Photorespiration and Improving Photosynthesis

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Contents

Abstract Improving photosynthesis has become a strategy to increase plant productivity. Current photosynthetic targets dealing with light and CO_2 capture will be briefly described and major breakthroughs dealing with photoprotection kinetics, CO_2-concentrating mechanisms, and ribulose-1,5-bisphosphate regeneration will be highlighted before focusing on photorespiration. This metabolic process

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M. Hodges (✉)
Université Paris-Saclay, CNRS, INRAE, Université Evry, Université Paris Cité, Institute of Plant Sciences Paris-Saclay (IPS2), Gif sur Yvette, France
e-mail: michael.hodges@cnrs.fr

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occurs when ribulose-1,5-bisphosphate carboxylase/oxygenase, the major CO_2-assimilatory enzyme, uses O_2 thereby producing toxic 2-phosphoglycolate that has to be removed. This is achieved by the photorespiratory cycle, a high energy cost pathway that competes with photosynthetic CO_2 assimilation and releases both CO_2 and ammonium that can be lost to the atmosphere if not re-assimilated. This wasteful metabolic pathway cannot be knocked out, as photorespiratory mutants are unable to develop normally in air and require high CO_2 atmospheres that limit photorespiration for normal growth. Surprisingly, little is known about the regulation of this important metabolic cycle even though photorespiratory enzymes are associated with several post-translational modifications. Current progress in the use of photorespiratory mutants to better understand photorespiration and its interactions with other metabolic pathways, and in proteomics to identify potential regulatory mechanisms will be described before moving onto how manipulating the photorespiratory cycle has led to the improvement of photosynthesis and plant productivity. This has been achieved either by over-expressing photorespiratory proteins or by creating alternative glycolate catabolism routes within the chloroplast.

1 Increasing Agricultural Productivity

In the past, the development of new breeding techniques and the green revolution enabled food production to mirror population growth but these advances have reached their limits and new strategies are required to feed the growing world population. A 25–70% increase in productivity is necessary to meet the predicted food requirements in 2050. This goal could be hampered by farmland losses, bio-economical requirements (such as feed for animals, bioenergy, and biopharmaceuticals) (Tilman et al. 2011), and the need to protect the environment by reducing pesticide and fertilizer uses as well as limiting greenhouse gas emissions (Sayer et al. 2013; Hunter et al. 2017). Future crop production will also be limited by extended periods of drought and high temperatures (Battisti and Naylor 2009) that are predicted from climate change models.

Plant yield is determined by the efficiencies of light capture and conversion of intercepted light into biomass and then the proportion of biomass partitioned into harvested plant parts. Both light capture and biomass partitioning are near to theoretical expectations due to plant breeding over the past decades. The determinant that has not yet reached its biological limit is light conversion to biomass. Since this is mainly dependent on photosynthesis, it has become a target for improving yield potential (Long et al. 2006; Ort et al. 2015). It is known that energy from sunlight is lost for a number of reasons. Not all wavelengths of light can be absorbed by photosynthetic pigments, a proportion of light is either reflected by or transmitted through the leaves, photosynthetic photochemistry is not 100% efficient, and a number of primary metabolic pathways (including carbohydrate synthesis,

photorespiration, and respiration) have a relatively high energy cost and/or lead to CO_2 losses (Zhu et al. 2010).

Knowledge and tools are now available to help achieve future global food sustainability by improving photosynthesis. Based on in silico modelling, potential bottlenecks related to key photosynthetic-related processes predicted to limit photosynthetic efficiency and biomass production were identified (Zhu et al. 2007, 2008), and targets to improve plant photosynthesis and productivity were selected, and some have been tested already. Amongst the principal limitations of efficient photosynthesis, plants absorb more light in full sunlight than they can use and they contain an inefficient ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) carboxylating enzyme that has an extremely slow catalytic rate. Both the "dark" reactions (CO_2 fixation by Rubisco, the regeneration of RuBP by the Calvin cycle and the production of starch and sugars) and the "light" reactions of photosynthesis (light absorption, photosynthetic electron transfer leading to the production of NADPH and ATP, as well as electrons for redox regulations of enzymes required for Calvin cycle functioning) contain potential targets for improvement (Long et al. 2006; Zhu et al. 2010; Raines 2011; Ort et al. 2015; Betti et al. 2016; Simkin et al. 2019).

2 A Brief Overview of How to Improve Photosynthesis

Before focusing on the major topic of this chapter which is photorespiration and how it is being manipulated to favour photosynthesis and yield, a brief overview of current strategies targeting specific photosynthesis-associated processes to improve photosynthesis, crop yield, and biomass will be given. They can be divided into different categories depending on the physiological processes to be optimized and they will be briefly mentioned below, after which a number of tested targets will be described in more detail.

2.1 The Light Reactions

"Light reaction" strategies are mainly aimed at improving the absorption and utilization of light and they are associated with the concept of "smart canopies": Designer plants that interact together at the canopy level to maximize light harvesting and biomass production per area of land (see Zhu et al. 2010; Ort et al. 2015).

- *Canopy optimization*: The idea is to create plants with vertical leaves in the upper canopy where high light intensities prevail, and horizontal leaves within the canopy where light is low. This also requires a redistribution of Rubisco with differing properties where upper leaves contain Rubisco with a high catalytic rate,

and lower leaves a Rubisco with a high CO_2 specificity. A redistribution of antenna and reaction centre complexes is also needed with upper canopy leaves containing reduced light harvesting antenna systems and more reaction centres, and a reversed situation in lower canopy leaves. It might be advantageous to make upper canopy leaves lighter with less chlorophyll (Chl) while making lower leaves with more Chl and with far red-shifted absorption properties since lower canopy leaves receive more infra-red light and so this will help increase light-harvesting capacity. Such canopies would allow higher levels of photosynthesis to occur throughout the plant.

- *Photoprotection:* When light absorption by photosynthetic antenna complexes exceeds photosynthetic electron transfer capacity (for example, in high light situations), excess excitation energy is dissipated as heat (qE) within a process called non-photochemical quenching (NPQ) (see Murchie and Ruban 2020). This involves conformational changes within the light harvesting antenna of PSII and requires a combination of a transmembrane proton gradient, the PSII subunit S (PsbS), and the xanthophyll cycle. Optimizing qE relaxation kinetics is important since photosynthetic efficiency decreases as a plant readjusts to low light intensities after periods of high light (see Kromdijk et al. 2016). This will be described further in Sect. 3.2 Photoprotection.

2.2 Improving the Dark Reactions

"Dark reaction" strategies are mainly aimed at improving the capacity of Rubisco to fix CO_2. Rubisco is an inefficient enzyme (Carmo-Silva et al. 2015) with a low *k*cat for CO_2 and so plants produce extremely high amounts of Rubisco protein to sustain adequate photosynthesis but this represents a large N investment (Zhu et al. 2007). Furthermore, Rubisco has an oxygenase activity competing with the CO_2 assimilatory carboxylase activity. This is the starting point for an energy consuming metabolic process called photorespiration, the major topic of this chapter.

- *Improving Rubisco*: This includes the identification of natural Rubisco forms or engineered Rubisco with kinetic properties that improve carboxylation rate and reduce oxygenase activity (Parry et al. 2007). The improvement of Rubisco activation could also help increase crop productivity (Carmo-Silva et al. 2015).
- *CO_2 concentrating mechanisms (CCM)* (see Hennacy and Jonikas 2020): This involves creating plants that can substantially increase CO_2 concentrations in the vicinity of Rubisco thus improving its carboxylation activity. One strategy is based on cyanobacterial CCMs and requires the engineering of active bicarbonate pumps, carbonic anhydrases, and carboxysome structures in C3 plants. However, many proteins are required to form carboxysomes and active pumps and therefore it is a high-risk strategy. Another excessively challenging strategy is to express a C4 photosynthetic pathway in C3 plants by manipulating both anatomical and biochemical traits to introduce a Kranz anatomy and specific enzymes. If

successful, a functional CCM in C3 plants would eventually allow endogenous Rubisco to be replaced by a Rubisco with a lower CO_2 affinity, but a higher kcat (Zhu et al. 2004). This strategy would eventually require lower amounts of Rubisco and thus improve N-use-efficiency (NUE). Further details concerning recent advances in engineering CCM mechanisms into C3 plants are given below in Sect. 3.3 CO_2 concentrating mechanisms**.**

- *Mesophyll Conductance*: This targets components involved in CO_2 diffusion into (stomatal conductance) and within the leaf to improve CO_2 availability for Rubisco.
- *RuBP Regeneration*: RuBP limitations can be lowered by manipulating rate-limiting Calvin cycle enzymes (see Simkin et al. 2019) and details concerning recent advances in this area are given below in Sect. 3.4 RuBP regeneration.
- *Photorespiratory bypasses*: To date, this has involved the engineering of artificial glycolate catabolism pathways in the chloroplast to lower the energy cost of photorespiration by removing N losses and limiting C-losses (for example, see South et al. 2018). Section 4.6 is focused on this topic.
- *Source-sink relationships*: The idea is to improve the export of photosynthetic products from source leaves to sink organs. The build-up of carbohydrates in the leaf can feed-back inhibit photosynthesis as seen by photosynthetic acclimation under elevated CO_2 conditions (Moore et al. 1999).

It is recommended to check out Ort et al. 2015 for an interesting perspective about improving photosynthetic efficiency and performance by redesigning plant systems at various scales to improve plant yield. These designs range from rather straightforward modifications, already supported by a proof-of-concept, to substantial conceptual changes that might become possible 1 day with the development of synthetic biology. A number of review papers are available that describe and discuss specific strategies to improve photosynthesis (examples include Zhu et al. 2010; Maurino and Peterhansel 2010; Raines 2011; Maurino and Weber 2013; Betti et al. 2016; Maurino 2019; Simkin et al. 2019; Baslam et al. 2020). Finally, on-going research projects aimed at improving photosynthesis using the strategies mentioned above can be visited at: https://ripe.illinois.edu/ (RIPE), https://www.capitalise.eu (Capitalise), http://www.photoboost.org/ (Photoboost), https://gain4crops.eu/ (Gain4crops) and https://c4rice.com/ (C4rice).

3 On-going Strategies for Improving Photosynthesis: Light Capture, Photoprotection, CO_2 Concentrating Mechanisms, and RuBP Regeneration

Before focusing on photorespiration, recent advances within a selection of current targets for improving photosynthesis will be described. While some of this work is still on-going, some strategies have been quite successful whereas others have not yet produced the expected improvements.

