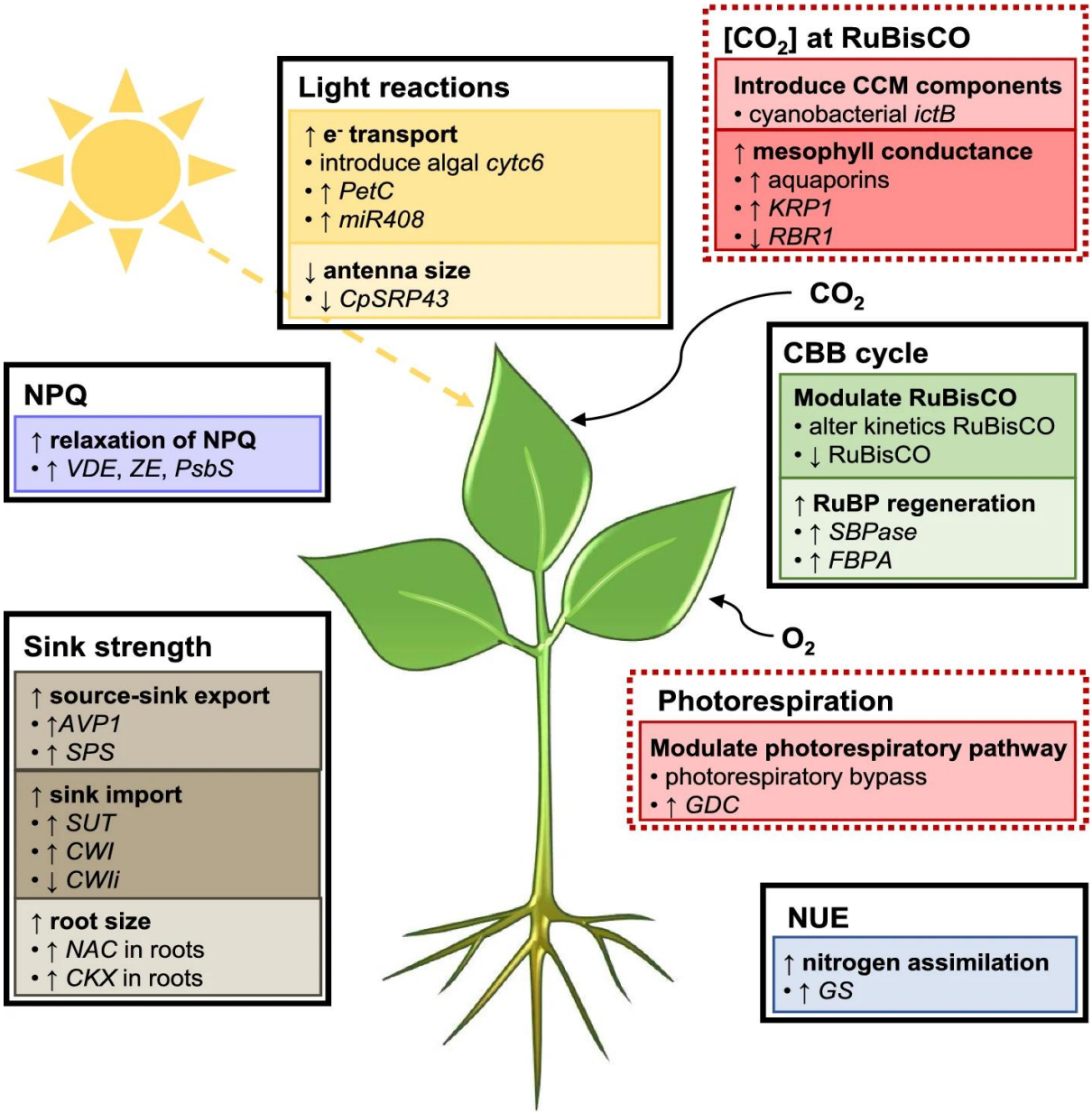

Figure 12 : Examples of biotechnological approaches for the improvement of photosynthetic efficiency in C_3 plants. Image taken from Singer et al. (2019).

