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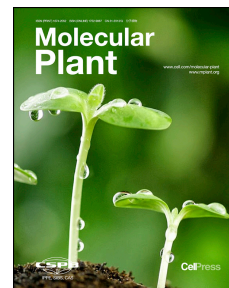
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1 **Improving photosynthetic efficiency toward food security: Strategies, advances, and**
2 **perspectives**

3
4 Edward N. Smith¹, Marvin van Aalst², Tiina Tosens³, Ülo Niinemets³, Benjamin Stich^{4*}, Tomas
5 Morosinotto⁵, Alessandro Alboresi⁵, Tobias Erb⁶, Paul A. Gómez-Coronado⁶, Dimitri Tolleter⁷,
6 Giovanni Finazzi⁷, Gilles Curien⁷, Matthias Heinemann¹, Oliver Ebenhöf², Julian M. Hibberd⁸,
7 Urte Schlüter⁹, Tianshu Sun⁸, Andreas P.M. Weber⁹

8
9 *Affiliations:*

10 ¹Faculty of Science and Engineering, Molecular Systems Biology — Groningen Biomolecular
11 Sciences and Biotechnology, Nijenborgh 4, 9747 AG Groningen, The Netherlands

12 ²Institute of Quantitative and Theoretical Biology, Cluster of Excellence on Plant Science
13 (CEPLAS), Heinrich-Heine-University, Universitätsstrasse 1, 40225 Duesseldorf, Germany

14 ³Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu,
15 51006 Estonia

16 ⁴Institute of Quantitative Genetics and Genomics of Plants, Cluster of Excellence on Plant Science
17 (CEPLAS), Heinrich-Heine-University, Universitätsstrasse 1, 40225 Duesseldorf, Germany

18 ⁵Department of Biology, University of Padova, 35131 Padova, Italy

19 ⁶Max Planck Institute for Terrestrial Microbiology, Department of Biochemistry & Synthetic
20 Metabolism, 35043 Marburg, Germany

21 ⁷Cell & Plant Physiology Laboratory, CEA-Grenoble, 38 054 Grenoble cedex 09, France

22 ⁸Molecular Physiology, Department of Plant Sciences, University of Cambridge, Cambridge, CB2
23 3EA, UK

24 ⁹Institute for Plant Biochemistry, Cluster of Excellence on Plant Science (CEPLAS), Heinrich-
25 Heine-University, Universitätsstrasse 1, 40225 Duesseldorf, Germany

26 *current address: Institute for Breeding Research on Agricultural Crops, Federal Research Centre
27 for Cultivated Plants, 18190 Sanitz, Germany

28
29 *Contact:*

30 Andreas P.M. Weber

31 aweber@hhu.de +49 211 8112347

32 Short Summary

33

34 The role of photosynthetic energy conversion efficiency in determining solar radiation
35 utilization for plant biomass generation is pivotal. Alongside other factors, it notably affects
36 crop yield. We here explore opportunities to enhance crop yields through modification of
37 photorespiration, optimizing light use, leveraging genetic variation in photosynthetic
38 parameters for breeding, and of adopting innovative new-to-nature synthetic biology
39 approaches.

Journal Pre-proof

40 Abstract

41 Photosynthesis in both crops and natural vegetation allows light energy to be converted into
42 chemical energy, and thus forms the foundation for almost all terrestrial trophic networks on Earth.
43 The efficiency of photosynthetic energy conversion plays a crucial role in determining the portion
44 of incident solar radiation that can be used to generate plant biomass throughout a growth season.
45 Consequently, alongside factors such as resource availability, crop management, crop selection,
46 maintenance costs, and intrinsic yield potential, photosynthetic energy use efficiency significantly
47 influences crop yield. Photosynthetic efficiency is relevant to sustainability and food security
48 because it impacts water-use efficiency, nutrient-use efficiency, and land-use efficiency. This
49 review focuses specifically on the potential for improvements in photosynthetic efficiency to drive
50 a sustainable increase in crop yields. We will discuss bypassing photorespiration, enhancing light
51 use efficiency, harnessing natural variation in photosynthetic parameters for breeding purposes,
52 and adopting new-to-nature approaches that show promise for achieving unprecedented gains in
53 photosynthetic efficiency.

54

55 Keywords

56 Photosynthesis; photorespiration; photorespiratory bypass; Natural variation; Synthetic biology;
57 plant metabolic engineering

58

59

60 Introduction

61

62 Photosynthesis harnesses the energy of visible light quanta to extract electrons from water,
63 utilizing them to convert atmospheric CO₂ into biomass. This crucial metabolic process originated
64 over 2 billion years ago in an atmosphere abundant in CO₂ and low in oxygen. Throughout
65 geological timescales, oxygenic photosynthesis caused a significant shift in the atmospheric O₂-
66 to-CO₂ ratio, resulting in a present 500-fold excess of oxygen over carbon dioxide. The enzyme
67 responsible for CO₂ fixation in the Calvin-Benson cycle (CBC), known as ribulose 1,5-
68 bisphosphate carboxylase/oxygenase (Rubisco), exhibits a higher affinity for CO₂ than for O₂.
69 However, under current atmospheric conditions, Rubisco frequently reacts with oxygen, leading
70 to the production of 3-phosphoglyceric acid (3PGA) and 2-phosphoglycolic acid (2PG). Notably,
71 2PG acts as an inhibitor for key enzymes in the CBC, namely triose phosphate isomerase and
72 sedoheptulose 1,7-bisphosphate phosphatase (Flügel et al., 2017).

73 The two carbon atoms present in 2PG cannot be further metabolized within the CBC. Instead, the
74 conversion of 2PG to 3PGA occurs via a metabolic pathway known as photorespiration (Bauwe,
75 2023). During photorespiration, one out of four carbon atoms contained in two molecules of 2PG
76 is released as CO₂, *i.e.*, previously fixed carbon is lost. Additionally, the process results in the
77 release of ammonia and the consumption of ATP and redox power. Overall, photorespiration
78 significantly diminishes the efficiency of carbon assimilation in C₃ plants, leading to yield losses
79 of approximately 30% or higher (Walker et al., 2016a). Despite its negative impact on
80 photosynthetic efficiency, photorespiration is an essential process that enables photosynthesis in
81 an oxygen-containing atmosphere through the breakdown of 2PG (Bauwe, 2023). Mutations
82 affecting photorespiration are typically lethal, even in the presence of carbon concentrating
83 mechanisms such as carboxysomes in cyanobacteria, pyrenoids in algae, or C₄ photosynthesis
84 in land plants (Eisenhut et al., 2008; Zelitch et al., 2009; Levey et al., 2019). The topic of
85 photorespiration has been extensively reviewed (Bauwe et al., 2010; Hodges et al., 2016;
86 Eisenhut et al., 2019; Fernie and Bauwe, 2020; Bauwe, 2023; Broncano et al., 2023), rendering
87 a detailed account of the pathway unnecessary in this work. Instead, we here focus on recent
88 approaches aiming at mitigating the impact of photorespiration on photosynthetic efficiency.

89 To mitigate the oxygenation reaction of Rubisco, land plants have evolved carbon concentrating
90 mechanisms, including C₄ and CAM photosynthesis. These pathways, however, are complex and
91 require specific leaf anatomy. Despite significant efforts, the introduction of these photosynthetic
92 subtypes into C₃ plants remains an unsolved challenge. Instead of reducing oxygenation, several
93 research groups have focused on improving efficiency of 2PG recovery designing and

94 implementing alternative pathways that bypass photorespiration, converting 2PG into CO₂,
95 intermediates of the CBC, or metabolites of C₄ photosynthesis. In this review, we will explore
96 these photorespiration bypasses and other strategies to enhance photosynthetic efficiency,
97 considering their potential contributions to increased crop yields. Additionally, we will examine
98 naturally occurring variations in photosynthetic efficiency and explore how such variations could
99 be leveraged for enhancing photosynthetic efficiency through breeding. Finally, we will discuss
100 the potential of new-to-nature pathways for increasing photosynthetic efficiency.

101

102 **Increasing Photosynthetic Efficiency through Photorespiratory Bypasses**

103

104 *Chloroplast-localized photorespiratory bypasses*

105

106 We will first describe the design and implementation of bypasses to photorespiration, focusing on
107 *in planta* experimentally validated approaches (Figure 1). We will then discuss how such
108 bypasses improve yield and novel strategies that have not yet been tested in plants. We will start
109 the discussion with the designs proposed by Kebeish et al. (2007) and Maier et al. (2012) since
110 most of the reported photorespiratory bypasses since 2012 are variations of these schemes.

111

112 *Intra-plastidic conversion of 2PG into 3PGA by glyoxylate carboligase and tartronate* 113 *semialdehyde reductase*

114

115 Kebeish et al. (2007) successfully introduced a bacterial glycolate metabolic pathway into the
116 chloroplasts of *Arabidopsis thaliana*, a process that involved the incorporation of five pathway
117 enzymes fused with chloroplast targeting peptides. The ability of certain bacteria to utilize
118 glycolate as the sole carbon source served as the basis for this implementation. Within the
119 bacterial glycolate pathway, glycolate dehydrogenase, composed of three subunits (GDH D, E,
120 and F), converts glycolate to glyoxylate. Subsequently, glyoxylate carboligase (GCL) catalyzes
121 the ligation of two glyoxylate molecules, resulting in the formation of tartronic semialdehyde and
122 the release of one molecule of CO₂. Tartronic semialdehyde is further transformed into glycerate
123 through the action of tartronic semialdehyde reductase (TSR). Native glycerate kinase converts
124 glycerate to 3PGA.