3.1 Light Capture

It has been postulated that low Chl containing leaves could be beneficial for overall canopy photosynthesis. The effects of reduced Chl on leaf and canopy photosynthesis were measured in the field using two Chl-deficient soybean (*Glycine max*) mutants (Y11y11 and Y9y9) and compared to the wild-type (WT) cultivar. Despite a >50% reduction of Chl, biomass accumulation and yield over the complete growing period was hardly impacted whereas photosynthetic efficiency (leaf-level photosynthesis per absorbed photon) appeared to be dependent on the time during the growing season (Slattery et al. 2017). Therefore, this study was unable to confirm an earlier published work where the Y11y11 mutant significantly out-yielded WT plants (Pettigrew et al. 1989). The strategy of reducing leaf Chl amounts was evaluated further using 67 soybean accessions with large variations in leaf Chl content including the soybean Y11y11 mutant. Leaf Chl amounts, leaf optical properties, and photosynthetic capacities were measured and modelled simulations suggested that canopy photosynthesis did not increase when Chl was reduced because of increased reflectance and a non-optimal N distribution within the canopy (Walker et al. 2018). Chl reduction did not improve net canopy CO_2 fixation capacity although higher net photosynthetic rates were suggested in lower canopy layers. Overall, photosynthesis was maintained in Chl-deficient canopies with a 9% saving in leaf N (Walker et al. 2018).

3.2 Photoprotection

Photosynthesis and crop productivity have been improved under fluctuating light conditions by accelerating leaf recovery from qE-associated NPQ photoprotection**.** Under full sunlight, leaves dissipate excess absorbed light energy as heat but when leaves become shaded, energy dissipation continues for many minutes and a lower photosynthesis is maintained. It had been calculated that the lag between changes in NPQ and irradiance can lead to a 20% yield penalty. By bioengineering tobacco (*Nicotiana tabacum)* to bring about an accelerated response to natural shading events, an increase of leaf net CO_2 uptake and biomass production of up to 15% was observed under fluctuating light conditions. This was achieved by targeting the xanthophyll cycle by over-expressing *Arabidopsis thaliana* violaxanthin de-epoxidase, zeaxanthin epoxidase, and *PsbS* in tobacco leaves. This led to an accelerated attenuation of NPQ after transfer from high light to shade and gave a more rapid restoration of maximum CO_2 assimilation efficiency (Kromdijk et al. 2016).

3.3 CO_2 Concentrating Mechanisms

3.3.1 Incorporating a C4 Plant CMM into a C3 Plant

Nature has evolved several strategies to suppress the Rubisco oxygenation reaction by sequestering the enzyme into compartments that can concentrate CO_2. This includes C4 plants that have different biochemical and anatomical properties when compared to C3 plants. C4 leaves contain two distinct layers of photosynthetic tissues (the so-called Kranz leaf anatomy) where mesophyll cells are in contact with atmospheric CO_2 whereas bundle sheath (BS) cells are less CO_2 permeable. Bicarbonate is assimilated in mesophyll cells via phospho*enol*pyruvate (PEP) carboxylase (PEPc) and oxaloacetate (OAA) is produced which is then converted to a more stable 4C organic acid (malate or aspartate depending on the type of C4 plant). After diffusion to BS cells, CO_2 is produced close to Rubisco by the decarboxylation of the mobile C4 acid. A coordinated international effort to introduce C4 metabolism into rice has begun to give some interesting results (see Hibberd et al. 2008; Von Caemmerer et al. 2012; Ermakova et al. 2020). To introduce Kranz anatomy into rice, vein spacing patterns must be altered so that leaf veins are closer together and chloroplast development in BS cells must be activated since they are non-photosynthetic in rice. However, the regulation of Kranz anatomy development in a C4 plant is still unknown and current work is aimed at identifying the regulatory genes in maize. Once identified, engineering in rice can begin. Recently it was found that BS chloroplast biogenesis was enhanced when the transcriptional activator, *Os*CGA1, was driven by a vascular specific promoter (Lee et al. 2021). Furthermore, a mutant screen of *Setaria viridis* (an NADP-malic enzyme (NADP-ME) type C4 monocot) has provided evidence that a functional suberin lamellae is an essential anatomical feature for efficient C4 photosynthesis in NADP-ME C4 plants like *S. viridis* (Danila et al. 2021). Manipulation of C4 pathway biochemistry is perhaps more straightforward because genes encoding C4 pathway enzymes and metabolite transporters are known. However, they must be turned on at the correct time, to the required level, and in specific cell types. It has been demonstrated that a promoter sequence of the C4-type PEPc gene from three different C4 plants can drive mesophyll-cell-specific reporter gene expression in rice (Gupta et al. 2020). Introducing C4 biochemistry into rice with a C3 anatomy is being carried out and a partial flux through the carboxylation part of NADP-ME C4 metabolism in transgenic rice has been demonstrated when transformed with maize NADP-ME, PEPc, NADP-malate dehydrogenase, and pyruvate phosphate dikinase (Lin et al. 2020). The expression of C4 photosynthesis enzymes (carbonic anhydrase, PEPc, NADP-malate dehydrogenase, pyruvate orthophosphate dikinase and NADP-ME from maize and driven by cell-preferential promoters) has also been achieved in rice using a single construct (Ermakova et al. 2021). Such encouraging results suggest that a functional C4 pathway will be 1 day achievable.

3.3.2 Incorporating a Cyanobacterial CMM or a Green Algal CMM into a C3 Plant

An alternative approach to creating C4 photosynthesis inside a C3 plant is to copy a cyanobacterial CCM composed of bicarbonate transporters and a Rubisco-containing proteinaceous compartment called a carboxysome (see Price et al. 2013). The cyanobacterial CCM is a system that generates a high HCO_3^- pool through the action of inorganic carbon transporters and CO_2-converting complexes. Bicarbonate and RuBP both diffuse into the carboxysome where the bicarbonate is converted back to CO_2 via a carboxysome localized carbonic anhydrase. To date, it has been demonstrated that β-carboxysome shell proteins can be assembled in tobacco (*Nicotiana benthamiana*) chloroplasts producing structures suggestive of self-assembled carboxysomes (Lin et al. 2014). The introduction of a functional CCM in C3 plants would allow native Rubisco to be replaced with the cyanobacterial enzyme that has a higher catalytic rate although at the expense of a lower affinity for CO_2 and a lower specificity factor when compared to plant Rubisco (Price and Howitt 2014). Simplified carboxysomes formed after the expression of two key α-carboxysome structural proteins have also been successfully produced in tobacco chloroplasts where the endogenous Rubisco large subunit gene was replaced by cyanobacterial form-1A Rubisco large and small subunits. Albeit demonstrating the formation of fully functional α-carboxysomes within chloroplasts using this reduced gene set, the tobacco plants had poor growth and a low CO_2 assimilation rate. Autotrophic growth was possible only at elevated CO_2 (Long et al. 2018). Attempts to increase tobacco plant photosynthetic efficiency and biomass by expressing and integrating individual components of a *Chlamydomonas reinhardtii* CCM (either carbonic anhydrase CAH3 or the bicarbonate transporter LCIA) into their chloroplasts demonstrated that biomass production could be increased in this way. This suggests that combining multiple CCM components could further increase the productivity and yield of C_3 crops (Nölke et al. 2019).

3.4 RuBP Regeneration

The accelerated rate of Rubisco-catalysed carboxylation at current atmospheric CO_2 levels (>400 ppm in 2021) has led to a kinetic limitation in the regeneration of RuBP, the CO_2 acceptor molecule, under non-limiting light conditions. This problem is expected to become more important in the future due to the continual increase in atmospheric CO_2 levels, a hallmark of the Anthropocene epoch and the significant impact of man on the climate and ecosystems of our planet. A proven strategy to limit this limitation has been the over-expression of rate-limiting enzymes of the Calvin cycle. Improvement of photosynthesis and yield using this approach has been reviewed recently (see, for example, Simkin et al. 2019; Baslam et al. 2020) and therefore only a brief account with be given. In 2005, it was shown that when sedoheptulose-1,7-bisphosphatase (SBPase) activity was increased in transgenic

N. tabacum plants expressing Arabidopsis SBPase both photosynthesis and growth were stimulated (Lefebvre et al. 2005). This has held true in other plant species where over-expression (OE) of SBPase has led to plants exhibiting increased photosynthetic activities and biomass. This includes *Arabidopsis thaliana* (Simkin et al. 2017), tobacco (Lefebvre et al. 2005; Rosenthal et al. 2011; Simkin et al. 2015), tomato (Ding et al. 2016), and wheat (Driever et al. 2017). However, beneficial effects were often dependent on plant developmental stage and/or growth conditions. For instance, higher photosynthetic rates were only seen in young tobacco leaves and they were found under short day periods and low light intensities (Lefebvre et al. 2005). The OE of cyanobacterial and green algal enzymes in higher plants has also been studied. OE of *Chlamydomonas reinhardtii* SBPase or cyanobacterial fructose-1,6-bisphosphatase (FBPase) led to increases in photosynthesis and biomass (Tamoi et al. 2006). A cyanobacterial bi-functional SBPase/FBPase enzyme has been over-expressed in tobacco (Miyagawa et al. 2001), lettuce (Ichikawa et al. 2010), and soybean (Köhler et al. 2017) with all transformed plants showing improved photosynthetic CO_2 assimilation rates and biomass production. When Arabidopsis fructose bisphosphate aldolase (FBPA) was over-expressed in the photosynthetic tissues of Arabidopsis using a Rubisco small subunit 2A promoter, similar increases in photosynthesis, dry weight (DW), and seed yield occurred (Simkin et al. 2017). Perhaps surprisingly, Arabidopsis plants over-expressing both SBPase and FBPA exhibited no additional increases in their maximal CO_2 assimilation rate, DW, and seed yield when compared to lines over-expressing the individual transgenes (Simkin et al. 2017).

4 Photorespiration

This chapter will now focus on photorespiration (see Bauwe et al. 2010; Eisenhut et al. 2019) as a target for improving plant performance. Before describing the photorespiratory cycle, its regulation, the use of photorespiratory mutants, and how photorespiration has been manipulated to improve photosynthesis, a concise explanation as to why photorespiration is bad for photosynthesis and crop yield will be given.

4.1 Why Is Photorespiration Bad for Photosynthesis and Crop Yield?

Photorespiration is essential for C3 (and C4) plant growth in air containing current CO_2 concentrations (normal air). However, this high flux metabolic process has an energy cost estimated to reduce the theoretical C3 photosynthetic efficiency by 48% at 30°C and an atmospheric CO_2 concentration of 0.038% (Zhu et al. 2008). In

normal air at 25°C, Rubisco undertakes approximatively ~2 oxygenation reactions for every 5 carboxylation reactions and the photorespiratory cycle requires 3.5 ATP and 2 NADH equivalents per oxygenation reaction to process the 2-phosphoglycolate (2PG) and 3-phosphoglycerate (3PGA) into RuBP (see Foyer et al. 2009; Walker et al. 2016). The energetic demands calculated for photorespiration suggests that 32% of total ATP and 28% of total NADH equivalents are consumed in an illuminated C3 leaf at 25°C and 0.035% CO_2 containing air (Walker et al. 2016). Of course, this will depend on environmental conditions that change chloroplast O_2 and CO_2 concentrations such as temperature.

