Synthetic Rewiring of Plant CO2 Sequestration Galvanizes Plant Biomass Production

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question and at stake and with transparency about financial or other interests shaping the conversations. Further, the outcomes of public deliberations need to be taken into account by policymakers and integrated into formal decision-making processes.

Robust public engagement must also be global and inclusive, involving a range of publics whose voices have, to date, been overlooked or minimized [8]. While scientists’ contributions are important, their voices should not dominate; social values and implications must be at the center. Thus, in addition to scholars in the social sciences and humanities, legal and policy specialists, and other experts, deliberations must include a broad swath of organized civil society, with special attention to public interest organizations focused on women’s health, reproductive rights and justice, racial justice, environmental justice, gender equality, disability rights, and human rights.

Concluding Remarks

No decision about whether to pursue heritable human genome modification can be legitimate without broadly inclusive and substantively meaningful public engagement and empowerment. Such deliberations may be challenging and messy. They will take time and organizing them will necessitate creativity, hard work, and significant human and financial resources [9]. The course correction proposed here is essential to these efforts.

We must in the meantime respect the predominant policy position against pursuing heritable human genome modification, if we are to prevent individual scientists or small committees from making this momentous decision for us all. This will preserve time to cultivate an informed and engaged public that can consider and discuss the societal consequences of altering the genes of future generations and make wise, democratic decisions about the shared future we aspire to build.

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Synthetic Rewiring of Plant CO₂ Sequestration Galvanizes Plant Biomass Production

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Synthetically designed alternative photorespiratory pathways in tobacco and rice plants have paved the way to enhanced plant biomass production. Likewise, some in vitro- and in vivo-tested carbon-concentrating cycles hold promise to increase plant biomass. We hypothesize a further increase in plant productivity if photorespiratory bypasses are integrated with carbon-concentrating cycles in plants.

Nearly 123 Gt carbon per year (C yr\(^{-1}\)) are absorbed as CO\(_2\) by terrestrial vegetation through photosynthesis. Soil and vegetation respiration release CO\(_2\) back to the atmosphere (120 Gt C yr\(^{-1}\)) [1]. Anthropogenic emissions of 10 Gt C yr\(^{-1}\) imply 7 Gt C yr\(^{-1}\) as net carbon emissions, suggesting and supporting carbon hypersequestration in plants. Synthetic rewiring of plant carbon-assimilatory pathways and synthetically designed circuits in plants may enhance yield in crop plants [2]. Newly designed in vitro and in vivo synthetic switches and circuits (Table 1) improve biomass production in plants and redesign CO\(_2\) sequestration by: (i) bypassing photorespiration using alternative routes (AP strategy) [4,10,12]; and (ii) carbon-concentrating mechanisms (CCMs) [6,8].

**Bypassing Photorespiration: Glycolate Oxidation and Decarboxylation Strategies**

C3 plants such as wheat, rice, and soybean lose 30–50% of their photosynthetic conversion efficiency of light into biomass due to photorespiratory metabolism with concomitant oxygenation of ribulose 1,5-bisphosphate (RuBP) by the enzyme RuBP carboxylase–oxygenase (RuBisCO) [1,13]. In nature, the penalties of photorespiration are overcome by C4 (producing stable 4-C compounds) and crassulacean acid metabolism (CAM) plants, which fix CO\(_2\) efficiently prior to the Calvin cycle [12]. Modulating photorespiration for high-yield crops has long been envisaged ([12]; Table 1). Thus, Arabidopsis biomass was increased through photorespiratory bypassing in the chloroplast, implying later crop improvements [4].