125

126 To establish the complete bacterial glycolate pathway, Kebeish et al. (2007) introduced the
127 corresponding pathway components using three different plasmids. Through genetic crossings of

128 lines expressing partial pathways, they eventually combined all the components to create the full
129 pathway. The transgenic Arabidopsis plants expressing the full pathway exhibited a two-fold
130 increase in shoot biomass and a three-fold increase in root biomass. The transgenics also
131 demonstrated a decrease in the glycine-to-serine ratio, which is indicative of reduced
132 photorespiratory flux. Moreover, the post-illumination burst of CO₂ release, a measure of
133 photorespiratory glycine in the light, was reduced.

134
135 Additionally, the lines expressing the complete bacterial pathway showed a minor reduction in
136 oxygen-inhibition of photosynthetic carbon assimilation and a slight decrease in the CO₂-
137 compensation point. These findings suggest that the implementation of the pathway resulted in a
138 modest elevation of CO₂ levels at the site of Rubisco. Intriguingly, transgenic lines expressing
139 only glycolate dehydrogenase D, E, and F also exhibited significant increases in biomass and
140 rosette diameter, along with decreased Gly/Ser ratios. However, the reason behind the improved
141 plant performance solely from the expression of bacterial glycolate dehydrogenase remains
142 unexplained.

143
144 *Intra-plastidic glycolate oxidation by glycolate oxidase*

145
146 Maier et al. (2012) developed an alternative photorespiratory bypass strategy by redirecting
147 peroxisomal enzymes to the chloroplasts. This pathway involves the complete oxidation of
148 glycolate to CO₂ within the chloroplast stroma. To achieve this, peroxisomal glycolate oxidase
149 was targeted to the chloroplasts, where it catalyzes the oxidation of glycolate to glyoxylate,
150 releasing hydrogen peroxide as a byproduct. The chloroplast-targeted catalase then dissipates
151 the hydrogen peroxide. Subsequently, glyoxylate and acetyl-CoA are condensed to malate by a
152 plastid-targeted malate synthase, an enzyme from the peroxisomal glyoxylate cycle. Malate is
153 decarboxylated by the native chloroplast NADP-malic enzyme, generating pyruvate and NADPH.
154 The resulting pyruvate is further decarboxylated by the native chloroplast pyruvate
155 dehydrogenase, yielding acetyl-CoA and NADH. Acetyl-CoA, along with another glyoxylate
156 molecule, re-enters the cycle through malate formation via malate synthase. Overall, this cycle
157 completely oxidizes the carbon present in glycolate and releases CO₂ within the chloroplast
158 stroma. Additionally, it generates NADPH and NADH as reducing equivalents. Unlike the pathway
159 described by Kebeish et al. (2007), the Maier et al. (2012) pathway requires the introduction of
160 only three transgenes.

161

162 Transgenic Arabidopsis plants expressing the glycolate oxidizing pathway showed significantly
163 increased rates of CO₂ assimilation and a decreased Gly/Ser ratio. However, in contrast to the
164 findings of Kebeish et al. (2007), the CO₂ compensation point remained unchanged. This result
165 is surprising considering that the local CO₂/O₂ ratio within the chloroplasts of these lines is
166 expected to be higher than in the lines of Kebeish et al. (2007). One of the two lines analyzed by
167 Maier et al. (2012) exhibited increased leaf fresh and dry weight, along with a reduction in leaf
168 thickness.

169

170 *Expression of glycolate dehydrogenase in chloroplasts*

171

172 Kebeish et al. (2007) observed that transgenic Arabidopsis plants expressing all three subunits
173 of *E. coli* glycolate dehydrogenase exhibited enhanced biomass accumulation and reduced flux
174 through the conventional photorespiration pathway. Building upon this discovery, Nölke et al.
175 (2014) created transgenic potato plants that were genetically modified to express a single
176 glycolate dehydrogenase polyprotein, wherein the D, E, and F subunits were linked by a
177 (Gly₄Ser)₃ linker sequence. The polyprotein was targeted to the chloroplasts using a Rubisco
178 small subunit targeting peptide (rbcS1).

179 The transgenic potato plants demonstrated elevated rates of CO₂ assimilation at a 400 ppm CO₂
180 concentration, decreased repression of CO₂ assimilation by O₂, and a lowered CO₂ compensation
181 point. Moreover, these transgenic lines exhibited increased above-ground biomass accumulation
182 compared to the control plants, with a more than twofold increase in tuber yield observed in lines
183 showing the highest glycolate dehydrogenase activity. The precise mechanism underlying the
184 augmented biomass production and yield in the transgenic plants was not extensively investigated
185 in this study. However, the authors put forward the hypothesis that chloroplast-produced glycolate
186 is decarboxylated by plastidial pyruvate dehydrogenase, resulting in a localized rise in CO₂
187 concentration at the Rubisco site.

188

189 *Combination of plastid-localized photorespiration bypasses with reduced export of glycolate from* 190 *chloroplasts*

191

192 South et al. (2019) conducted a comparative analysis of three alternative designs for
193 photorespiratory bypasses (AP1-3) in tobacco, a model crop, and evaluated the performance of
194 transgenic plants in field trials. AP1 corresponds to the pathway described by Kebeish et al.
195 (2007), AP2 is based on the pathway reported by Maier et al. (2012), and AP3 is a modified

196 version of the Maier et al. (2012) pathway. In AP3, the combination of peroxisomal glycolate
197 oxidase and catalase was replaced by mitochondrial glycolate dehydrogenase from *C. reinhardtii*,
198 which was retargeted to the chloroplasts. Unlike the bacterial glycolate dehydrogenase with its
199 three subunits, the algal mitochondrial enzyme consists of a single subunit and does not produce
200 H₂O₂ (the electron acceptor remains unknown), eliminating the need for co-expression of
201 catalase. Consequently, the AP3 design only requires two transgenes: glycolate dehydrogenase
202 and malate synthase.

203
204 Furthermore, all three alternative pathway designs were combined with a reduction in glycolate
205 export from chloroplasts by employing antisense repression of the chloroplastic
206 glycolate/glycerate transporter (PLGG1, Pick et al., 2013). T2 transformants were initially
207 screened under high light and low CO₂ conditions to identify lines that exhibited enhanced
208 protection against photorespiratory stress. This pre-selection step was crucial for identifying lines
209 with optimal expression levels and stoichiometry of the pathway components. In greenhouse
210 trials, all pathway designs were associated with increased biomass production, with AP2 and AP3
211 performing better when combined with repression of PLGG1. Subsequently, the AP3 design was
212 evaluated in replicated field trials, both with and without repression of PLGG1. Surprisingly,
213 contrary to the greenhouse experiments, the AP3 design performed best in the field without
214 repression of PLGG1. The field-grown AP3 lines exhibited significantly higher biomass
215 productivity, increased CO₂ assimilation rates, and decreased CO₂ compensation points.
216 Additionally, AP3 lines displayed elevated levels of glyoxylate, while serine and glycerate levels
217 were significantly reduced.

218
219 This study is significant as it validated previously reported bypass designs through a comparative
220 analysis in the same model system, demonstrating that growth benefits observed in a greenhouse
221 setting can be reproduced in the field. However, it is worth noting that lines expressing only a
222 chloroplast-targeted glycolate dehydrogenase from *C. reinhardtii* were not included in this
223 comparison. This omission is a limitation as it could have provided insights into whether the
224 improved performance observed for AP3 is truly dependent on the coordinated activity of glycolate
225 dehydrogenase and malate synthase or if, similar to Nölke et al. (2012), glycolate dehydrogenase
226 alone can confer growth benefits.

227
228 *Complete oxidation of glycolate via oxalate oxidase*

229

230 The study of Shen et al. (2019) describes a modified pathway, based on the work of Maier et al.
231 (2012), which enables complete intra-plastidic decarboxylation of glycolate. This pathway
232 involves the conversion of glycolate to oxalate through the action of rice glycolate oxidase isoform
233 3 (OsGLO3), previously demonstrated to oxidize both glycolate and glyoxylate (Zhang et al.,
234 2012). The resulting oxalate is then fully decarboxylated to CO₂ by oxalate oxidase (OsOXO3).
235 The accumulation of H₂O₂, a byproduct of glycolate and oxalate oxidation, is effectively eliminated
236 by a chloroplast-targeted catalase. Consequently, the implementation of this pathway
237 necessitates the incorporation of three transgenes encoding the chloroplast-targeted enzymes:
238 glycolate oxidase, oxalate oxidase, and catalase.

239 Rice plants expressing these genetic constructs exhibited enhanced photosynthetic performance,
240 characterized by a reduced CO₂ compensation point and a higher maximum photosynthetic rate
241 under saturating light conditions. Furthermore, the Gly/Ser ratio and glycolate levels decreased,
242 while glyoxylate and oxalate levels increased under ambient air conditions. Notably, the yield of
243 single plant seeds varied depending on the seeding season, with a 27% increase observed in
244 spring seeding but a yield penalty of 13-16% in fall seeding.