In the USA, photorespiration has been estimated to reduce wheat and soybean yields by 20% and 36%, respectively, and this is predicted to increase with expected higher temperatures and longer drought periods due to climate change (Walker et al. 2016). On the other hand, higher CO_2 concentrations should improve photosynthesis and C3 plant growth as photorespiration will be reduced due to an altered competition between O_2 and CO_2 at the active site of Rubisco in favour of CO_2 and a reduced waste of Calvin cycle flux to produce RuBP for Rubisco oxygenase activity (see Ainsworth and Long 2005 who reviewed 15 years of free-air CO_2 enrichment data). That said, photorespiration will continue to negatively impact crop yield under predicted future climates even though models suggest a 12–55% improvement in photosynthesis under different climate change scenarios in the absence of photorespiration (Walker et al. 2016). However, models predict photorespiratory yield penalties of 8% and 19% (for wheat and soybean, respectively) will occur at 0.1% CO_2 and with a 3.7°C increase in temperature (Walker et al. 2016). In conclusion, as both atmospheric CO_2 and temperature levels increase with the progression of climate change, crop yield losses due to photorespiration will remain significant. It is therefore not surprising that re-engineering the photorespiratory cycle has become a wide-spread strategy to improve photosynthesis and yield in both model plants and crops (see, for example, Hagemann and Bauwe 2016; South et al. 2018; Eisenhut et al. 2019), even though it is tightly embedded in a complex network of metabolic processes (Hodges et al. 2016) (see Fig. 1). That said photorespiration is believed to be an energy sink that can limit over-reduction of the photosynthetic electron transfer chain and thus protect against photoinhibition, especially under stress conditions that lead to stomatal closure (drought, salinity) and a reduction of photosynthetic CO_2 assimilation. It also produces glycine that can be used to make glutathione which is involved in the protection against reactive oxygen species that are often generated when plants are exposed to environmental stresses (see Wingler et al. 2000; Voss et al. 2013). A functional photorespiratory cycle is also important for plant tolerance against biotic stress, as seen from the reduced resistance to pathogens of certain photorespiratory mutants (Moreno et al. 2005; Rojas et al. 2012).

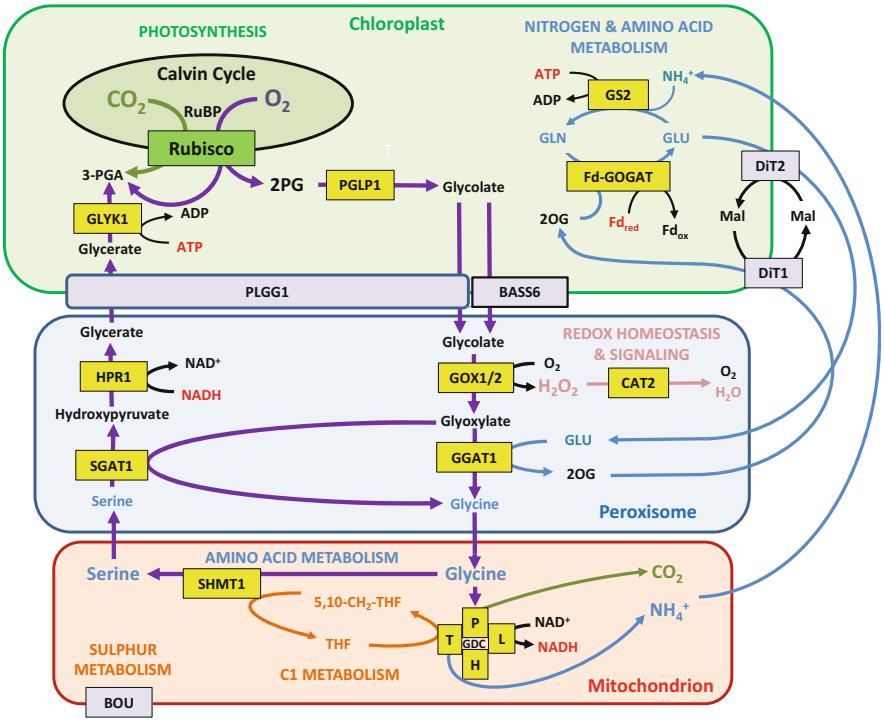

Fig. 1 The photorespiratory cycle and its interactions with other metabolic processes. Rubisco oxygenase activity produces 2PG that is transformed to 3PGA by the action of eight core photorespiratory enzymes. The photorespiratory cycle takes place in four cell compartments: chloroplasts, peroxisomes, mitochondria, and the cytosol. It interacts with photosynthesis, nitrogen and amino acid metabolisms, redox homeostasis and signalling, C1 and sulphur metabolisms. *BASS6* plastidial glycolate transporter, *CAT2* catalase 2, *DiT1* plastidial 2OG/malate transporter, *DiT2* plastidial glutamate-malate transporter, *Fd-GOGAT* ferredoxin-dependent glutamate synthase, *GGAT1* glutamate glyoxylate aminotransferase 1, *GDC* glycine decarboxylase complex (composed of P, T, L, H subunits), *GLYK1* glycerate kinase 1, *GOX1/2* glycolate oxidase 1/2, *GS2* plastidial glutamine synthetase, *HPR1/2* hydroxypyruvate reductase 1/2, *PGLP1* 2PG phosphatase 1, *PLGG1* plastidial glycolate/glycerate transporter, *Rubisco* RuBP carboxylase/oxygenase, *RuBP* ribulose-1,5-bisphosphate, *SGAT1* serine glyoxylate aminotransferase 1, *SHMT1* serine hydroxymethyltransferase 1, *THF* tetrahydrofolate, *2OG* 2-oxoglutarate, *2PG* 2-phosphoglycolate, *3PGA* 3-phosphoglycerate

4.2 What Is Photorespiration?

Photorespiration is a high flux metabolic pathway, essential to all O_2-producing photosynthetic organisms living in aerobic environments. It is often seen as a closed metabolic cycle that removes and recycles toxic 2PG to make 3PGA to fuel the Calvin-Benson cycle but this view is over-simplified since this C2-cycle interacts with several primary metabolic pathways. These include photosynthesis, N-assimilation, amino acid metabolism, C1 metabolism, sulphur metabolism, the Krebs cycle, respiration, cell redox balance, and signalling (see Fig. 1, and Eisenhut

et al. 2019). Indeed, as mentioned below in Sect. 4.3, a dysfunctional photorespiratory cycle leads to a reduction of photosynthetic CO_2 assimilation and an increase in NPQ when photorespiratory mutants are transferred from high CO_2 (low photorespiration) to normal air (see, for example, Dellero et al. 2015, 2016a). The accumulation of photorespiratory 2PG inhibits the Calvin cycle and reduces starch production (Flügel et al. 2017). A reduction in photorespiratory activity under elevated CO_2 has been shown to reduce nitrate uptake and assimilation (Rachmilevitch et al. 2004; Bloom et al. 2010). Photorespiratory ammonium is re-assimilated by the glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle in the chloroplast. The importance of plastid GS2 in photorespiratory ammonium assimilation and ferredoxin (Fd)-GOGAT in Glu production for both GS2 activity and photorespiratory cycle aminotransferase activity is highlighted by the respective mutant phenotypes (Somerville and Ogren 1980a; Wallsgrove et al. 1987; Coschigano et al. 1998; Ferreira et al. 2019). Photorespiration is a major source of leaf Gly and Ser production and their levels are modulated with photorespiratory flux as seen, for instance, under low 0.01–0.02% CO_2 levels (Dellero et al. 2021). It was also observed that glutamate glyoxylate aminotransferase (GGAT) activity is important for Ser homeostasis (Dellero et al. 2015). Photorespiration is also linked to C1 metabolism because tetrahydrofolate (THF) is needed for Gly decarboxylase (GDC) activity. Indeed, the accumulation of extremely high Gly levels and severe developmental phenotypes were observed in air-grown Arabidopsis 10-formyl tetrahydrofolate [THF] deformylase mutants (Collakova et al. 2008). A link with sulphur metabolism has been identified during the phenotypic characterization of a *bou* mutant that exhibits a low GDC activity and lacks a mitochondrial carrier of unknown function. There was also a down-regulation of Calvin Cycle and N-assimilation genes, whereas key enzymes of glycolysis and the TCA pathway were up-regulated and the accumulation of sugars and TCA intermediates was modified (Samuilov et al. 2018). Photorespiration produces NADH in mitochondria that can be used for respiration but if NADH accumulates this can lead to the inhibition of TCA cycle dehydrogenases (Tcherkez et al. 2008; Nunes-Nesi et al. 2013). Photorespiratory glycolate oxidase (GOX) produces H_20_2, an important redox signal and component of cell redox homeostasis (Foyer and Noctor 2020), and low GOX activities reduce plant resistance to pathogens (Rojas et al. 2012). These examples clearly highlight the complex interactions between the photorespiratory cycle and other plant primary metabolisms that could hinder the successful re-engineering of photorespiration to improve plant performance.

As stated several times already, photorespiration begins when Rubisco oxygenase activity produces a molecule of 2PG (and a molecule of 3PGA) from the oxygenation of RuBP. 2PG is toxic to the cell because it inhibits the Calvin-Benson cycle enzymes triosephosphate isomerase (TPI) and SBPase and the glycolytic enzyme phosphofructokinase (PFK) (Anderson 1971; Kelly and Latzko 1976; Flügel et al. 2017). Therefore, it must be removed and its carbon salvaged as 3PGA by the photorespiratory cycle, a pathway that spans four cell compartments: chloroplasts, peroxisomes, mitochondria, and the cytosol (see Fig. 1). The photorespiratory cycle recovers three quarters of the carbon contained in two molecules of 2PG by making

one 3PGA molecule. It is composed of eight core enzymes excluding Rubisco. First, 2PG is dephosphorylated to glycolate by phosphoglycolate phosphatase (PGLP, Schwarte and Bauwe 2007) in the chloroplast. Glycolate is then exported by two transporters, PLGG1 (a glycolate-glycerate transporter, Pick et al. 2013) and BASS6 (a glycolate transporter, South et al. 2017) and oxidized to glyoxylate in peroxisomes by GOX (Dellero et al. 2016a, b) that also produces a molecule H_20_2 which is subsequently removed by the action of a catalase. In Arabidopsis leaves this is carried out by CAT2 (Queval et al. 2007). Peroxisomal glyoxylate is then converted to Gly by a glutamate glyoxylate aminotransferase (GGAT, Igarashi et al. 2006; Liepman and Olsen 2003; Dellero et al. 2015) that also produces 2-oxoglutarate (2OG) from Glu. This reaction links photorespiration to the chloroplast GS2/Fd-GOGAT cycle. Glycine is imported into mitochondria where two Gly molecules are converted to one molecule of Ser by the joint action of GDC and serine hydroxymethyltransferase (SHMT). GDC catalyses the oxidative decarboxylation and deamination of Gly and generates CO_2, ammonium and NADH. The remaining methylene carbon of Gly is transferred to THF to form methyl-THF that reacts with a second Gly in a reaction catalysed by SHMT to form Ser. GDC is a multimeric complex composed of four subunits (H, P, T and L). H is a lipoamide-containing protein that has a pivotal role in the complete sequence of GDC reactions as it undergoes a cycle of reductive methylamination (catalysed by the P-protein), methylamine transfer (catalysed by the T-protein), and electron transfer (catalysed by the L-protein). The P-protein is responsible for the decarboxylation of Gly and the liberation of CO_2, the T-protein produces ammonium and methyl-THF and the L-protein generates NADH from NAD (see Douce et al. 2001). Serine then enters the peroxisome to be converted to hydroxypyruvate (with the production of Gly) by serine glyoxylate aminotransferase (SGAT, Liepman and Olsen 2001) and subsequently to glycerate by hydroxypyruvate reductase (HPR, Timm et al. 2008, 2011) that oxidizes NADH to NAD. Glycerate is imported into the chloroplast via PLGG1 where it is converted to 3PGA by glycerate kinase (GLYK, Boldt et al. 2005). The complete photorespiratory cycle and interacting metabolisms are shown in Fig. 1.