Synthetic bypasses (Figure 1A) for alternative glycolate oxidation in tobacco (South strategy) [10] and glycolate decarboxylation in rice (Shen strategy) [11] have been used to enhance growth rates in crop plants. South and colleagues [10] designed three different bypasses (AP1, AP2, and AP3) in tobacco. Tobacco plants with a synthetic AP3 pathway are more efficient than those with native photorespiration (10; Figure 1A,B), relying on two different enzymes: glycolate dehydrogenase from Chlamydomonas reinhardtii and malate synthase from Cucurbita maxima (Figure 1B). The AP3 pathway utilizes endogenous pyruvate dehydrogenase for glycolate oxidation and release of CO\(_2\) close to the RuBisCO enzyme inside the chloroplast. The AP3 bypass is glycolate supplemented by inhibition of chloroplast glycolate export by RNAi suppression of plastidial glycolate/glycerate transporter 1 (PLGG1). This resulted in higher photosynthetic efficiency (around 40% compared with wild type; there is no general fixed rate for all plants) [10] and increased growth under field conditions (Figure 1B). However, the bypass promotes early vigor with no indication of how well it might work in mature leaves and individual variations are not yet known. Compared against the number of synthetic biology biomass augmentation studies done already in the laboratory, this study [10] is nevertheless the most relevant example of synthetic biology in plants in a field study. Shen and colleagues [11] implemented an alternative decarboxylation strategy, combining glycolate oxidase, oxalate oxidase, and catalase (GOC strategy). They redirected native enzymes to the chloroplast that ordinarily

| Table 1. Overview of the Design and Engineering of Synthetic CO\(_2\)-Fixating Carboxylases and Artificial Circuits |
| --- |
| **Strategy** | **Underlying effects** | **Refs** |
| Synthetically designed preventors of CO\(_2\)-release | Design of synthetic routes that bypass photorespiration without CO\(_2\) release | [3] |
| Engineering the CO\(_2\)-fixation pathway by increasing the CO\(_2\)-supply | Chloroplastic photorespiratory bypass increases CO\(_2\)-supply and hence increases photosynthesis and biomass production Increase in the intracellular acetyl-CoA pool provides a strategy to improve carbon fixation efficiency | [4] [5] |
| Engineering CO\(_2\)-fixation by modifying carboxylases | Replacing the native RuBisCO with cyanobacteria RuBisCO in tobacco enhanced growth rate of tobacco under elevated CO\(_2\) concentrations Enhanced thermotolerance of RuBisCO activase from Arabidopsis thaliana increased the stability of RuBisCO | [6] [7] |
| Synthetically designed CCM tested cycles | CETCHO comprises 17 enzymes originating from nine different organisms from all three kingdoms of life; synthetically designed alternative carboxylases are more efficient than naturally occurring CO\(_2\)-fixation cycles PyrS-PyrC-glyoxylate cycle and C4-PyrC-alanine MOG cycle are 2–3 times faster than the Calvin cycle | [8] [9] |
| Photorespiratory bypasses in crop plants | Three synthetically designed alternate pathways were introduced into the tobacco chloroplast for efficient recycling of glycolate. Also, RNAi suppressed the transport of glycolate out of the chloroplast Glycolate oxidase, oxalate oxidase, and catalase-based bypass culminates in decarboxylation of glycolate with the production of CO\(_2\) in rice | [10] [11] |
localize to the peroxisome in rice. These enzymes catalyze the decarboxylation of glycolate with production of CO₂. The plastid glycolate exporter was not silenced in this decarboxylation strategy; nevertheless, the biomass production bypassed native rice plants [11]. These bypasses culminate in higher biomass production yet show inconsistent outcomes for crop yield: calculations show that conversion of glycolate to CO₂ by bypasses underperforms compared with plants with native photorespiration [12]. However, the rice bypass