245 In another variation of the pathways proposed by Kebeish et al. (2007) and Maier et al. (2012),
246 Wang et al. (2020a) substituted the bacterial glycolate dehydrogenase component of the
247 glyoxylate carboligase route with the glycolate oxidase/catalase system in transgenic rice plants.
248 This approach effectively combined aspects of the two previously reported bypasses. The
249 transgenic plants exhibited improved photosynthetic parameters and yield in replicated field trials,
250 further confirming the potential of photorespiratory bypasses to enhance crop performance under
251 field conditions.

252

253 *Peroxisome-localized photorespiratory bypasses*

254

255 In the native photorespiration process, glycolate is transported from chloroplasts to peroxisomes.
256 Within the peroxisomes, glycolate undergoes oxidation by glycolate oxidase, resulting in the
257 production of glyoxylate. Subsequently, glyoxylate is transaminated to form glycine. The glycine
258 molecules are then transported to the mitochondria, where glycine decarboxylase and serine
259 hydroxymethyltransferase catalyze the conversion of two glycine molecules into serine, CO₂, and
260 ammonia. The generated serine is subsequently transported back to the peroxisomes, where it is
261 converted into hydroxypyruvate by the action of serine:glyoxylate aminotransferase.
262 Hydroxypyruvate is further reduced to glycerate, which is then transported back to the
263 chloroplasts, thereby completing the photorespiratory cycle. Given the crucial role of peroxisomes

264 in photorespiration, researchers have endeavored to develop strategies for bypassing the
265 ammonia and CO₂-releasing step that occurs in the mitochondria, through alternate glycolate
266 conversion within peroxisomes. Two such attempts have been documented in the literature.

267

268 *Peroxisomal conversion of glyoxylate to hydroxypyruvate*

269

270 This pathway design capitalizes on the conversion of glycolate to glyoxylate by the inherent
271 peroxisomal glycolate oxidase, aiming to redirect glyoxylate towards hydroxypyruvate through the
272 involvement of glyoxylate carboligase and hydroxypyruvate isomerase. Similar to the pathway
273 design proposed by Kebeish et al. (2007), bacterial glyoxylate carboligase is employed to convert
274 glyoxylate into tartronate semialdehyde, which is subsequently transformed into hydroxypyruvate
275 by hydroxypyruvate isomerase. Essentially, this strategy enables the retrieval of 75% of the
276 carbon content present in two glyoxylate molecules, while mitigating the release of ammonia
277 mediated by mitochondrial glycine decarboxylase. Transgenic tobacco plants carrying constructs
278 encoding peroxisome-targeted versions of these bacterial enzymes were generated.

279 Under non-photorespiratory conditions at high-CO₂, the transgenic plants exhibited robust growth.
280 However, when exposed to current ambient CO₂ conditions, the leaves displayed yellow lesions,
281 and the plants exhibited a chlorotic phenotype. Metabolic labeling experiments employing [¹⁴C]-
282 glycolate revealed that glycolate was still predominantly converted into glycine and subsequently
283 serine, suggesting that only a minor fraction of glycolate entered the engineered pathway.
284 Surprisingly, amino acid analysis indicated that the leaves of the transgenic plants contained
285 higher levels of glycine and serine compared to wild-type plants, contrary to initial expectations.
286 Notably, the researchers were unable to detect the presence of the hydroxypyruvate isomerase
287 protein in the transgenic plants through immunoblotting, despite the detectable expression of the
288 transgene confirmed by RNA gel blots. These findings suggest that the pathway may have been
289 incomplete, and the observed phenotypes could arise from the accumulation of undesired
290 tartronate semialdehyde in peroxisomes, exerting adverse effects.

291

292 *Peroxisomal glyoxylate to oxaloacetate conversion via the beta-hydroxyaspartate shunt*

293

294 In this study (Roell et al., 2021), a recently discovered microbial pathway involved in the
295 metabolism of glyoxylate was introduced into plant peroxisomes. The pathway, known as the β -
296 hydroxyaspartate cycle, encompasses four enzymatic steps that convert glyoxylate and glycine
297 into oxaloacetate. The sequential actions of β -hydroxyaspartate aldolase, β -hydroxyaspartate

298 dehydratase, and iminosuccinate reductase transform glyoxylate and glycine into β -
299 hydroxyaspartate, iminosuccinate, and aspartate, respectively. Finally, aspartate:glyoxylate
300 aminotransferase converts glyoxylate into glycine, and releases oxaloacetate as the end product
301 of the pathway. Transgenic Arabidopsis plants were engineered to express the pathway enzymes
302 fused with peroxisomal targeting signals. Promoters that drive gene expression specifically in
303 photosynthetic tissues were employed to prevent undesired pathway activity in non-
304 photosynthetic plant organs.

305 Roell et al. (2021) hypothesized that reducing the conversion of glyoxylate to glycine would
306 enhance metabolic flux through the β -hydroxyaspartate cycle. Therefore, apart from wild-type
307 Arabidopsis plants, the pathway was introduced into the genetic background of the *ggt1-1* mutant,
308 which lacks peroxisomal glutamate:glyoxylate aminotransferase 1 and exhibits a photorespiratory
309 phenotype under ambient air conditions. This enabled the investigation of the β -hydroxyaspartate
310 cycle's function by assessing its ability to complement the visual phenotype of the *ggt1-1* mutant.
311 Similar to the findings of Carvalho et al. (2011), wild-type plants expressing the β -
312 hydroxyaspartate cycle exhibited reduced growth and photosynthetic rates in ambient air.
313 However, when exposed to elevated CO₂ concentrations that suppress photorespiration, their
314 growth was comparable to that of wild-type controls. Importantly, the expression of the β -
315 hydroxyaspartate cycle in the *ggt1-1* mutant partially rescued the photorespiratory phenotype,
316 indicating that the introduced pathway fulfilled its expected function to some extent.

317 Metabolic analysis was conducted to investigate the underlying reasons for the impaired growth
318 observed in wild-type transgenic plants expressing the β -hydroxyaspartate cycle. These plants,
319 as well as the transformed *ggt1-1* mutants, exhibited elevated levels of aspartate and malate,
320 while glycine levels were reduced. Furthermore, intermediates of the CBC, such as 3-
321 phosphoglycerate and sedoheptulose 7-phosphate, were depleted, suggesting a decrease in the
322 availability of CBC intermediates and unproductive metabolic flux into C₄ acids.

323
324 Collectively, the studies by Carvalho et al. (2011) and Roell et al. (2021) indicate that perturbing
325 the peroxisomal steps of the canonical photorespiration pathway does not yield the anticipated
326 improvement in photosynthetic performance.

327
328 Although not extensively discussed in this review, we note that bypasses to photorespiration often
329 led to a multitude of pleiotropic changes, including alterations in leaf shape and anatomy,
330 metabolic changes, and developmental effects. For example, changes in leaf anatomy can have
331 a multitude of effects on leaf photosynthetic activities. Changes in leaf thickness alone can result

332 in increased photosynthetic activity due to more photosynthetic biomass per unit leaf area (Onoda
333 et al., 2017). Changes in leaf anatomy such as modifications in leaf thickness, intercellular
334 airspace volume, mesophyll cell wall thickness and chloroplast size can also affect mesophyll
335 conductance for CO_2 (g_m , see Figure 2 for a schematic explanation of g_m), and thereby alter the
336 CO_2 concentration in chloroplasts (Flexas et al., 2012; Flexas et al., 2013; Knauer et al., 2022).
337 Given that observed changes in the CO_2 compensation points have often been small in the above-
338 mentioned studies, consideration of possible changes in g_m is important. We also note that the
339 CO_2 compensation point does not only depend on Rubisco characteristics and CO_2 and O_2
340 concentrations, but is also affected by the rate of mitochondrial respiration in light (R_d). R_d may
341 change as a consequence of pathway engineering, for example, generation of extra respiratory
342 substrate such as malate transported out of chloroplasts.

343 The precise relationship between modified photorespiration and its impact on plant structure and
344 function, beyond photosynthesis, remains incompletely understood. It is important to
345 acknowledge that native photorespiration does not operate as a closed cycle in which 75% of the
346 carbon derived from glycolate is reincorporated into the CBC as glycerate and 25% are released
347 as CO_2 . Instead, considerable amounts of carbon can be diverted towards the synthesis of amino
348 acids glycine and serine (Samuilov et al., 2018; Abadie and Tcherkez, 2019; Fu et al., 2023a),
349 which serve as building blocks for protein biosynthesis and other plant metabolites. Moreover,
350 photorespiration indirectly transfers redox equivalents from chloroplasts to mitochondria (Heber
351 and Krause, 1980; Heber et al., 1996), where they are oxidized by the mitochondrial electron
352 transport chain, contributing to ATP biosynthesis. The observed effects extending beyond
353 photosynthetic metabolism may be linked to these additional, albeit less extensively studied and
354 recognized, functions of the photorespiratory pathway. In the following paragraphs, we will provide
355 a critical assessment of the assumptions as to how photorespiratory bypasses function and we
356 highlight unresolved questions on these pathways.

357

358 *A critical qualitative assessment of mechanistic hypotheses*

359

360 Despite the fact that some of the genetic implementations of photorespiratory bypasses increased
361 photosynthetic efficiency and even yield under some conditions, many questions remain
362 unresolved. In particular the precise molecular mechanisms that are responsible for higher
363 performance are not yet understood. In order to develop informed hypotheses which are also
364 backed up by theoretical considerations, we give some qualitative and quantitative arguments
365 about attempts to explain increased photosynthetic rates.