4.3 Regulation of the Photorespiratory Cycle

To date, little is known about the regulation of the photorespiratory cycle. That said, recent reviews dealing with the redox regulation of photorespiration (Keech et al. 2016) and the role of regulatory proteins and metabolites (Timm and Hagemann 2020) are available. Advances in proteomics have led to the identification of a large number of peptides containing putative post-translational modifications (PTM) including protein phosphorylation, ubiquitination, acetylation, and different redox modifications such as nitrosylation, glutathionylation, methionine oxidation, cysteine sulfenylation, and reversible cysteine oxidation. Potential PTMs associated with photorespiratory enzymes can be found by examining public databases that include PhosPhAt (https://phosphat.uni-hohenheim.de/), Athena (http://athena.proteomics.

wzw.tum.de) and the Plant PTM viewer (https://www.psb.ugent.be/webtools/ptm-viewer/). Certain data concerning the phosphorylation and redox regulation of core photorespiratory enzymes have been extracted from these three databases and presented in Table 1.

It can be seen that photorespiratory enzymes are associated with many putative phosphorylation sites (see Table 1 and Hodges et al. 2013). All of them appear to undergo protein phosphorylation at multiple phosphorylation sites (Table 1). However, these potential phosphorylations have not been confirmed either in vitro or *in planta* by non-proteomic approaches and their roles remain unknown. The production and characterization of phospho-mimetic recombinant proteins have been used to study the impact on the enzyme kinetics of several photorespiratory enzymes. In such experiments, identified phospho-amino acids were replaced by a negatively charged Asp molecule to mimic the negative charge of the phosphate group. It was found that Arabidopsis HPR1-T335D (where Thr335 was replaced by an Asp) exhibited a lower NADH-dependent HPR activity and an improved NADPH-dependent activity. When introduced into the Arabidopsis *hpr1-1* mutant line under the control of an SHMT1 promoter, HPR1-T335D was unable to fully complement the *hpr1-1* growth phenotype in normal air. HPR1-T335D-containing *hpr1-1* plants remained smaller and had lower photosynthetic CO_2 assimilation rates. Rosette leaf metabolite analyses of the transformed lines suggested that there were subtle perturbations in photorespiratory cycle functioning when compared to WT plants and HPR1-T335A (where Thr335 was replaced with an Ala, as a control) expressing lines (Liu et al. 2020). In a similar approach, the Arabidopsis *shm1-1* mutant was complemented with a phospho-mimetic SHMT1-S31D protein to generate *Compl-S31D* lines. In response to either a salt or a drought stress, *Compl-S31D* lines showed a lower tolerance when compared to WT and the control *Compl-S31A* plants. The poorer salt sensitivity of *Compl-S31D* plants appeared to correlate with their lower SHMT1-S31D protein amounts and SHMT activities that led to Pro under-accumulation. The phospho-mimetic S31D mutation of *Compl-S31D* lines also led to a reduction in salt-induced and ABA-induced stomatal closure (Liu et al. 2019). Several putative GOX phosphorylation sites (Thr4, Thr158, Ser212, and Thr265) were tested using recombinant Arabidopsis GOX1 and GOX2, and maize GO1 and three different substrates (glycolate, lactate and 2-hydroxy-octanoate) (Jossier et al. 2020). The phosphopeptides had been identified either in PhosPhAt or in the literature (Thr4 (Reiland et al. 2009 and PhosPhAt), Thr158 (PhosPhAt, Umezawa et al. 2013; Choudhary et al. 2015; Abadie et al. 2016), Ser212 (Umezawa et al. 2013 and PhosPhAt) and Thr265 (Aryal et al. 2012)). Several phospho-mimetic mutations (T4D, T158D, and T265D) led to a severe inhibition of recombinant enzyme activity without altering the Km values for the tested substrates. This was associated with a loss of flavin mononucleotide (FMN) cofactor within the T4D and T158D proteins. Phospho-dead versions exhibited different modifications according to the phospho-site and/or the GOX mutated. All T4V and T265A enzymes had kinetic parameters similar to WT GOX, all T158V proteins showed reduced activities while S212A and S212D mutations had no effect on GOX1 activity but GOX2 and GO1 activities were 50% reduced (Jossier et al. 2020). Taken together, these

Table 1 Potential phosphorylated and redox modified amino acids of photorespiratory core enzymes

	Phosphorylation			REDOX				
	PhosPhAt	Athena	PTM	NO	GO	SO	CYS OX	MET OX
PGLP1 At5g36700	T322 S356	S121 T122 S356	S38 T122 S356	C239 C320				M106
GOX1 At3g14420	T4 T155 T158 T355	S197 S201 S364 T61 T355 T360	S201 S212 T355 T360 S364		N195			
GOX2 At3g14415	S212		T158 S201 S212		N195			
GGAT1 At1g23310	Y8 S275 T339	S275 T399	S275 T399	C226 C377 C382 C417		C149 C226 C239 C417	C149	
GDC-T At1g11860	S174 S268 S330 S331 S337 S339 S393	S393 S268 S330 S331	S174 S268 S331 S337 S393	C75 C151		C75	C75 C88 C151 C276	M163 M367
GDC-P1 At4g33010	S267 Y557 S1002	S48 S69 S85 S476	T49 T92 S476 S1002	C98 C245 C463 C777 C943		C463 C777 C943	C777	M120 M863
GDC-P2 At2g26080	S44 S47 S1008	S46 S47	S46 S47 S1008	C251 C783 C949		C783 C949 C1028	C783	M125 M869
GDC-L1 At1g48030	S19 S190 S211 T318 S319	S19 S31 S190 S319	S19 S31 S190 T318 S319	C372		C87 C372	C71 C82 C87 C372	M423
GDC-L2 At3g17240	S190 T318 S319 S426		S190 T318 S319	C372 C483		C87 C372	C71 C82 C87 C372	M423 M437
GDC-H1 At2g35370	S140 S141	S140 S141	S120 S140 S141				C158	M149
	S141 S142	S21 S30 S32 S121 S141 S142	S21 S30 S121 S142				C159	M150

(continued)

Table 1 (continued)

	Phosphorylation			REDOX				
	PhosPhAt	Athena	PTM	NO	GO	SO	CYS OX	MET OX
GDC-H3 At1g32470								
SHMT1 At4g37930	S12 S13 S26 Y28 S31 S34 T46 T470	S12 S13 S24 S26 Y28 S232 T348	S12 S13 S26 T46 S232	C125				
SGAT At2g13360	S37 S215 S387 T388	S37	S37 S215	C142 C297			C181	
HPR1 At1g68010	S178 S229	S6 S228 S229 S365	S228 S229 S365	C271 C38				M261 M262
HPR2 At1g79870	S45	T41 S45	S45			C86		
GLYK At1g80380			T222 T223					

Data was retrieved from PhosPhAt, Athena, and Plant PTM Viewer (PTM)
NO nitrosylation, *GO* glutathionylation, *SO* sulphenylation, *CYS OX* reversible cysteine oxidation, *MET OX* oxidized methionine. Enzyme abbreviations are given in the text. Note that PGLP1 is also annotated as At5g36790

observations suggest that phosphorylation of photorespiratory enzymes has the potential to modulate their activities and impact plant growth and response to abiotic stresses.

Proteomics have also indicated that photorespiratory enzymes can be redox regulated and acetylated. GDC-P, GDC-H, GDC-T, GDC-L, and SHMT1 were found in analyses aimed at identifying thioredoxin (Trx)-regulated proteins in mitochondria isolated from pea and spinach leaves, and potato tubers using Trx-affinity chromatography and mass spectroscopy (Balmer et al. 2004). In a similar study using Arabidopsis extracts, GDC-H1 was identified (Marchand et al. 2004). Arabidopsis PGLP1 was found to interact with two chloroplastic redox actors, a 2-Cys-peroxiredoxin (2-Cys PRX) (Cerveau et al. 2016) and the NADPH-Trx reductase NTRC (González et al. 2019). The oxidation of 3,845 cysteines within the proteome of *Phaeodactylum tricornutum* was quantified and PGLP, GDC-P, and SHMT were amongst the identified H_20_2-sensitive proteins (Rosenwasser et al. 2014). A Lys acetylome of Arabidopsis mitochondrial proteins identified SHMT1, and SHMT2, GDC-H1, GDC-P1, GDC-P2, and GDC-T subunits (König et al. 2014) whereas all core photorespiratory enzymes are found annotated as Lys acetylated in Plant PTM Viewer. A modified biotin-switch method was used to detect Arabidopsis proteins modified by S-sulfhydration under physiological conditions and this led to the identification of GGAT1, GDC-L1, GDC-H1, and SHMT1 (Aroca et al. 2015). A site-specific nitrosoproteomic approach allowed the identification of 1,195 endogenously S-nitrosylated peptides in 926 proteins from *Arabidopsis thaliana* including nitrosylated HPR1 and PGLP1 (Hu et al. 2015). Arabidopsis PGLP1 was also identified in the guard cell nitrosoproteome after *flg22*-treatment (Lawrence et al. 2020). A site-specific nitrosoproteomic approach allowed the identification of 1,195 endogenously *s*-nitrosylated peptides in 926 proteins from *Arabidopsis thaliana* including C320-nitrosylated PGLP1 (Hu et al. 2015). On the other hand, when genomes of nine representative model species from streptophyte algae to angiosperms were analysed, no photorespiratory enzymes were identified in the plastid glutathione-dependent redox network (Müller-Schüssele et al. 2021). Peroxisomal GOX1 and GOX2 are both annotated as glutathionylated in the plant PTM viewer (Table 1).