(Figure continued on next page.)
Figure 1. Integration of Synthetic Bypass for the Alternative Glycolate Oxidation Pathway (AP3) and the Artificially Engineered CO₂-Fixation Crotonyl-CoA/Ethylmalonyl-CoA/Hydroxybutyryl-CoA (CETCH) Cycle Holds Promise for Increased Plant Yield. Native photorespiration (A) competes with the Calvin cycle where RuBisCO acts on oxygen instead of CO₂, photorespiration requires ATP, and CO₂ is released from a carbon previously fixed through the Calvin cycle (left). This inefficiency impacts the yield potential of C₃ plants (right). Yield losses can be prevented through bypassing the photosynthetic pathway that should promote carboxylation by RuBisCO inside the chloroplast. The South strategy [10] (B) employs glycolate dehydrogenase from Chlamydomonas reinhardtii, malate synthase from Cucurbita maxima, and endogenous malic enzyme and pyruvate dehydrogenase for glycolate oxidation with release of CO₂ inside the chloroplast. Knockdown (black cross, left) of plastidial glycolate/glycerate transporter 1 (PLGG1) reduces the export of glycolate from the chloroplast and thus enhances glycolate consumption by the alternative bypass allowing carboxylation of CO₂ through RuBisCO with a concomitant yield increase (right). AP3-w/plgg1-FNA-CETCH plants (C) are expected to fix even more CO₂ at a faster rate. We anticipate a further increase in plant yield if the AP bypass (B) is integrated with an in vitro-realized CETCH cycle (adapted from [8]; third panel, right), which is a set of synthetically designed, efficient carboxylases (originating from nine different organisms of all three domains of life) that can fix CO₂ manifold times more than the native Calvin cycle (A). A feedback loop is expected where glycolate synthesized by CETCH will be utilized by the AP with release of CO₂ (blue dots). The released CO₂ will be efficiently re-fixed by CETCH-based RuBisCO in the combined cycle. This combination will liberate more carbon skeletons required for higher plant biomass production (C). Stoichiometries of the CETCH and AP3 cycles (D). Combining a highly efficient synthetic CO₂-fixation pathway, the CETCH cycle (adapted from [8]), with photosynthetic bypass pathways/synthetic glycolate pathways (adapted from [10]) is expected to increase biomass production and decrease the energy cost of photosynthesis. The CETCH cycle is designed to increase the rate of CO₂ fixation and this cycle can be connected to AP3 by its byproduct glyoxylate, which can be utilized back in pyruvate metabolism by the enzymes of AP3. In return, CO₂ produced in pyruvate metabolism can be fixed by the CETCH cycle. In this manner, two synthetic pathways are expected to feed each other and make use of their byproducts. Integration of these two pathways in plants holds the promise of higher CO₂ fixation rates and biomass production without the wasteful execution of photorespiration. Arrows indicate connectivity of reactions and summarize the balanced stoichiometry (details in [8,10]). Abbreviations: ccr, crotonyl-CoA carboxylase/reductase; ecm, ethylmalonyl-CoA mutase; epi, emC/mmC epimerase; fdh, formate dehydrogenase; hbd, 4-hydroxybutyryl-CoA dehydratase; kat, katalase; mco, methylmalonyl-CoA mutase; mct, methylsuccinyl-CoA oxidase; mco, propionyl-CoA oxidase; mcl, methylmalonyl-CoA lyase; pco, propionyl-CoA oxidase; PGA, 3-phosphoglycerate; pkk, polyphosphate kinase; RuBP, ribulose-1,5-biphosphate; ssr, succinic semialdehyde dehydrogenase.

[11] of glycolate decarboxylation resulted in a 40% increase in the size of mesophyll cells and almost doubled the chlorophyll content, unexplained and unexpected increases in biomass production. Synthetic bypasses shed light on unexplored functional roles of plant photorespiration. Ideally, bypassing should not negatively impact other metabolic processes; specifically, N cycling, which has a profound impact on crop production. However, the interdependence of photorespiration with nitrogen, carbon, and sulfur metabolism has pertinent consequences for plant growth and yield production. Apparently, in the case of the AP3 and GOC bypasses, the metabolic flux through endogenous photorespiration in the presence of the introduced cycles is sufficient to satisfy the demands of C₁, N, and S metabolism.
Detailed transcriptomics and metabolomics of plants (transgenic or mutants) with photorespiratory bypass will further explain the signaling and transcriptional basis of biomass increase in these plants. Moreover, these studies will help to elucidate the metabolic crosstalk in photorespiration.

The Introduction of CETCH in Plants as a Major CCM
A promising development for efficient fixation of CO$_2$ is the in vitro realization of the crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle ([8]; Figure 1). These 17 synthetically designed enzymes convert CO$_2$ into organic molecules at a rate of 5 nmol CO$_2$ min$^{-1}$ mg$^{-1}$ of core CETCH protein. The natural CBB cycle fixes CO$_2$ with a rate of only 1–3 nmol CO$_2$ min$^{-1}$ mg$^{-1}$ of the CBB proteins. The CETCH cycle was established with enzymes originating from nine different organisms of all three domains of life and optimized in several rounds by enzyme engineering and metabolic proofreading. The CETCH cycle requires less energy to operate than other aerobic CO$_2$-fixation pathways. One limitation of CETCH is the production of glyoxylate, a less active metabolic intermediate that requires acetyl-CoA (AcCoA) or propanoyl-CoA (PPCoA) for conversion into other metabolites. Also, glyoxylate is not well connected to other metabolic pathways. Despite functional impediments associated with any synthetically designed pathway, CETCH is the most efficient artificial cycle that fixes (in vitro) several-fold more CO$_2$ than does the natural CBB. The incorporation of CETCH-based enoyl-CoA carboxylase/reductase (ECRs) should be an excellent alternative to the native Calvin cycle. It can sequester approximately 80 CO$_2$ molecules per second (in vitro) compared with RuBisCO, which fixes two to five CO$_2$ molecules per second in plants.