366 First, it should be noted that an observation of biomass increases of a certain percentage after a
367 - usually several weeks long - growth period, cannot be directly translated into increased fluxes.
368 Even marginal differences in flux will, over time, lead to an exponential difference in overall
369 biomass accumulation. For example, in South et al. (2019) it was shown that under saturating
370 CO₂ the assimilation rate is increased by approximately 10% (cf. South et al., 2019, Fig. 5A).
371 However, this increase is considerably lower than the reported 24% increase in biomass after a
372 growth period of 6 weeks. This example illustrates that yield gains in percent stated for the diverse
373 experimental approaches are not comparable on a quantitative level.

374 Other observations in South et al. (2019) are also challenging to explain. For example, it has been
375 suggested that an increase in CO₂ *locally* in the chloroplast could explain a higher carbon fixation
376 rate. However, an increased CO₂ assimilation was observed even for saturating conditions, which
377 entails that increasing the local concentration is not the primary cause for higher fixation rates. A
378 similar result was observed when a complete glycolate decarboxylation pathway was expressed
379 in rice with several transgenic lines showing increased maximum rates of Rubisco carboxylation
380 under saturating CO₂ (Shen et al., 2019). Another hypothesis was that as a result of the glycolate
381 oxidase activity, glycolate levels should be reduced, and thus reducing its toxic effects. However,
382 in various lines the glycolate levels were actually increased, making also this explanation unlikely.
383 Similarly, although not explicitly measured, 2PG levels might be reduced as a result of the newly
384 introduced pathways, but because the dephosphorylation of 2PG to glycolate is highly irreversible,
385 there is no convincing argument why 2PG levels should actually be reduced. We note, however,
386 that it was previously shown that more efficient removal of 2PG by over-expression of 2-
387 phosphoglycolate phosphatase improved photosynthetic performance in *Arabidopsis thaliana*
388 under stress conditions (Timm et al., 2019).

389 Possibly, the growth promoting effect is of a more indirect nature. A possible explanation could
390 be connected with the algal glycolate dehydrogenase, which transfers electrons not to H₂O₂ but
391 to a so far unidentified electron acceptor. If this acceptor is one of the common electron carriers
392 of the photosynthetic electron transport chain, such as plastoquinone or ferredoxin, then this new
393 pathway would contribute to the generation of redox equivalents and thus directly support the
394 photosynthetic electron transport chain. The observation that the transformed plants exhibit a
395 higher apparent quantum efficiency (ϕ , cf. South et al., 2019, Fig. 6) is in line with this hypothesis.
396 An experimental test would be to measure how the observed increase in growth depends on the
397 light intensity under which plants are grown. If this speculation is correct, the growth increase
398 should be less pronounced the more saturating the light intensity.

399 In summary, these considerations illustrate the complexity of the system and the necessity to use
400 mathematical models, which are based on clear mechanistic hypotheses and are designed to
401 quantitatively reproduce experimental results and thus provide a platform to test different
402 mechanistic hypotheses and, by making novel predictions, support the experimental design to
403 confirm or falsify these.

404

405 *Modeling at different scales can inform photorespiration engineering*

406

407 The mechanisms of yield improvement caused by photorespiratory bypasses can be divided into
408 four categories: improved stoichiometry and energy efficiency, improved kinetics (*i.e.*, altered
409 Rubisco carboxylation-to-oxygenation ratio), relief of inhibition by toxic intermediates, and indirect
410 effects of altered physiology or development. Models at different scales, from simple cofactor
411 accounting and stoichiometric models, to kinetic models of photosynthesis, have provided some
412 insight into how these mechanisms allow photorespiratory bypasses to be effective but further
413 work is still required if models are to explain all observed experimental results (Peterhansel et al.,
414 2013; Xin et al., 2015; Basler et al., 2016; Trudeau et al., 2018; Khurshid et al., 2020; Osmanoglu
415 et al., 2021).

416 A common feature of photorespiratory bypasses is to avoid the energetic cost of ammonium
417 refixation or to capture the reducing power from glycolate oxidation, thus decreasing the ATP and
418 NADPH cost of photorespiration. Models for energy cofactor accounting can quantify these direct
419 energetic benefits of bypassing photorespiration, as well as indirect benefits such as avoiding the
420 cost of CO₂ refixation via the CBC in carbon fixing bypasses (Table 1) (Peterhansel et al., 2013;
421 Trudeau et al., 2018). Larger, genome scale stoichiometric models can also calculate energy
422 efficiency and have the advantage of predicting flux into biomass rather than just rates of carbon
423 fixation, placing bypasses in the wider context of the plant metabolic network (Basler et al., 2016).
424 For example, a curated stoichiometric model was able to predict a decrease in photorespiratory
425 flux and a biomass output increase of ~6.2%, qualitatively consistent with experimental data
426 (Basler et al., 2016; Kebeish et al., 2007). However, stoichiometric models predict no benefit for
427 bypasses that are energetically more costly than photorespiration, such as those that completely
428 decarboxylate glycolate in the chloroplast. Experimentally, such bypasses still show increased
429 yields when expressed in plants, suggesting either incorrect prediction of energy costs, or benefits
430 that are beyond just direct ATP and NADPH savings (Maier et al., 2012; Peterhansel et al., 2013;
431 Shen et al., 2019; South et al., 2019; Xin et al., 2015).

432 Photorespiration involves the transport of metabolites between three compartments, as well as
433 the movement of reducing equivalents from the chloroplasts to the mitochondria. Photorespiratory
434 bypasses can relocate reactions to different compartments, potentially avoiding the energetic
435 costs of metabolite transport. Therefore, more complete modeling of transport reactions, including
436 thermodynamic constraints may improve the accuracy of energy accounting. However, even if
437 all energetic costs were accurately modeled, stoichiometric models alone cannot account for
438 changes in metabolite concentrations or reaction kinetics.

439 Several of the bypasses validated in plants aim to relocate the release of photorespiratory CO₂
440 from the mitochondria to the chloroplasts which has two potential advantages: increasing the CO₂
441 concentration at the site of Rubisco, and recapturing photorespiratory CO₂ that could otherwise
442 be lost from the cell by diffusion out of mitochondria. Predicting such effects requires the use of
443 kinetic models or additional constraints on Rubisco carboxylation and oxygenation fluxes (Basler
444 et al., 2016). A kinetic model predicted that under high light conditions, the entire benefit of the
445 Kebeish et al., (2007) bypass is the relocation of CO₂ release, not the reduced ATP cost, which
446 only contributes under low light conditions (Xin et al., 2015). However, the same kinetic model
447 failed to explain the benefit of complete glycolate decarboxylation in the chloroplast, predicting
448 that photosynthetic rate would be 31% lower than in the wild-type, despite reported increases of
449 >30% in carbon assimilation rate and biomass (Maier et al., 2012; Xin et al., 2015). Additionally,
450 the kinetic model demonstrated that any benefit of relocating CO₂ release is dependent on the
451 CO₂ permeability of the chloroplasts; predicting that if >30% of photorespired CO₂ is already
452 recaptured and refixed in wild-type plants, then there is no benefit to the bypass (Xin et al., 2015).
453 It is therefore surprising that a glycolate decarboxylation bypass in rice, where wild-type plants
454 already refix 38% of photorespiratory CO₂, still showed some biomass gains, suggesting
455 additional benefits not captured by the kinetic model (Busch et al., 2013; Xin et al., 2015; Shen et
456 al., 2019; Wang et al., 2020a; Zhang et al., 2022).

457 In contrast to Xin et al. (2015), a different kinetic model of a cyanobacterial complete glycolate
458 decarboxylation bypass expressed in the chloroplast predicted a 10% increase in photosynthetic
459 rate, although this model did not explicitly account for the movement of CO₂ between
460 compartments and is yet to be experimentally validated by expression of the full pathway in plants
461 (Bilal et al., 2019; Khurshid et al., 2020; Abbasi et al., 2021). Kinetic models have therefore been
462 useful for estimating the potential effect of relocation of CO₂ release and distinguishing this from
463 energy benefits, but they are still unable to explain all currently observed experimental results.

464 Reducing the concentration of inhibitory intermediates of photosynthetic metabolism could
465 account for some of the benefit of photorespiratory bypasses, and this can be simulated using

466 kinetic models. However, kinetic models of photorespiratory bypasses did not include parameters
467 for the inhibitory effects of 2-phosphoglycolate on the CBC or the inhibitory effect of glyoxylate on
468 Rubisco activation and Rubisco oxygenase activity (Oliver and Zelitch, 1977; Oliver, 1980;
469 Campbell and Ogren, 1990; Xin et al., 2015; Flügel et al., 2017; Khurshid et al., 2020). Increasing
470 the scale of kinetic models to include more inhibition terms may provide additional explanation for
471 benefits, although this can be limited by the availability of accurate kinetic parameters.

472 As pointed out previously, a higher apparent quantum efficiency could be responsible for yield
473 improvements in the transformed plants. Modeling the effect of bypasses on quantum efficiency
474 would require a high-fidelity kinetic model of the photosynthetic electron transport chain and the
475 CBC, such as Saadat et al. (2021), to be combined with a model of photorespiration.