The impact of mitochondrial Trx (Trxo1 and Trxh2) on photorespiration has been studied (Reinholdt et al. 2019; Da Fonseca-Pereira et al. 2020). Combining in vitro enzymatic activity measurements and metabolomics, a redox-regulation of GDC-L activity by either a dithiothreitol treatment or by an NTRA-TRXo1 or NTRA-TRXh2 system were observed, that led to a reduced activity in vitro (Reinholdt et al. 2019; Da Fonseca-Pereira et al. 2020). However, single *trxo1* and *trxh2* T-DNA mutants did not display a growth phenotype, although there was evidence of altered photorespiration based on Gly accumulation. In vitro studies of recombinant GDC proteins revealed that Trxo1 and Trxh2 modulated the activity of mitochondrial lipoamide dehydrogenase (GDC-L) (Reinholdt et al. 2019; Da Fonseca-Pereira et al. 2020). A double mutant lacking TRXo1 and up to 95% of GDC-T protein exhibited a severe growth phenotype whereas the original *gldt1* knockdown line showed only a mild growth reduction (Reinholdt et al. 2019). It was proposed

that TRXo1 regulation of GDC was necessary to allow for the rapid induction of mitochondrial photorespiratory cycle steps to facilitate the light-induction of photosynthesis (Reinholdt et al. 2019). However, other regulatory processes or regulators cannot be excluded, and more work is still required to elucidate whether the functions of Trxo1 and Trxh2 are redundant or have specific targets controlled by specific environmental and physiological cues. Indeed Arabidopsis plants lacking TRXh2 also showed delayed seed germination, reduced respiration, impaired stomatal and mesophyll conductance without impacting photosynthesis, alterations in key metabolites of photorespiration, respiration and amino acid metabolism as well as a decreased abundance of SHMT and GDC-H and -L subunits (Da Fonseca-Pereira et al. 2020). This work has recently been reviewed in the wider context of Trx-mediated regulation of (photo)respiration and central metabolism (Da Fonseca-Pereira et al. 2021). A C4-specific Trx-dependent regulation of maize GLYK has also been reported (Bartsch et al. 2010). A short C-terminal extension containing two strategically positioned Cys residues forms a disulphide bridge at night when GLYK becomes less active, whereas this bridge is reduced by a chloroplastic Trx-f and full activity is restored in the light.

When Arabidopsis leaf mitochondria were S-nitrosoglutathione (GSNO)-treated, the biotin-switch method coupled to nano-liquid chromatography and mass spectrometry allowed the identification of GDC-H1, GDC-L1/2, and GDC-P1/2 subunits as well as SHMT as either *S*-nitrosylated and/or glutathionylated (Palmieri et al. 2010). A GSNO treatment of a partially purified Arabidopsis GDC-P protein led to a 70% inhibition of GDC activity and showed multiple Cys glutathionylations rather than nitrosylations. A decrease in activity was also observed in the presence of sodium nitroprusside (a NO donor), thus suggesting that nitrosylation could also be responsible for GDC-P protein inhibition (Palmieri et al. 2010). Two independent studies with pea have also shown that several photorespiratory enzymes can be subject to S-nitrosylation. In a study of salt-stress and mitochondrial protein nitrosylation, GDC-P, GDC-T, GOX, and SHMT were identified (Camejo et al. 2013). When pea peroxisome proteins were treated with GSNO, HPR, GDC-L, GDC-H, SGAT, and GOX were found to be S-nitrosylated and this inhibited GOX activity (Ortega-Galisteo et al. 2012).

It can be seen from these studies that when photorespiratory enzymes undergo a PTM (either phospho-mimetic, Trx-associated or S-nitrosylation) their respective activities are always reduced. Further work is required to validate the potential PTMs identified by proteomics (Table 1) and to understand the *in planta* roles of such PTMs. Perhaps in the future, verified PTMs could be manipulated to modify photorespiratory cycle flux and thus impact favourably plant metabolism and performance.

4.4 Characterization of Photorespiratory Mutants

Photorespiratory cycle mutants have been identified in many plant species from plant models like *Arabidopsis thaliana* to crops including rice, barley, pea, and potato (Timm and Bauwe 2013). For the model C3 plant *Arabidopsis thaliana*, an almost complete set of genetically defined mutant lines (by chemical treatment using ethyl methanesulfonate (EMS), by the insertion of a T-DNA, and by the use of antisense and RNAi strategies) for core photorespiratory enzyme genes and for several transporter genes is now available (see Table 2). It should be noted that Table 2 only contains core photorespiratory enzyme mutants whereas mutants involved in "associated processes" such as ammonium assimilation can be found elsewhere (Timm and Bauwe 2013; Eisenhut et al. 2019). The characterization of mutants was primordial in identifying photorespiratory genes and highlighting the presence of redundant gene functions and compensatory pathways. Mutants have also indicated that photorespiration is important even in CCM-containing plants (Zelitch et al. 2009; Levey et al. 2019), cyanobacteria (Eisenhut et al. 2008), and green algae (Suzuki et al. 1990). Above all, the identification and characterization of photorespiratory mutants showed that it was not possible to improve plant performance by knocking out (or slowing down) the photorespiratory cycle. Indeed, mutations of photorespiratory core cycle genes are characterized by either lethality or stunted growth when grown under normal air although most of them become viable when grown under elevated CO_2 conditions (see Fig. 2 and Table 2). This comportment has been described as "the photorespiratory phenotype" and it was used by Somerville and Ogren in the late 1970s and early 1980s to isolate the first photorespiratory mutants of *Arabidopsis thaliana* (see Somerville 2001). Indeed, to accomplish this they took advantage of the photorespiratory phenotype. EMS-treated Arabidopsis seeds were produced; they were germinated under high 1% CO_2 conditions and then transferred to ambient air that led to leaf bleaching. Recovery under high CO_2 was then carried out and this led to the identification of mutants affected in the following core photorespiratory functions: PGLP (Somerville and Ogren 1979), SGAT (Somerville and Ogren 1980b), SHMT (Somerville and Ogren 1981), and GDC (Somerville and Ogren 1982). This strategy also led to the identification of mutants lacking associated photorespiratory functions: Fd-GOGAT (Somerville and Ogren 1980a) and a plastid dicarboxylate transporter (Somerville and Ogren 1983). Since these pioneering experiments, photorespiratory mutants have continued to be isolated from a multitude of plant species (see Timm and Bauwe 2013) mainly by homology searches using model plant gene sequences but also more recently by co-expression analyses. This has highlighted distinct photorespiratory phenotypes according to the place of the protein within the cycle and this has often been studied by comparative analyses after transferring mutants from elevated CO_2 to normal air (see, for example, Timm et al. 2012b; Timm and Bauwe 2013; Dellero et al. 2015, 2016a). The precise reasons for mutant-specific phenotypes are mostly unknown; however, they could be associated with photorespiratory cycle interactions with other metabolic pathways (as discussed

Table 2 *Arabidopsis thaliana* photorespiratory genes and their corresponding mutant lines

Enzyme	Mutant	Arabidopsis locus	Growth recovery % CO_2	Mutant type/comment	References
PGLP	*pcoA* *pglp1-1*	At5g36700	2	EMS T-DNA Antisense	Somerville and Ogren (1979) Schwarte and Bauwe (2007) Flügel et al. (2017)
GOX	*gox1–1*	At3g14420	0.038	T-DNA	Dellero et al. (2016a, b) Rojas et al. (2012)
	gox2-1	At3g14415	0.038	T-DNA	Dellero et al. (2016a)
	gox2-2	At3g14415	0.038	T-DNA	Dellero et al. (2016a) Rojas et al. (2012)
	gox1gox2		0.3	RNAi	Dellero et al. (2016a)
GGAT	*aoat1-1* *ggt1-1* *ggt1-2*	At1 g23310	0.3	T-DNA	Igarashi et al. (2003) Dellero et al. (2015)
GDC-T	*gldt1-1*	At1g11860	0.038	T-DNA KD & RNAi	Engel et al. (2008)
GDC-P	*gldp1-1*	At4g33010	0.038	T-DNA	Engel et al. (2007)
	gldp2-2	At2g26080	0.038	T-DNA	Engel et al. (2007)
	gldp1-1gldp2-2		Lethal	T-DNA/>2% CO_2 does not recover mutant phenotype	Engel et al. (2007)
GDC-L	*No mutant lines*	LPD1 At1g48030		Proposed photorespiratory gene	
	mtlpd2	LPD2 At3g17240	0.038	T-DNA	Lutziger and Oliver (2001)
GDC-H	*No mutant lines*	GDH1 At2g35370 GDH3 At1g32470		Potential photorespiratory GDC-H genes	
SHMT	*shm1-1 (shm)* *shm1-2*	At4g37930	0.15–0.3	(EMS) T-DNA	Somerville and Ogren (1981) Voll et al. (2006)

(continued)

Table 2 (continued)

Enzyme	Mutant	Arabidopsis locus	Growth recovery % CO_2	Mutant type/comment	References
					Engel et al. (2011)
	shm2-2	At5g26780	0.038	T-DNA	Engel et al. (2011)
	shm1-2shm2-2		Lethal (soil)	Viable in vitro 0.9% CO_2 and sucrose	Engel et al. (2011)
SAGT	*sat1* (*agt1*)	At2g13360	1%	EMS	Somerville and Ogren (1980a, b) Liepman and Olsen (2001)
HPR	*hpr1-1* *hpr1-2*	At1g68010	0.3–1	T-DNA	Timm et al. (2008) Liu et al. (2020) (hpr1–1)
	hpr2-1 *hpr2-2*	At1g79870	0.04	T-DNA	Timm et al. (2008)
	hpr3-1 *hpr3-2*	At1g12550	0.04	T-DNA	Timm et al. (2011)
	hpr1-1hpr2-1		0.3–1	T-DNA	Timm et al. (2008)
	hpr1-1hpr3-1		0.3–1	T-DNA	Timm et al. (2011)
	hpr2-1hpr3-1		0.04	T-DNA	Timm et al. (2011)
	hpr1hpr2hpr3		0.3–1	T-DNA	Timm et al. (2011)
GLYK	*glyk1-1*	At1g80380	0.12–0.2	T-DNA	Boldt et al. (2005)
PLGG	*plgg1-1*	At1g32080	0.2	T-DNA	Pick et al. (2013)
BASS6	*bass6-1*	At4g22840	0.2	T-DNA	South et al. (2017)
BOU	*bou-2*	At5g46800	0.3	T-DNA/impaired GDC activity	Eisenhut et al. (2013) Samuilov et al. (2018)

above, and Fig. 1). Interestingly, mutant phenotypes can also be modulated by environmental factors other than atmospheric CO_2 levels, including photoperiod, light intensity, and pathogens.

Photorespiratory core enzyme mutants have been classed into several categories based on the severity of their phenotypes in normal air (Timm and Bauwe 2013).