However, for in vivo implementation, the cloning of heterologous genes encoding the 17 CETCH enzymes originating from nine different organisms is a tremendous task. Challenges include expression levels, enzyme activity, stability, localization, and regulation, as well as silencing of transgenes. It is much easier to test CETCH in vivo first in simpler organisms such as Escherichia coli to establish the kinetics, thermodynamics, and metabolic burden arising from the toxicity of engineered proteins or their reaction products and side products/byproducts. More recently, synthetic autotrophy in E. coli has been established by engineering a Calvin cycle as a source of carbon fixation in a normal heterotrophic strain [14]. Together with other recent successes in the area [3,8,10,11,15], this will pave the way to introduce CETCH into E. coli as a first step in the in vivo realization of the CETCH cycle. Next, model plants such as Arabidopsis or tobacco can be transformed with genes under the control of constitutive promoters (or native promoters of the genes of Calvin-cycle enzymes) to test the in planta efficiency of CETCH. To comprehend the effects of CETCH in plants, it should replace the endogenous Calvin cycle by the application of either RNAi or CRISPR–Cas9. Shutting down vital Calvin cycle enzymes is deleterious for plant growth, so it is possible only after successfully implantation of the CETCH cycle. Substantial optimization pertaining to CO$_2$ fixation efficiency, regulation of heterologous genes, and the nature of nutrients in soil would be needed to adjust the metabolic capacity of the plant after the introduction of CETCH.

Combining Bypasses with CCMs: Challenges and Prospects
The efficiency of C4 plants provides an impetus to design and implant efficient CO$_2$-fixation cycles and circuits to reduce losses due to futile cycles in plants. We hypothesize that the combination of a highly efficient CETCH cycle [8] and robust AP3 bypassing [10] will further enhance metabolic capacity and plant yield (Figure 1C) by producing carbon skeletons that will enhance both primary and secondary metabolism of the plant. We reassembled the stoichiometries pertaining to the CETCH and AP3 bypassing to explain the spatiotemporal compatibility between these two cycles inside the chloroplast of the transformed cell (Figure 1D). Accordingly, the CO$_2$ released by the conversion of glyoxylate to pyruvate as a function of the photorespiratory bypasses [10] will be efficiently fixed by the CETCH cycle. Likewise, the glyoxylate and malate produced by the CETCH cycle will be metabolized by the AP3 bypass. The two pathways should reinforce each other by consuming their products reciprocally, creating a positive feedback loop and reducing metabolic burden and extra energy expenditure to run the cycles in coherence, so they should be introduced into the chloroplast together (Figure 1D).

The anticipated AP3-w/Plgg1-RNAi-CETCH combinatorial design will be expected to quickly fix more CO$_2$ with better bypassing from photorespiration, resulting in higher fluxes of carbon metabolites, fast photosynthetic rates, and more biomass production. Despite it being fast in operation and far more efficient in fixing CO$_2$, establishing CETCH in plants is still a complicated task. However, there have been many successful attempts regarding the transformation of various plants with artificial synthetic cycles in recent years (Table 1). Alternatively, the high-efficiency CETCH cycle may be replaced with another, comparatively less energetic cycle, the Pyrs-Pyrc-glyoxylate or C4-Pyrc-alanine MOG (malonyl-CoA-oxaloacetate-glyoxylate) cycle [9], which contains fewer reactions than CETCH and still is 2–3 times faster than the Calvin cycle. The integration of the Pyrs-Pyrc-glyoxylate or C4-Pyrc-alanine MOG cycle with the AP3 cycle should be easier due to the smaller number of enzymatic reactions. However, all of these combinatorial
possibilities demand robust computational analysis, such as metabolic flux analysis (MBA) or flux variability analysis to theoretically model the whole concept and its metabolic feasibility in a complex biological system like plants before attempting its in vivo implementation.

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