476 Additional effects beyond the cellular metabolic changes described by current models such as
477 altered gene expression, signaling, physiology, and pleiotropic effects could also explain a large
478 proportion of observed growth benefits (Maier et al., 2012; Shen et al., 2019). Using larger scale
479 integrated models could potentially provide more accurate prediction of the effect of engineering
480 photorespiration in the field by accounting for interactions across scales (Wu, 2023). Additionally,
481 attempting to model dynamic processes under non-steady state conditions may also help identify
482 advantages of photorespiratory bypasses not captured by current models (Fu et al., 2023b).
483 Finally, extending current metabolic models to account for diurnal cycles could explain
484 advantages of bypass reactions beyond altered photosynthetic metabolism, such as altered dark
485 respiration and sucrose export during the night (Dalal et al., 2015).

486 No single modeling strategy can explain all of the observed phenotypes of plants expressing
487 photorespiratory bypasses and instead multiple models at different scales should be used as tools
488 to help explain the underlying mechanisms of growth benefits. Questions still remain to be tested
489 by exploring models: why does expression of glycolate dehydrogenase alone also increase
490 photosynthetic performance and yield in Arabidopsis, potato, and Camelina (Abbasi et al., 2021;
491 Dalal et al., 2015; Kebeish et al., 2007; Nölke et al., 2014); and how does the anatomy and
492 physiology of different crop species affect CO₂ diffusion between cells and subcellular
493 compartments? Future implementation of new bypass designs based on carbon fixing, rather than
494 decarboxylating, reactions will also provide valuable data for exploring the effect of a
495 fundamentally different bypass mechanism (Trudeau et al., 2018; Scheffen et al., 2021) (see
496 discussion of *new-to-nature pathways* below). With future modeling efforts and better
497 understanding, it may be possible to further increase yields and improve the transferability of
498 benefits between different crop species.

499

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501 **Table 1.** Photorespiratory bypass energy costs (adapted from Trudeau et al., 2018). Based on
 502 the consumer model of photosynthesis which describes the processes of photorespiration and
 503 the Calvin-Benson cycle as independent cycles that are able to regenerate ribulose 1,5-
 504 biphosphate using ATP (β) and reducing equivalents (γ) and either consume or produce CO₂
 505 and glyceraldehyde 3-phosphate (GAP) (α) (Trudeau et al., 2018). This description allows
 506 bypasses to be compared directly and separated from the Calvin-Benson cycle. Positive values
 507 represent consumption, negative values represent production. See supplementary table 1 for
 508 detailed calculations. *3OHP bypass assuming pyruvate is converted to GAP via pyruvate
 509 phosphate dikinase. **BHAC bypass assuming oxaloacetate is converted to GAP via
 510 phosphoenolpyruvate-carboxykinase in order to regenerate RuBP. † assuming 2.5 ATP per
 511 reducing equivalent. †† assuming Calvin-Benson cycle compensates for carbon lost by PR
 512 bypasses.

513

Reference	Pathway name	CO ₂ (α)	ATP (β)	Red. equiv. (γ)	Total ATP equiv.†	Total ATP equiv.† normalised to net 0 carbon lost/gained††
Trudeau et al., 2018	TaCo	1	7	4	17	9
Kebeish et al., 2007	Chloroplast TSS	-0.5	3	1	5.5	9.5
Shih et al., 2014	3OHP*	1	8	4	18	10
Carvalho et al., 2011	Peroxisome TSS	-0.5	3	2	8	12
Roell et al., 2021	BHAC**	-0.5	3	2	8	12
	Photorespiration	-0.5	3.5	2	8.5	12.5
South et al., 2019	AP3	-2	2	-2	-3	13
Maier et al., 2012	GMK	-2	2	-1	-0.5	15.5
Shen et al., 2019	GOC	-2	2	1	4.5	20.5
	Calvin cycle	1	3	2	8	

514

515 *Abbreviations: 3OHP, 3-hydroxypropionate; Red equiv, reducing equivalents; TaCo, tartronyl-CoA; TSS, tartronic-*
 516 *semialdehyde shunt; BHAC, beta-hydroxyaspartate cycle; GMK, glycolate oxidase, malate synthase, catalase (KatE);*
 517 *AP3, alternative pathway 3; GOC, glycolate oxidase, oxalate oxidase, catalase.*

518

519

520

521 Combined Optimization of Light Use and Carbon Assimilation Efficiency to Enhance Plant
522 Productivity

523

524 The intricate relationship between light conversion, carbon fixation, and their impact on plant
525 productivity calls for a comprehensive exploration of their interdependencies. Through combining
526 modifications to light utilization and carbon fixation, we have the potential to achieve synergistic
527 enhancements. We here discuss the interplay between these pathways and evaluate the
528 possibility of maximizing overall productivity by improving the efficiency of converting light energy
529 into ATP and NADPH while optimizing their utilization for carbon fixation.

530

531 In plants, pigments absorb light, and the excitation energy is utilized by photosystems to facilitate
532 the synthesis of ATP and NADPH, which are essential for all metabolic reactions, including carbon
533 fixation. This initial phase of photosynthetic reactions effectively transforms light into chemical
534 energy, and its efficiency significantly influences crop productivity (Zhu et al., 2010).

535 In highly dynamic environments, the amount of light absorbed by the photosynthetic apparatus
536 can exceed the metabolic capacity of the cell, resulting in the over-reduction of the photosynthetic
537 electron transport chain and the production of harmful reactive oxygen species (ROS).
538 Photosynthetic organisms have evolved various mechanisms to regulate light utilization efficiency
539 and photosynthetic electron transport, aiming to minimize the likelihood of over-reduction and
540 cellular damage by safely dissipating excess excitation or electrons (Li et al., 2009). While the
541 protection of the photosynthetic apparatus plays a crucial biological role, it comes at the expense
542 of reduced efficiency in converting sunlight into chemical energy (Alboresi et al., 2019).

543 Fluctuations in light intensity represent a particular challenge to the regulation of photosynthesis
544 and significantly impact primary productivity (Long et al., 2022). Abrupt increases in illumination
545 can be detrimental as they do not allow sufficient time for the activation of regulatory responses
546 and modulation of metabolic reactions. Conversely, when light levels decrease from excessive to
547 limiting, the photoprotective mode remains active for several minutes, leading to unnecessary
548 energy dissipation and a subsequent reduction in carbon fixation efficiency (Wang et al., 2020b).

549

550 To address this limitation, modifications were made to the kinetics of Non-Photochemical
551 Quenching (NPQ), which is one of the mechanisms involved in light harvesting regulation. By
552 overexpressing key proteins such as PsbS, VDE, and ZEP, the activation and relaxation kinetics
553 of NPQ were accelerated. This overexpression allowed tobacco and soybean plants to effectively

554 respond to changes in light and minimize potential damage during sudden increases in sunlight.
555 Additionally, it facilitated faster relaxation of NPQ when illumination intensity decreased. This
556 approach has demonstrated positive effects on biomass productivity not only in the field for crops
557 like tobacco and soybean (Kromdijk et al., 2016; Souza et al., 2022), but also in high-density
558 culture photobioreactors for the microalga *Nannochloropsis* (Perin et al., 2023). However, in
559 *Arabidopsis*, the same approach was not as successful, suggesting that species-specific traits,
560 including light distribution within the canopy, significantly impact plant productivity and the optimal
561 balance between light harvesting and photoprotective responses (Garcia-Molina and Leister,
562 2020).

563 Another strategy to enhance the efficiency of sunlight utilization is the modification of the light
564 harvesting apparatus. Leaves have evolved to efficiently capture light and outcompete other
565 organisms for sunlight. However, in densely cultivated fields, this high light harvesting efficiency
566 can have negative consequences. Light is primarily absorbed by the uppermost leaves in the
567 canopy, leaving fewer photons available for the lower layers. While high light harvesting efficiency
568 provides a competitive advantage in natural environments where individuals vie for a limited
569 resource like light, it proves detrimental in cultivated fields. In this context, plants with paler leaves
570 can enable a more uniform distribution of light within the canopy, which has been shown to
571 enhance overall productivity (Rotasperti et al., 2022; Cutolo et al., 2023). Also, extra nitrogen that
572 is no longer needed for construction of pigment-binding machinery of photosynthesis, could be
573 invested in Rubisco and rate-limiting proteins of photosynthetic machinery, thereby increasing
574 leaf photosynthetic capacity (Walker et al., 2017; Niinemets, 2023). It is interesting here to discuss
575 if these efforts in modulating regulation of light harvesting could be combined with improvements
576 in carbon fixation efficiency. In principle, a higher efficiency conversion of light energy into ATP
577 and NADPH could be combined with a more efficient utilization of these molecules for carbon
578 fixation, with a potentially additive effect on productivity.

579 One possible implication to be considered is that photorespiration has been shown to be a major
580 sink for photosynthetic electron transport, and that this energy loss can have a beneficial effect in
581 conditions where photosynthetic apparatus is over-excited (Heber and Krause, 1980; Kozaki and
582 Takeba, 1996; Hanawa et al., 2017). Light saturation occurs when electron transport is faster than
583 the metabolic capacity of consuming ATP and NADPH produced and, in these conditions,
584 photorespiration can be beneficial in reducing over-saturation. If efficiency in carbon fixation is
585 improved by introduction of photorespiratory bypasses, however, this would also drive to a
586 stronger ATP and NADPH consumption with a similar protecting effect. This suggests that the
587 critical point is that the new or modified pathways have a sufficient capacity to compensate also

588 for ATP and NADPH consumption associated with photorespiration. If this is the case their
589 introduction will increase CO₂ fixation while also complementing the role of photorespiration in
590 protection from light excess.