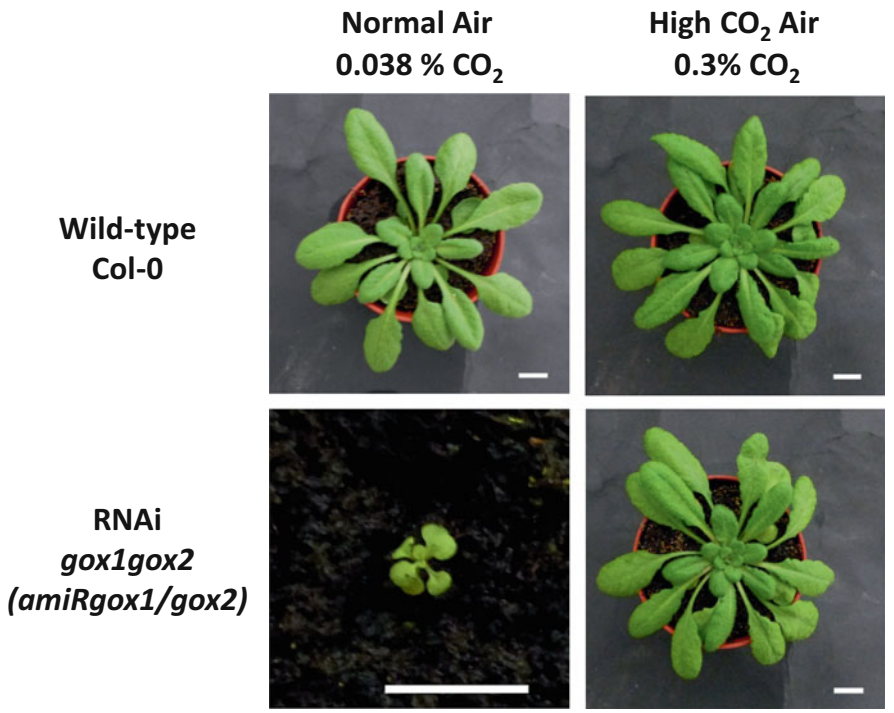

Fig. 2 The photorespiratory phenotype. The RNAi suppressed *Arabidopsis thaliana gox1gox2* mutant (*amiRgox1/gox2*) shows a severe retarded growth phenotype in normal air (0.038% CO_2) whereas this is absent when grown under high 0.3% CO_2-containing air. This CO_2-dependent phenotype is not observed with WT Arabidopsis Col-0 plants. This is an example of a photorespiratory phenotype. It should be noted that these photos were used in Dellero et al. (2016a)

However in this chapter, photorespiratory core enzyme mutants will be described in order of their appearance within the C2-cycle and this will be followed by known photorespiratory transporter protein mutants.

PGLP1: Identification of the Arabidopsis photorespiratory *PGLP* gene was achieved by the characterization of plants with a T-DNA inserted at the At5g36700 locus. The mutant was not viable in normal air but grew better in 0.9% CO_2-enriched air; however, it required 2% CO_2 for a complete phenotypic complementation (Schwarte and Bauwe 2007). It was also shown that the high CO_2-requiring phenotype of the EMS-derived *pcoA* mutant (Somerville and Ogren 1979) was due to the aberrant splicing of At5g36700 pre-mRNA (Schwarte and Bauwe 2007). When the original *pglp1* mutant line (*pcoA*) was transferred from high CO_2 conditions to normal air, net CO_2 assimilation decreased within minutes, while this was not observed in air containing 2% O_2 (Somerville and Ogren 1979). Extremely low photosynthetic CO_2 assimilation rates were also observed in *pglp1-1* plants transferred to air (Timm et al. 2012b; Flügel et al. 2017). Radiolabelled $^{14}CO_2$ feeding showed that rosette leaf 2PG became highly labelled while glycolate,

Ser, and Gly were far less labelled compared to the WT (Somerville and Ogren 1979). It was suggested that most of the properties of the *pcoA* mutant could be explained by the inhibitory effect of 2PG on TPI activity thus blocking the conversion of glyceraldehyde-3-phosphate (GAP) to dihydroxyacetone phosphate (DHAP) (Somerville and Ogren 1979). Leaf transpiration rates were also seen to be lower in *pglp1-1* plants transferred to air from 1% CO_2 when compared to WT leaves. Furthermore, leaf transpiration was higher in *pglp1-1* plants under high CO_2 conditions when compared to the WT. These observations suggest an effect of photorespiratory cycle functioning on stomatal movements (Eisenhut et al. 2017). Low or absent PGLP1 activities (using antisense lines and a T-DNA mutant, respectively) showed a modified C-allocation between RuBP regeneration and starch synthesis and this was attributed to 2PG inhibiting not only TPI but also SBPase (Flügel et al. 2017). The importance of removing toxic 2PG was also seen in CCM-containing photosynthetic organisms with limited photorespiration. PGLP RNAi lines of the C4 plant *Flaveria bidentis* showed a photorespiratory stunted growth phenotype when <5% of normal PGLP protein amounts were present. The 2PG-accumulating lines also exhibited C-starvation characteristics with accelerated leaf senescence, and accumulation of branched-chain and aromatic amino acids (Levey et al. 2019). A mutant strain of *Chlamydomonas reinhardtii* with only 10–20% of normal PGLP activity required a high CO_2 concentration for photoautotrophic growth in spite of a functional CCM (Suzuki et al. 1990). In low 2% O_2, the photosynthetic characteristics of mutant and WT cells were similar but net CO_2 uptake was severely inhibited when mutant cells were exposed to normal air for more than a few minutes. When compared to WT cells, radiolabelled 2PG accumulated within several minutes of photosynthetic activity in PGLP mutant cells (Suzuki et al. 1990).

GOX: *Arabidopsis thaliana* contains five potential GOX genes (*GOX1*, *GOX2*, *GOX3*, *HAOX1,* and *HAOX2*) (see Dellero et al. 2016b). Single *gox1* and *gox2* Arabidopsis mutants exhibit no photorespiratory phenotype (Dellero et al. 2016a). However, when they were transferred to low CO_2 atmospheres (0.01–0.02% CO_2), a reduction in photosynthetic CO_2 fixation rates was observed and a reconfiguration of soluble amino acid levels was triggered with higher increases in Gly and Ser in *gox1-1* and *gox2-2* mutant rosettes compared to *gox2.1* and WT plants (Dellero et al. 2021). Previously Rojas and colleagues had compared the effect of *P. syringae* on *gox* mutant plants and found that all mutant lines were less resistant to non-host pathogen infection by the avirulent strains *P. syringae* pv *tomato* strain DC3000 (*AvrB*) (Rojas et al. 2012). Surprisingly, all of the five Arabidopsis GOX genes were tested and each mutant exhibited a similar 80% reduction in seedling GOX activity and a similar response to pathogens even though GOX3 is probably a lactate dehydrogenase expressed mainly in roots (Engqvist et al. 2015) and HAOX1 and HOAX2 are medium- and long-chain hydroxyacid oxidases expressed in seed (Esser et al. 2014). The redundant role in photorespiration of GOX1 and GOX2 was shown from the photorespiratory phenotype of *gox1gox2* RNAi (*amiRgox1/2*) plants that retained only 5% of normal leaf GOX activity (Dellero et al. 2016a). After a high (0.3%) CO_2 to normal air transfer, the net CO_2 assimilation rate of *amiRgox1/2* was

rapidly reduced to 50% of control levels, and a high NPQ was maintained. ^{13}C-labelling coupled to NMR analyses revealed that daily assimilated carbon accumulated in glycolate and this led to a reduced C-allocation to sugars, organic acids, and amino acids in *amiRgox1/2* rosettes. This altered C-allocation provoked the early senescence of old rosette leaves while younger leaves remained green (Dellero et al. 2016a). The inducible antisense suppression of GOX activity in rice using *OsGLO1* also revealed a strong regulation over photosynthesis. These plants exhibited a typical photorespiratory phenotypes and a positive and linear correlation between GOX activity and net photosynthetic rates (Xu et al. 2009). When *OsGLO4* was used to down-regulate rice GOX activity using an RNAi approach, again a photorespiratory stunted growth phenotype and a reduction in net CO_2 assimilation rate were observed in normal air-grown plants. It was proposed that the accumulation of glyoxylate inhibited photosynthesis by deactivating Rubisco (Lu et al. 2014). Surprisingly, maize plants with reduced GOX activities due to an *activator*-insertion in *ZmGO1* led to a seedling lethal phenotype in plants with only 5–10% of WT GOX activity. When *go1* mutants were transferred from high CO_2 to normal air, they accumulated glycolate in the light. Low GOX activity lines also exhibited a severely reduced net CO_2 assimilation rate in air containing 50% O_2 (Zelitch et al. 2009).

GGAT: Deletion of Arabidopsis GGAT1 led to a retarded growth and development phenotype that was not seen in high 0.3% CO_2 (Igarashi et al. 2003; Dellero et al. 2015). It was also observed that low light and 3% sucrose addition to in vitro grown plants reduced the photorespiration-dependent growth phenotype of the GGAT1 (named *aoat1-1*) mutant (Igarashi et al. 2003). Growth of two independent *ggat1* lines *(ggt1-1* and *ggt1-2)* in normal air was rather similar to that of *amiRgox1gox2* (Dellero et al. 2015, 2016a). When compared to WT plants, normal air-grown *ggt1* mutant rosettes showed glyoxylate accumulation, global changes in amino acid amounts, lower organic acid levels, and modified ATP/ADP and NADP/NADPH ratios (Dellero et al. 2015). Their net CO_2 assimilation rates were 50% lower when compared to WT plants and this mirrored a similar decrease in Rubisco protein content per leaf area. When high CO_2-grown *ggt1* plants were transferred to normal air, a rapid decrease of photosynthetic CO_2 assimilation and electron transfer was observed while a high NPQ was maintained. Again, these characteristics were similar to *amiRgox1gox2* plants. After a short-term transfer to normal air from high CO_2, *ggt1* leaves accumulated glyoxylate whereas amino acid levels were not modified. That said, *ggt1* leaves contained extremely low soluble Ser levels compared to the WT, but this was not dependent on air CO_2 levels (Dellero et al. 2015). Rubisco content, activity, and activation state were not altered after a short-term transfer to normal air; however, Rubisco levels and plant growth were both reduced in *ggt1* leaves after a long-term (12 days) acclimation to normal air from high CO_2 when compared to WT plants. It was proposed that the low photosynthetic activity in *ggt1* leaves limited N-assimilation and thus decreased leaf Rubisco content until they attained a new homeostatic state to maintain a constant C/N balance and this led to smaller, slower growing plants (Dellero et al. 2015).