Light-use strategies illustrate this interdependence. Reducing antenna size (Section 3.2) improves canopy light penetration but increases reliance on dynamic photoprotection, making accelerated NPQ kinetics and recovery (Section 3.3) essential. Spectrum broadening (Section 3.1) further complements these traits by allowing shaded leaves to exploit far-red photons,

and sufficient CBB cycle capacity. Likewise, enhancing Calvin cycle enzymes (Section 4.1), Rubisco (Section 4.3), or Rubisco activase (Section 4.2) will not deliver their full benefits unless coupled with mechanisms that reduce photorespiratory losses (Section 5.2) or concentrate CO_2 around Rubisco via emerging CCMs (4.4). Lastly, strengthening sinks and modulating source-sink balance (Section 5.4) will be

indispensable to effectively translate improvements in light-use efficiency and carbon assimilation into consistent, large-scale biomass and yield gains. However, each intervention carries inherent trade-offs: faster NPQ recovery may increase oxidative stress, while open stomata improve CO_2 uptake at the expense of water use. Such costs explain why evolution has favoured robustness and adaptability over maximum efficiency, and why engineering must anticipate risks as well as benefits when combining traits. These cross-links highlight the importance of multigene stacking and coordinated engineering (Section 5.3), which integrate multiple levers of efficiency into a balanced redesign, rather than incremental fixes.

Evidence confirms this systems logic. Field trials show that accelerating NPQ recovery, enhancing Calvin cycle enzymes, and installing synthetic bypasses can boost yield in real crops, while systems modelling predicts that relieving multiple bottlenecks simultaneously could raise efficiency by up to 60% (Zhu *et al.*, 2010; Ort *et al.*, 2015). Large initiatives such as the C_4 Rice Project and the RIPE program exemplify the scale of integration and coordination required to realize durable productivity gains. These multinational efforts combine multiple traits and approaches in field environments, demonstrating that photosynthesis can be engineered as a system. Yet outcomes vary sharply across species—antenna reduction succeeds in rice but fails in soybean, NPQ optimization helps tobacco but not potato—highlighting that context-specific optimization is indispensable (Leister, 2023). Together, experimental and theoretical work point to the same conclusion: durable gains will not come from isolated improvements, but from integration and coordinated redesign. Future perspectives build on this principle. In the near term, stacking validated traits with sink and canopy improvements offers realistic opportunities for yield gains. Medium-term prospects lie in partial and full CCMs, re-domestication, and canopy-level redesigns, while longer-term visions include synthetic carbon cycles and modular chloroplasts. As highlighted in recent reviews, the real challenge is to integrate these strategies in ways that not only raise efficiency but also enhance resilience to elevated CO_2, nutrient limitations, and climate stress (Simkin *et al.*, 2019; Singer *et al.*, 2019; Croce *et al.*, 2024). Predictive and systems modelling approaches will therefore be essential to identify synergistic combinations, balance trade-offs, and guide translation from lab to field.

Beyond specific traits, achieving these visions will also require advances in the tools that enable integration, as progress will depend on genomics and systems-level research. Genome sequencing and genome-wide association studies can reveal natural variation in photosynthetic traits, while transcriptomic, proteomic, and metabolomic datasets help identify regulatory bottlenecks and cross-talk with stress responses (Li *et al.*, 2023). Coupling these resources with genome editing and synthetic biology will allow precise tuning of multiple pathways simultaneously (Batista-Silva *et al.*, 2020). Combining omics with predictive models thus provides a roadmap to prioritize engineering targets and design balanced, resilient photosynthetic systems (Matthews, 2023).

The evidence is clear: the strategies reviewed in this monograph show that engineering photosynthesis is not about isolated breakthroughs, but about designing integrated systems that directly target the inefficiencies constraining crop productivity. The most promising path forward is to combine complementary traits across light capture, carbon fixation, and whole-plant physiology, guided by modelling and integrative omics

approaches, and enabled by synthetic biology and breeding. If pursued in this manner, photosynthetic engineering can deliver crops that are not only higher yielding but, most importantly, resilient and sustainable in the face of global change—securing one of the most powerful levers for future agriculture and global food security, and standing as a cornerstone for a second Green Revolution aimed at sustaining a growing global population.

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