591 It is anticipated that altering crucial pathways in plant metabolism, including light conversion and
592 carbon fixation, will inherently possess interdependent effects that require additional investigation.
593 Furthermore, there exists the possibility of enhancing the efficiency of converting light energy into
594 ATP and NADPH, while concurrently optimizing the utilization of these molecules for carbon
595 fixation. Such combined improvements could potentially yield an additive outcome, thereby
596 maximizing the overall impact on productivity.

597

598 **Leveraging Naturally Occurring Variation in Photosynthetic Parameters**

599

600 *Exploiting the natural variation of Rubisco kinetic traits, Γ^* , and photorespiration in coordination*
601 *with CO₂ diffusion*

602

603 Leveraging the inherent diversity in essential photosynthetic traits presents two notable
604 advantages: first, it provides engineering with essential knowledge about potential trade-offs, and
605 secondly, it facilitates the breeding process, allowing for accelerated improvements in crops. The
606 crucial traits related to photorespiration in Rubisco include the Michaelis-Menten constant for CO₂
607 (K_c), the Michaelis-Menten constant for O₂ (K_o), and Rubisco specificity to CO₂ over O₂ ($S_{c/o}$), and
608 maximum turnover rates (specific activity per active center per mass or protein, s⁻¹) for
609 carboxylase (V_c) and oxygenase (V_o). Several authors have investigated the environmental and
610 evolutionary trends of natural variation in Rubisco's kinetic properties and their temperature
611 responses, including several recent meta-analyses (Galmés et al., 2019; Bouvier et al., 2021;
612 Tcherkez and Farquhar, 2021). These studies have revealed substantial variability in major kinetic
613 traits, which are highly sensitive to factors such as temperature, CO₂ availability, and
614 photosynthetically active quantum flux density. Moreover, this variability is inherent among
615 species adapted to different environments. There are several key trade-offs among Rubisco
616 kinetic traits, most notably the reverse relationships between $S_{c/o}$ and V_c (Galmes et al. 2014,
617 2019), which are important to consider in Rubisco engineering.

618 Recent findings have demonstrated a strong co-regulation between the natural diversity of
619 Rubisco kinetics and mesophyll conductance (g_m) as well as in underlying anatomical traits. For
620 instance, plants adapted to drought exhibit reduced g_m and chloroplast CO₂ concentration (C_c)
621 due to higher mesophyll cell wall thickness. Consequently, these plants possess Rubisco with

622 higher $S_{c/o}$ but lower turnover rates (Galmés et al., 2019). This highlights the substantial influence
623 of the C_c associated with species adaptation on the diversity of Rubisco's kinetic properties and
624 the trade-offs observed. Importantly, this regulation is significantly influenced by g_m , which exhibits
625 considerable variation among species and is responsive to environmental stressors. (Elferjani et
626 al. 2020; Knauer et al. 2022)

627 It is noteworthy that g_m can limit photosynthesis, accounting for approximately 10-70% of the
628 limitation, thereby exerting an equally significant impact on photosynthetic assimilation (A) as
629 stomatal conductance (g_s) does (Knauer et al., 2020).

630 The CO_2 compensation point (Γ) has traditionally served as a parameter reflecting Rubisco
631 functionality and photosynthetic efficiency. It represents the equilibrium between the A and leaf
632 respiration, making it a useful characteristic for categorizing crops and herbaceous species based
633 on their inherent photosynthetic efficiency and stress resilience. In C_3 plants, Γ is typically highest
634 (40 - 100 $\mu\text{mol mol}^{-1}$), intermediate in C_3 - C_4 intermediates (20 - 30 $\mu\text{mol mol}^{-1}$), and low in C_4
635 plants (3 - 10 $\mu\text{mol mol}^{-1}$) (Nobel, 1991; Schlüter et al., 2023).

636 However, Γ is a complex trait and can be estimated in a multitude of ways. To incorporate Rubisco
637 kinetics, photorespiration, and more accurately, an alternative parameter called the
638 photorespiratory CO_2 compensation point in absence of day respiration (Γ^*) has been proposed.
639 Γ^* can be determined from A/C_c curves by using the common interception method measured at
640 different light intensities, and represents the C_c at which photosynthetic carbon uptake equals
641 photorespiratory CO_2 release (Walker et al., 2016b). Γ^* and Γ differ significantly, the magnitude
642 of difference depends on the variable g_m (Figure 2) and the fate of the mitochondrial CO_2 fluxes,
643 as both estimates affect the resulting C_c and Γ^* . The understanding of partitioning of respiratory
644 fluxes between the proportion directed into the chloroplast and what diffuses into the cytoplasm
645 and intercellular airspace is also crucial for correct g_m estimations. However, the fate of
646 respiratory fluxes and g_m largely depend on similar anatomical traits, such as the proportion of
647 mesophyll cell surface area lined with chloroplasts, and physical dimensions of cell wall,
648 chloroplasts, and cytoplasm (Figure 2B). All these traits vary significantly across species referring
649 to substantial variability in partitioning of respiratory fluxes between the chloroplasts and
650 cytoplasm (von Caemmerer et al., 1994; Tosens et al., 2012; Ubierna et al., 2019). Thus,
651 considering the physiological nature of CO_2 compensation points estimated through different
652 methods, including Γ^* , offers valuable insights into how mitochondrial CO_2 effluxes curb C_c
653 (Walker et al., 2016b; Busch, 2020; Sage et al. 2022).

654

655

656 Achieving optimal photosynthetic efficiency requires precise coordination between traits that
657 regulate CO₂ diffusion efficiency and the functionality of Rubisco. Consequently, a major focus
658 in improving photosynthesis, whether through breeding or engineering, is to explore and
659 comprehend the natural diversity of key kinetic traits of Rubisco and their relationship with CO₂
660 availability in the chloroplast stroma (Walker et al., 2016b; Galmés et al., 2019; Flexas and
661 Carriquí, 2020; Evans, 2021; Knauer et al., 2022; Iqbal et al., 2023). In angiosperms with
662 elevated rates of turnover, the constraints of photosynthesis, including stomata, mesophyll
663 conductance, and photo/biochemical processes, typically coexist in a harmonious equilibrium,
664 unless stress-induced alterations disrupt this balance. Consequently, attaining optimal
665 enhancements in photosynthesis and resource utilization efficiency requires the simultaneous
666 manipulation of all three constraints or a shift in focus towards augmenting the g_m/g_s ratio. This
667 approach enables a simultaneous increase in intrinsic water-use efficiency (Gago et al., 2019;
668 Flexas and Carriquí, 2020; Knauer et al., 2020; Clarke et al., 2022; Kromdijk and McCormick,
669 2022).

670

671 Previous studies have often addressed the kinetic components of A/C_i curves (Rubisco kinetics,
672 RuBP turnover rate, electron transport limitations; R_d , respiration due to photorespiration [R_p],
673 CO₂ compensation point) separately, combining *in vivo* and *in vitro* estimations, resulting in
674 fragmented information (Bernacchi et al., 2013). However, employing state-of-the-art fast
675 response gas-exchange and optical diagnostic systems, as described by Laisk et al. (2002),
676 allows for the simultaneous measurement of all necessary parameters to comprehensively assess
677 the photosynthetic apparatus in leaves. This approach enables a holistic understanding of how
678 photosynthesis is optimized. A primer on measuring photorespiration is given as Supplementary
679 File S1.

680

681 *Natural genetic variation in photosynthetic parameters as a basis for breeding*

682

683 One way to adapt plants to human requirements is by harnessing the natural genetic variation
684 that has been generated through random mutations over historical time spans. This genetic
685 variation can occur at both, the intraspecific and the interspecific level.

686 The first evidence of natural genetic variation in the context of photorespiration was provided by
687 Jorda and Ogren (1981). They observed differences in the specificity factors towards the
688 substrates CO₂ and O₂, $S_{c/o}$, of Rubiscos purified from seven different species, ranging from 77
689 to 82. Subsequent studies, as reviewed by Hartmann and Harpel (1994), revealed changes in the
690 specificity factor due to replacement of the active-site metal, random and site-directed
691 mutagenesis, chemical modification, and hybridization of heterologous subunits. The highest
692 reported specificity factor for Rubisco is 238, found in the red alga *Galdieria partita* (Uemura et

693 al., 1996), which is about three times higher than that reported for Rubisco from most crop plants
694 (Parry et al., 1989).

695 Even before the discovery of the oxygenase activity of Rubisco (Bowes et al., 1971), selection
696 experiments aiming to manipulate the specificity factor of Rubisco through selection were
697 conducted (*cf.* Cannell et al., 1969; Menz et al., 1969). In these experiments, plants were kept at
698 or slightly above the compensation point, and plants with high rates of photorespiration were
699 expected to perish.

700 As discussed in the previous section, over the recent years, the number of studies exploring the
701 natural plasticity and inherent variability of Rubisco's key catalytic traits has significantly
702 increased. Information about the interspecific variation in photorespiration has also been
703 observed through carbon isotope fractionation (Lanigan et al., 2008). However, to the best of our
704 knowledge, only the study by Cai et al. (2014) examined and observed natural genetic variation
705 in CO₂ compensation points and evaluated these differences in the context of leaf anatomy
706 variations among three *Rhododendron* species.