GDC: Engel et al. (2007) established that an Arabidopsis mutant lacking both GDC-P protein genes (*gldp1gldp2*) was not viable, even when grown in high CO_2

conditions. Air-grown *gldp1gldp2* double mutants could only reach the cotyledon stage even in 0.9% CO_2-enriched air. This was explained by the important role of GDC in metabolic functions other than photorespiration such as C1 metabolism. On the other hand, single *gldp* mutants (*gldp1* and *gldp2*) showed no significant alterations in growth, metabolism, and photosynthetic performance, thus indicating their functional redundancy (Engel et al. 2007). The knockdown of the unique Arabidopsis GDC-T gene (*gldt*) either in a T-DNA insertional mutant or RNAi lines revealed a disturbed photorespiratory metabolism including very high leaf Gly and glyoxylate levels. Growth of these plants was impaired and their leaves were chlorotic (Engel et al. 2008). An Arabidopsis mutant lacking GDC activity (*glyD*) was isolated by Somerville and Ogren (1982) but to date the mutated GDC gene has not been identified. In air, the photosynthetic activity of this mutant was decreased; however, this was not seen under non-photorespiratory conditions. Potato (*Solanum tuberosum*) plants with an antisense-induced 60–70% reduction of GDC-P-protein were used to study the interaction between respiration and photorespiration. In the light, photosynthetic activity was reduced in the anti-sensed lines and GDC-deficient leaves showed increased respiratory decarboxylations probably to compensate for the low photorespiratory activity. This latter observation supported the idea that TCA cycle decarboxylations are restricted in the light by a high mitochondrial photorespiratory activity (Bykova et al. 2005). GDC activity has also been reduced in transgenic rice (*Oryza sativa*) by knocking down the most abundant mature leaf GDC H-subunit (Os10g37180) in leaf mesophyll cells using a targeted RNAi approach (Giuliani et al. 2019). This led to *gdc-h* lines where GDC-H and GDC-P proteins were undetectable in leaves. Such plants exhibited photorespiratory-dependent stunted growth, accelerated leaf senescence, reduced levels of Chl, soluble proteins and sugars, and higher levels of leaf Gly (Lin et al. 2016). In normal air conditions, *gdc-h* knockdown plants showed lower leaf net CO_2 assimilation rates compared to the WT (Giuliani et al. 2019).

SHMT1: Three allelic SHMT (*stm*) mutants were isolated and described by Somerville and Ogren (1981). They showed a typical photorespiratory phenotype and their CO_2 assimilation capacity was reduced in normal air conditions. One of the original *stm* mutants (now named *shm1-1*) was mapped to the Arabidopsis SHMT1 (At4g37930) gene and a new SHMT1 T-DNA mutant line, *shm1-2*, was identified, characterized, and tested for allelism with *shm1-1* (Voll et al. 2006). The new SHMT1 mutant was used in high CO_2 to normal air transfer studies where it was observed that *shm1-2* rosette leaves did not become completely bleached and younger leaves of the inner rosette survived and remained green in normal air and *shm1-2* plants went on to produce fertile seed (Timm et al. 2012b). A similar developmental phenotype was found for *amiRgox1gox2* plants (Dellero et al. 2016a). In contrast to *shm1* single mutants (Voll et al. 2006), a double *shm1shm2* mutant was not viable even under rather high CO_2 concentrations of up to 1% (Engel et al. 2011). In this case, the phenotype was similar to *gldp1gldp2* plants (Engel et al. 2007) and this was again interpreted by the role of GDC/SHMT1 in C1 metabolism. Recently, by comparing *shm1-1* lines complemented with mutated versions of SHMT1, it was found that SHMT1 activity impacted stomatal movements induced

by in vitro salt treatments and ABA addition to epidermal peels (Liu et al. 2019). A stomatal effect was also observed in *shm1-2* leaves by measuring leaf transpiration using a gas exchange system. In this case, as for the *pglp1-1* mutant, *shm1-2* stomata remained open under high 1% CO_2 and closed upon transfer to air while the opposite was observed for WT plants (Eisenhut et al. 2017).

SGAT: Three allelic mutants lacking SGAT activity (*sat*) were isolated by Somerville and Ogren (1980b). They were viable and had normal photosynthetic activities under conditions suppressing photorespiration. In normal air, however, they were not viable (stunted at the 2-cotyledon stage and chlorotic) and Ser and Gly accumulated at the expense of starch and sucrose (Liepman and Olsen 2001). DNA sequence analysis of SGAT (At2g13360) from a *sat* mutant line showed a single mutation giving rise to a Pro-to-Leu substitution that led to an inactive enzyme (Liepman and Olsen 2001).

HPR1: There are three putative HPR genes in Arabidopsis: *HPR1* (peroxisomal), *HPR2* (cytosolic), and *HPR3* (plastidial). The complete loss of HPR1 led to only minor phenotypic alterations when *hpr1* plants were grown in normal air even though their photosynthetic CO_2 assimilation rates were reduced by approximatively 30% (Timm et al. 2008). However, it should be noted that more significant differences in *hpr1* plant growth in normal air have been reported (Timm et al. 2011; Liu et al. 2020). Unlike several photorespiratory mutants, CO_2-associated stomatal movements did not appear to be affected in *hpr1* plants (Eisenhut et al. 2017). The limited photorespiratory phenotype of *hpr1* plants reflected a cytosolic bypass involving HPR2 whereas HPR3 contributed to a much lesser extent (Timm et al. 2008, 2011). Indeed, HPR2 and HPR3 mutants resemble WT plants when grown in normal air (Timm et al. 2011). However, *hpr1hpr2* double mutants and *hpr1hpr2hpr3* triple mutants show a more pronounced sensitivity to normal air when compared to single *hpr1* mutants. This was associated with a reduced photosynthetic capacity of 60% and 75%, respectively (Timm et al. 2011). The absence of a significant photorespiratory phenotype was also reported for a barley (*Hordeum vulgare*) mutant deficient in NADH-dependent HPR activity that was capable of carrying out 75% of WT photosynthetic CO_2 assimilation rates in normal air (Murray et al. 1989).

GLYK: Plants defective in the single Arabidopsis glycerate kinase gene (*GLYK*) were unable to grow in normal air (as they were unable to develop primary leaves) but they fully recovered at an elevated 0.12% CO_2 level (Boldt et al. 2005). Unlike *shm1*-2 and *amirgox1gox2* mutants, *glyk1-1* mutants became completely chlorotic within 1 week after their transfer from high CO_2 to normal air (Timm et al. 2012b). When *glyk1-1* plants were transferred to normal air from 1% CO_2-containing air, there was a 75% decrease in CO_2 assimilation rates after the first day and this continued to decrease during the following days (Timm et al. 2012b). Furthermore, like *pglp1-1* and *shm1-2* mutants, *glyk1-1* plants also exhibited altered transpiration properties compared to WT plants at 1% CO_2 and when transferred to air (Eisenhut et al. 2017). Again suggesting that normal photorespiratory cycle functioning is somehow involved in CO_2-dependent stomatal movements.

PLGG: A plastidal glycolate-glycerate translocator 1 gene (*PLGG1*) was identified using mRNA co-expression analyses (Pick et al. 2013). The *plgg1-1* mutant only exhibited WT-like growth in a high 0.3% CO_2 atmosphere whereas in normal air it developed yellow and bleached lesions on leaf lamina but not along leaf veins (Pick et al. 2013). It also showed retarded growth and a reduced photosynthetic activity in normal air (South et al. 2017). The mutant accumulated both glycolate and glycerate and its role as a photorespiratory plastid glycolate/glycerate transporter was supported by in vivo and in vitro transport assays and metabolic flux profiling (Pick et al. 2013). In a similar manner, rice PLGG1 mutants showed retarded growth in normal air with pale-green leaves, reduced tiller number, and reduced seed grain weight as well as a reduced photosynthetic CO_2 assimilation rate and an accumulation of glycolate and glycerate. The rice *plgg1* mutant phenotypes were rescued under a high CO_2 condition (Shim et al. 2020).

BASS6: Arabidopsis plants containing a T-DNA disruption of the bile acid sodium symporter gene *BASS6* showed decreased photosynthesis and slower growth under normal air, but not in an elevated CO_2 atmosphere (South et al. 2017). Metabolite analyses and genetic complementation of yeast showed that BASS6 transported glycolate. An Arabidopsis *bass6plgg1* double mutant line had a more severe growth phenotype in normal air that was accompanied by increased glycolate levels, and further reductions in photosynthetic activities when compared to the individual single mutants. Therefore, BASS6 and PLGG1 export photorespiratory glycolate from chloroplasts, whereas PLGG1 alone accounts for glycerate import into chloroplasts. In this way, the two transporters can balance the expected glycolate and glycerate movements required for photorespiratory cycle functioning (South et al. 2017).

A BOUT DE SOUFFLE: The *BOU* gene encoding a mitochondrial carrier of unknown function was identified as a potential photorespiratory gene by a co-expression analysis. The *bou-2* mutant showed a photorespiratory growth phenotype and an accumulation of Gly in normal air due to a reduced GDC activity associated with GDC-P-protein degradation (Eisenhut et al. 2013). Perhaps surprisingly, *bou-2* plants exhibited a reduction in light-saturated CO_2 assimilation rates in elevated CO_2 atmospheres and after their transfer to normal air. It was seen that air-grown *bou-2* photorespiratory defects had dramatic consequences at the early seedling stage of development (Eisenhut et al. 2013). Previously, in vitro air-grown *bou-1* mutant seedlings germinated under continuous light stopped growing at a pale-green cotyledon stage unless supplemented with sucrose (Lawand et al. 2002). Recently, *bou-2* was used to elucidate the significance of photorespiratory Ser production for Cys biosynthesis. In the mutant, despite enhanced leaf Ser levels, the reduced GDC activity led to a decrease in sulphur flux to major sulphur-pools due to a deregulation of sulphur reduction and assimilatory gene expression. Leaf Cys and glutathione contents were enhanced while Cys used for Met and glucosinolates synthesis was reduced. The low GDC activity also led to the down-regulation of Calvin Cycle and N-assimilation gene expression, while upregulating glycolytic and TCA cycle enzymes thus altering the accumulation of sugars and TCA intermediates. This showed that photorespiratory Ser production could be

replaced by other Ser sources; however, S, N, and C metabolism cross-talks become deregulated (Samuilov et al. 2018).

4.5 Over-Expression of Photorespiratory Cycle Enzymes

It can been seen that plant performance cannot be improved by knocking out specific photorespiratory genes since this leads to a panoply of growth defects (from lethal to slower growing) and altered plant metabolism including a reduction of photosynthetic CO_2 assimilation rates when mutant plants are grown in air containing current CO_2 levels. So the question arises, can the OE of a photorespiratory gene lead to improved photosynthesis and growth? There are several examples in the literature that show that this approach can indeed improve plant performance under certain environmental conditions and one case where growth is impaired. They will now be described in the following paragraphs.

PGLP1: Arabidopsis over-expressing PGLP1 led to lower 2PG contents that appeared to be beneficial for maintaining photosynthesis under abiotic stresses such as drought, elevated temperature as well as a combination of high light and temperature. It was proposed that an adequate photorespiratory flux is required for organic carbon production and allocation under environmental stress conditions. A faster photorespiratory metabolism improved plant stress tolerance (Timm et al. 2019).

GOX: Rice over-expressing either *GLO1* (Os03g0786100) or *GLO4* (Os07g0152900) showed that growth could be improved when GLO activities were increased by 60% or 100% (in the OE *GLO4* lines), whereas growth was impaired when GOX activity was further increased by 150% or 210% (in the OE *GLO1* lines) (Cui et al. 2016). However, all OE plants exhibited significantly improved net CO_2 assimilation rates under high light (900 $\mu mol.m^{-2}.s^{-1}$) and temperature (40°C day/30°C night) conditions when compared to WT plants but this was not seen under "normal" conditions. In addition, OE plants were more resistant to photo-oxidative stress and this was associated with an induction of H_2O_2 and salicylic acid. This suggests that GOX activity has a role in plant tolerance to high light and temperatures (Cui et al. 2016).