707 The genes or alleles responsible for the advantageous photorespiratory phenotype can be
708 transferred through interspecific hybridization, protoplast fusion, transformation, or genome
709 editing. To facilitate these processes, it is necessary to identify the underlying genes or alleles.
710 One approach to achieve this is through large-scale comparative genomics, which incorporates
711 phenotypic information and is referred to as phylogenetic association mapping (*e.g.*, Collins and
712 Didelot, 2018).

713 However, with the exception of Schlüter et al. (2023), no earlier study has considered natural
714 variation in characters related to photorespiration in a densely sampled phylogenetic tree, making
715 the application of phylogenetic association mapping challenging for identifying the underlying
716 genomic features.

717 An alternative approach for identifying the genomic features responsible for interspecific
718 differences is the utilization of segregating genetic material derived from interspecific hybrids.

719 Comparative transcriptomics is another approach that can aid in identifying the *cis* and *trans*
720 factors responsible for interspecific differences. By comparing transcriptomes of accessions
721 adapted to specific conditions or subjected to environmental perturbations, strategies for crop
722 improvement can be guided. For example, in rice, RNA sequencing identified a transcription factor
723 with the potential to enhance photosynthetic capacity and increase yield (Wei et al., 2022).
724 However, when using bulk RNA sequencing of an organ, it becomes challenging to detect
725 differentially expressed genes specific to rare cell types, as their expression is diluted. In the case
726 of C₃-C₄ or C₄ photosynthesis, where the pathways operate in specific cell types of the leaf,

727 obtaining cell-type-specific transcriptomes is crucial. Approaches such as mechanical separation
728 of cell types (John et al., 2014) and laser capture microdissection followed by microarray/RNAseq
729 have been used previously, but their scale and resolution are limited by the speed of sampling,
730 and separating low abundance cell types can still be challenging (Zhang et al., 2007; Aubry et al.,
731 2014; Hua et al., 2021; Xiong et al., 2021).

732 The adoption of single-cell methods, particularly droplet-based technology, is gaining increasing
733 acceptance in plant research. This approach allows the capture of individual transcriptomic
734 profiles of cells from a wide range of plant species, providing valuable insights at a remarkably
735 low cost per cell. For example, comparative single-cell analysis of roots from three grass species
736 provided significant insights into the evolution of cellular divergence in these crops (Guillot et
737 al., 2023). As such, this approach therefore must hold potential for better understanding the
738 compartmentation of gene expression in C_3 - C_4 and C_4 species (Cuperus, 2021; Seyfferth et al.,
739 2021). Currently the number of single-cell or single nuclei datasets generated from leaf tissues is
740 limited and most studies have tended to focus on a single model species under one condition
741 (Berrío et al., 2021; Bezruczyk et al., 2021; Kim et al., 2021; Lopez-Anido et al., 2021; Procko et
742 al., 2022; Sun et al., 2022). To enhance photosynthetic efficacy in crops, understanding the
743 changes in gene expression associated with closely related C_3 , C_4 or/and C_3 - C_4 intermediate
744 species is likely to provide insights into the molecular signatures of each of these traits, and
745 therefore how they might be rationally engineered. Comparative analysis of leaf anatomy, cellular
746 ultrastructure and photosynthetic traits between species within a genus, for example in
747 *Gynandropsis* (Marshall et al., 2007; Koteyeva et al., 2011), *Moricandia* (Schlüter et al., 2017), or
748 *Flaveria* (McKown and Dengler, 2007; Kümpers et al., 2017) have provided insights into traits that
749 engender higher photosynthesis efficiency. Therefore, acquiring transcriptome data from
750 individual bundle sheath and mesophyll cells of these closely related plants should provide new
751 insights into how the patterns of transcript abundance alter in association with modifications to
752 photosynthetic efficiency. We also anticipate that single-cell RNAseq will contribute to crop
753 improvement by providing insights into underlying molecular mechanisms. Lastly, additional
754 advantages can be obtained from single-cell RNAseq if the data are associated with a complex
755 phenotype (single-cell TWAS) and genotype (cell-type specific eQTL) at a population level (Perez
756 et al., 2022). It seems likely that such approaches will be adopted for high resolution genotyping
757 of bioengineered plants in synthetic biology projects. In summary, the combination of scRNAseq
758 technology with comparative transcriptomics, population genetics, and synthetic biology is likely
759 to serve as a powerful tool for crop improvement.

760 A technically simpler procedure to the above-described approach of exploiting interspecific natural
761 genetic variation would be the exploitation of the intraspecific variation within the species under
762 consideration for photosynthetic properties as the transfer or the enrichment of the positive alleles
763 is much easier to realize in comparison to the interspecific variability. Within species, natural
764 variation in leaf photosynthesis has been reported for model species (e.g., (Tomeo and
765 Rosenthal, 2018) but also for major crops (e.g., Gu et al., 2012; Driever et al., 2014). However,
766 the improvements that can be realized in that way are up to now smaller compared to the
767 approaches exploiting interspecific variability.

768

769 **New-to-Nature Approaches to Improve Photosynthetic Efficiency**

770

771 Most efforts of improving carbon capture in plants have focused on engineering naturally existing
772 enzymes and pathways (Kebeish et al., 2007; South et al., 2019). However, the emergence of
773 synthetic biology has opened up the possibility to radically (re)draft plant metabolism to overcome
774 the limitations of natural evolution, which is driven by co-linearity, tinkering, epistatic drift, and
775 purifying selection, rather than by “design” (Wurtzel et al., 2019). Thus, such engineering
776 approaches have the potential to expand the biological solution space and provide new-to-nature
777 pathways that outcompete their natural counterparts in respect to thermodynamics and/or
778 kinetics, because they are drafted from first principles.

779 As an example, while Nature has evolved seven different pathways for CO₂ fixation, more than
780 30 synthetic CO₂ fixation pathways have already been designed, which are all superior to the
781 CBC (Bar-Even et al., 2010). Some of these new-to-nature solutions have even been successfully
782 realized *in vitro* (Schwander et al., 2016; Luo et al., 2022; McLean et al., 2023), and are awaiting
783 their transplantation *in vivo*.

784 The concept of designer metabolism has also been extended to photorespiration lately (Figure
785 3). Two studies have proposed alternative ways to use the photorespiratory metabolite glyoxylate
786 to feed into synthetic carbon fixation cycles (Figure 3, pathways 1 & 2). The first cycle is the malyl-
787 CoA-glycerate pathway (MCG). In this cycle, the bacterial glyoxylate assimilation route condenses
788 two molecules of glyoxylate to form a C₃ compound, releasing CO₂ in the process. The resulting
789 tartronate semialdehyde is then reduced and phosphorylated into 2-phosphoglycerate (2PG).
790 2PG is converted to phosphoenolpyruvate and further carboxylated to oxaloacetate, subsequently
791 reduced to malate and activated with coenzyme A, followed by cleavage into glyoxylate and
792 acetyl-CoA. Glyoxylate is then available to initiate the next cycle, while acetyl-CoA can be utilized

793 for biosynthesis (Yu et al., 2018). The MGC pathway, therefore, does not result in a net loss of
794 CO₂.

795
796 A second study proposed the 3-hydroxypropionate (3OHP) photorespiratory bypass, inspired by
797 the naturally occurring 3OHP bi-cycle (Zarzycki et al., 2009). The 3OHP bypass exploits the malyl-
798 CoA lyase to produce β-methylmalyl-CoA from glyoxylate and propionyl-CoA. Methylmalyl-CoA
799 undergoes a series of interconversion that yield citramalyl-CoA, which in turn serves as a substrate
800 for the malyl-CoA lyase and produces pyruvate and acetyl-CoA. Acetyl-CoA is carboxylated to
801 malonyl-CoA, which is then reduced and further activated into the starter compound of the cycle:
802 propionyl-CoA. Pyruvate on the other hand can be further converted to phosphoglycerate and re-
803 enter the CBC (Shih et al., 2014) and resulting in a net gain in carbon.

804 Both studies implemented their proposed cycles in cyanobacteria. Regardless of the CO₂ fixing
805 ability of 3OHP bypass, strains carrying the cycle did not present a conclusive phenotype.
806 However, the authors set an example for the future *in vivo* implementation of carbon capturing
807 photorespiratory bypasses. On the other hand, strains containing the MCG pathway showed
808 increased bicarbonate assimilation, acetyl-CoA accumulation, and achieved higher optical
809 densities than strains lacking MCG.

810 The aforementioned cycles, represent what has been described in literature as “mix and match”
811 synthetic pathways, as they described novel pathways, relying on known reactions and enzymes
812 (Erb et al., 2017). Nevertheless, a recent study has identified several new-to-nature pathways by
813 systematically developing reaction sequences from the pool of feasible biochemical
814 transformations that could convert (phospho)glycolate back into a central intermediate of the CBC
815 cycle (Trudeau et al., 2018). The design of these solutions was guided by two additional principles.
816 First, the new reaction sequences should require as little energy as possible, and second, these
817 pathways should be CO₂-neutral (i.e., do not release CO₂) or even capture CO₂.

818 Through these efforts, the authors proposed four “carbon neutral pathways” in order to
819 reincorporate the C2 compound product of the oxygenation reaction of Rubisco into the CBC
820 (Figure 3, pathways 3-6). These pathways rely on a novel reduction of glycolate to glycolaldehyde
821 via engineered enzymes. Taking advantage of the high reactivity of glycolaldehyde, it is further
822 combined with sugar phosphates present on the CBC such as: Glyceraldehyde 3-phosphate
823 (GAP), dihydroxyacetone phosphate (DHAP), fructose 6-phosphate or sedoheptulose 7-
824 phosphate (S7P) via aldolase, transketolase or transaldolase reactions. In all cases, a C5
825 compound is produced and further converted into the substrate of rubisco, ribulose 1,5-
826 biphosphate. In the case of the transketolase and transaldolase reactions side products are

827 formed that are also part of the CBC and therefore, can directly be reused. Trudeau et al. proved
828 the *in vitro* feasibility of one of their proposed carbon neutral bypasses, however, to-date no further
829 improvement or *in vivo* implementation has been reported (Trudeau et al., 2018).

830 Furthermore, in the same study, another photorespiration bypass comprising novel reactions was
831 proposed, the tartronyl-CoA (TaCo) pathway (Figure 3, pathway 7). The TaCo pathway is a five-
832 reaction sequence that first converts photorespiratory glycolate into glycolyl-CoA, which is
833 subsequently carboxylated into tartronyl-CoA, the namesake compound of the pathway. In two
834 subsequent steps, tartronyl-CoA is then reduced to glycerate, which can re-enter the CBB cycle
835 at the level of phosphoglycerate. Compared to natural photorespiration, the TaCo pathway
836 sequence is about 50% shorter, requires about 20% less ATP and 30% less reducing power,
837 does not release ammonia, and notably also not CO₂, but instead captures additional CO₂ during
838 photorespiration. In other words, through the TaCo pathway, photorespiration can be turned into
839 a carbon-capturing process, in which the oxygenation reaction of Rubisco will still lead to a fixation
840 of carbon subsequently.

841 Although this pathway outcompetes natural photorespiration, a challenge has been that the
842 central TaCo pathway sequence relies on enzyme reactions that have not been described, thus
843 far. This apparent challenge was addressed through re-engineering the active sites of enzyme
844 candidates that catalyze similar reactions to establish and/or improve the desired reactions
845 (Scheffen et al., 2021). The full pathway was reconstituted and prototyped *in vitro*.

846 Yet, this proof-of-principle is only the starting point for further developments to successfully realize
847 the TaCo pathway in the context of the living plant. This could be achieved through a combination
848 of complementary approaches. The *in vitro* prototyping efforts could be expanded by combining
849 high throughput combinatorics with machine learning guided experimentation to optimize the
850 enzyme stoichiometry and robustness of the pathway (Pandi et al., 2022; Vögeli et al., 2022). The
851 optimized network could then be (partially) transplanted in a suitable (micro)organism to test for
852 its feasibility and use adaptive laboratory evolution (ALE) to further improve the network and its
853 cellular integration. The latter strategy has been recently successfully used to establish the CBC,
854 the reverse glycine cleavage pathway or a modified serine cycle in *E. coli* (Antonovsky et al.,
855 2016; Yishai et al., 2017; Gleizer et al., 2019; Luo et al., 2022), in which selection strains were
856 designed, which need to form (part) of their biomass through the new pathways. Having
857 established a functional cycle inside of a microorganisms will guide further efforts to integrate this
858 new reaction sequence in plants.

859

860

861 **Concluding Remarks and Perspectives**

862 Sustainably increasing crop yields is crucial to meet the growing demands of a rising global
863 population for food, feed, and other plant-derived products. Maximizing photosynthetic efficiency
864 is hence an important factor for food security in the context of anthropogenic climate change and
865 resource limitations. In the quest for increasing crop productivity through improvement of
866 photosynthetic efficiency, bypasses to canonical photorespiration and modifications of excess
867 light protection have been designed, implemented in several model species and in crops and
868 have been tested in the field. Jointly, these data provide proof-of-concept for yield increases
869 through engineering of photorespiration and light harvesting.

870 While progress has been made, there are still unanswered questions that need to be addressed,
871 essential to fully gather the potential improvements achievable from these approaches. For
872 example, how do photorespiratory bypasses actually work? What are the mechanisms that
873 underpin the observed yield increases? Resolving questions such as these will require additional
874 experiments and comprehensive analysis by mathematical models of plant metabolism.

875 Available data suggest that combining photorespiratory bypasses with optimized energy
876 dissipation holds promise for maximizing the energy available for CO₂ assimilation. New-to-nature
877 pathways show potential to exceed the yield improvements that have been achieved by current
878 photorespiratory bypasses. To date, these pathways have been prototyped *in vitro* and in
879 bacteria, and they are awaiting testing in plants.

880 Furthermore, natural variation in photosynthetic efficiency exists in both domesticated crops and
881 their wild relatives, and so provides a valuable resource for breeding strategies. Techniques such
882 as intra- and inter-specific hybridization and genome editing could leverage promising alleles
883 identified through pan-genomic association mapping and systems biology approaches to
884 introduce beneficial genetic variants into crops. Knowledge of the natural variation in
885 photosynthetic parameters should be combined with synthetic photorespiratory bypasses for
886 synergistic effects. For example, alternative forms of Rubisco with higher V_c at the cost of lower
887 V_c/V_o could be combined with more energy efficient or CO₂ concentrating photorespiratory
888 bypasses, resulting in additive benefits. Also, it should be possible to combine more performant
889 Rubisco variants with better sourcing of CO₂ via optimization of g_m . The CO₂ compensation point
890 serves as a key parameter in screening for photosynthetic efficiency; multiple methods have been
891 employed for its determination, but its precision relies on accurate estimation of cellular CO₂
892 effluxes and influxes.

893 The efficacy of increasing photosynthetic efficiency in crops in the field has been clearly
894 demonstrated, but further improvements, or reliable transfer of traits from model organisms to

895 crop species, or between crop species, will likely require additional modifications of sink tissues
896 or harvestable biomass to reap the full benefits of increased photosynthetic efficiency. Only if sink
897 strength can keep up with source capacity it will be possible to capitalize on gains in efficiency.
898 The practicality of implementing different strategies for improving photosynthetic efficiency that
899 range from selective breeding to new-to-nature pathways depends on government regulation of
900 genetic modification, which must be also considered when applying scientific discoveries to solve
901 real world problems.

902

903

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910

911 **Supplementary Materials**

912

913 Supplementary Text S1: A primer on measuring photorespiration

914

915 Supplementary Table 1: Energy budget accounting for photorespiratory bypasses

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1297 **Figure Legends**

1298

1299 **Figure 1.** Bypasses to photorespiration discussed in this review. 1) Wild type photorespiration; 2)
1300 Maier et al., 2012; 3) South et al., 2019; 4) Kebeish et al., 2007; 5) Shen et al., 2019; 6) Carvalho
1301 et al., 2011; 7) Roell et al., 2021.

1302

1303 **Figure 2.** Semi- and ultrathin cross-sections of *Helianthus occidentalis* leaf mesophyll (A) and
1304 palisade cells (B) to illustrate the CO₂ diffusion pathway from ambient air (C_a) to substomatal
1305 cavities (C_i); through intercellular airspaces to the outer surface of mesophyll cell wall ($C_{i,w}$) and
1306 further into the chloroplast (C_c). The CO₂ concentration drawdown, $C_a - C_i$, is modulated by
1307 stomatal conductance. Mesophyll conductance (g_m) is determined by both gas- and liquid-phase
1308 conductance. The $C_i - C_{i,w}$ drop is modulated by gas phase diffusion conductance (g_{ias}),
1309 depending on mesophyll thickness and effective porosity of mesophyll airspace. The CO₂
1310 drawdown from outer surface of cell walls to chloroplasts, $C_{i,w} - C_c$, is determined by liquid-phase
1311 diffusion conductance (g_{liq}) that is determined by multiple liquid and lipid phase barriers: cell wall
1312 (cw), plasma membrane (pm), cytoplasm (cyt), chloroplast envelope (env), and chloroplast stroma
1313 (chl). Thus, the physical dimensions of each anatomical component of g_m determine its partial
1314 conductance, largely setting the maximum g_m in a given species (Tosens et al., 2012). In this
1315 context, the cell periphery facing the intercellular airspace (IAS) is largely enveloped by
1316 chloroplasts ($S_c/S_{mes} \sim 1$, b). On a global scale, S_c/S_{mes} varies from 0.3 to 0.98. A high S_c/S_{mes}
1317 signifies the direct passage of CO₂ fluxes from the IAS into the chloroplasts, while, this
1318 configuration also facilitates the efficient recycling of respiratory CO₂ fluxes as chloroplasts are
1319 covered by mitochondria (M) (Busch et al., 2013). Scale bars: (A) 0.03 mm, (B) 2 μ m. Unpublished
1320 images by Tiina Tosens.

1321

1322 **Figure 3. Designed (new-to-nature) photorespiratory bypasses.** 1) Malyl-CoA-glycerate
1323 pathway (MCG) (Yu et al., 2018); 2) 3-hydroxypropionate bypass (3OHP) (Shih et al., 2014); 3),
1324 4), 5) & 6) carbon neutral bypasses (Trudeau et al., 2018); 7) tartronyl-CoA pathway (TaCo)
1325 (Scheffen et al., 2021).

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Journal Pre-proof