GGAT1: Over-expression of *GGAT1* in Arabidopsis modulated amino acid steady-state values but neither growth nor photosynthesis was improved (Igarashi et al. 2006). In this study, 42 OE *GGAT1* lines were analysed for their amino acid contents. Free Ser, Gly, and citrulline increased in OE *GGAT1* plants when compared to WT levels and these changes were strongly correlated with leaf GGAT activity. Accumulation started after exposure to light and it was repressed in high CO_2 air (Igarashi et al. 2006).

GDC: Arabidopsis mtLPD OE lines have been made using a pea (*Pisum sativum*) mtLPD (*PsL*) cDNA driven by a leaf mesophyll promoter (Timm et al. 2015). Mitochondrial dihydrolipoyl dehydrogenase (mtLPD) is a component of several multi-enzyme systems involved in the TCA cycle, photorespiration (GDC-L subunit

of GDC), and branched-chain amino acid degradation. OE lines displayed decreased steady-state amounts of TCA cycle and photorespiratory intermediates such as Gly, 2OG, malate and succinate. These plants also exhibited increased CO_2 assimilation rates, photorespiration, and plant growth but day respiration remained unaffected. Enhanced sucrose biosynthesis was observed in the light and this was combined with a lower starch accumulation and breakdown (Timm et al. 2015). OE of GDC-H in Arabidopsis using a *Flaveria pringlei* GDC H-protein cDNA (*GLDH*) considerably enhanced net CO_2 assimilation rate, photosynthetic electron transfer and plant growth (rosette diameter, leaf number, dry and fresh weights) (Timm et al. 2012a). Increases in leaf area, DW, and photosynthesis of these Arabidopsis OE GDC-H plants were also observed by Simkin et al. (2017). An increased GDC activity was suggested from lower Gly levels (Timm et al. 2012a). Based on this work, GDC-H was over-expressed in tobacco by a targeted OE of the Arabidopsis H-protein gene (*GDC-H1*, At2g35370) using a leaf-specific promoter (López-Calcagno et al. 2019). This had a positive impact on plant biomass under controlled environmental conditions even in the absence of higher photosynthetic activities. However, higher OE levels driven by a constitutive promoter led to reduced plant growth (López-Calcagno et al. 2019). Leaf-specific OE of this H-protein also improved yield under field conditions although variable results were obtained over the two studied growing seasons. A small positive effect on photosynthetic CO_2 assimilation rate was observed under field conditions, and OE lines had higher DW, leaf area, and height and a better photosynthetic performance when outdoor light intensities were high (López-Calcagno et al. 2019). Transgenic Arabidopsis plants with altered combinations of Arabidopsis SBPase, Arabidopsis FBPA, and *Flaveria pringlei* GDC-H have also been studied under controlled greenhouse conditions (Simkin et al. 2017). A cumulative impact on biomass (+71%) was found with the SBP-FBPA-GDH-H plants which was significantly higher than GDC-H (+50%) and SBPase-FBPA (+41%) OE plants under low light (Simkin et al. 2017).

SHMT1: Rice plants that OE *OsSHMT1* had increased photosynthetic efficiencies and improved plant productivity (Wu et al. 2015). At the heading stage, OE SHMT1 lines showed higher photosynthetic activities and stomatal conductance that led to higher intercellular CO_2 concentrations and transpiration rates when compared to WT rice. Seed number per OE plant was 5% greater than that of the WT. The OE SHMT1 plants grew faster, produced more shoots with a greater panicle biomass, and contained more soluble sugars. The observed increase in biomass production of the transgenic plants could be correlated with their improved photosynthesis (Wu et al. 2015).

SGAT: Elevated SGAT activities in Arabidopsis were obtained by the constitutive OE of a *Flaveria pringlei SGAT* cDNA sequence (Modde et al. 2017). This led to a slight reduction of photosynthetic CO_2 assimilation, rosette diameter and area, leaf number, fresh weight but not DW. These changes were not observed under a high 1% CO_2 atmosphere. Faster photorespiratory Ser turnover led to lower day-time leaf Ser amounts and the induction of phosphoserine pathway gene expression. The higher SGAT activity also provoked the increased use of Asn as an external amino donor to the photorespiratory pathway. The authors suggested that leaf SGAT

activity has to be adjusted to ensure a minimal Asn consumption and to maintain Ser levels for cell metabolism (Modde et al. 2017).

4.6 *Photorespiratory Bypasses to Improve Photosynthesis and Plant Productivity*

A successful strategy to improve photosynthesis has been to reengineer photorespiration by introducing novel metabolic pathways that use less energy, do not release ammonium, and release photorespired CO_2 within the chloroplast in close proximity to Rubisco. Since 2007, there have been a number of different alternative photorespiratory metabolic pathways introduced into various plant species and tested (Fig. 3). These pathways will now be described and discussed and this will be done by type of photorespiratory bypass (and chronologically, when relevant). It should be noted that the majority of these synthetic bypasses are located to chloroplasts and begin at the step of photorespiratory glycolate removal.

4.6.1 A Bacterial Glycolate Catabolic Pathway

The first published work concerning a photorespiratory bypass was reported for *Arabidopsis thaliana* where five *E. coli* genes encoding the three subunits (*glc*D, *glc*E, *glc*F) of glycolate dehydrogenase (GlcDH), glyoxylate carboligase (GCL) and tartronic semialdehyde reductase (TSR) were used to express three functional enzymes in the chloroplast (Kebeish et al. 2007). This pathway converts glycolate to glycerate in three enzymatic steps with the liberation of a molecule of CO_2 (Fig. 3). It does not release ammonium. This strategy reduced, but did not eliminate, endogenous photorespiratory flux. Transgenic Arabidopsis plants grew faster, produced more shoot and root biomass, and contained more soluble sugars reflecting reduced photorespiration and enhanced photosynthesis. This was explained by an increase in chloroplastic CO_2 concentration close to Rubisco. Perhaps surprisingly, similar changes were also present in plants that only expressed the three bacterial GlcDH subunits, although most of the positive effects were enhanced when the complete pathway was present. Although the increase in rosette area was the same between GlcDH and complete pathway plants, increases in shoot and root DW and net CO_2 assimilation rates were higher in the presence of the complete pathway (Kebeish et al. 2007).

Camelina sativa plants have also been transformed with either a partial (GlcDH only) or a full bypass (GlcDH, GCL, and TSR) with the aim of improving this oilseed crop with a potential for biofuel production (Dalal et al. 2015). Transgenic plants showed an increase of photosynthetic CO_2 fixation activity whether they contained a partial or a full bypass with 20–25% and 14–28% increases per unit leaf area, respectively, compared to WT plants. The transgenic plants also showed an

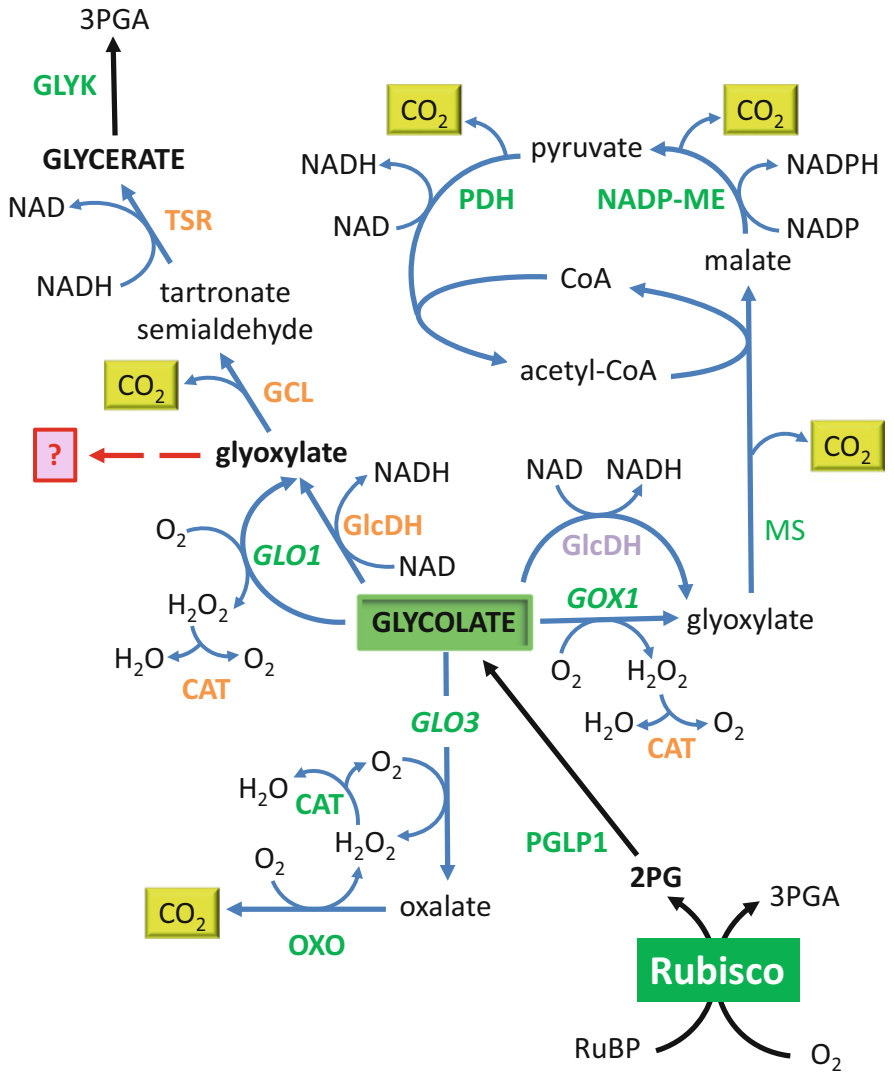

Fig. 3 Common synthetic chloroplastic photorespiratory bypasses. Common chloroplast bypasses using alternative glycolate catabolic pathways to short-circuit the naturally occurring photorespiratory cycle. In each case, ammonium is no longer produced and they liberate differing quantities of CO_2 in the chloroplast. Bacterial enzymes are shown in brown, plant enzymes are shown in green. Purple GlcDH is either a bacterial or a green algal GlcDH. The fate of glyoxylate produced by a partial bypass consisting of plastidial GlcDH is unknown. *Upper left-hand side*: The bacterial glycolate pathway composed of glycolate dehydrogenase (GlcDH), glyoxylate carboligase (GCL), and tartronate-semialdehyde reductase (TSR) that was tested in *Arabidopsis thaliana* (Kebeish et al. 2007), *Camelina sativa* (Dalal et al. 2015), cucumber (Chen et al. 2019), rice (Nayak et al. 2022), and tobacco (South et al. 2019). A modified version of this pathway was tested in rice where GlcDH was replaced with rice glycolate oxidase (*GLO1*) and a bacterial catalase (CAT) (Wang et al. 2020). It should be noted that a partial bypass (addition of GlcDH only) has often been tested including in potato where a GlcDH polyprotein was used (Nölke et al. 2014). *Upper right side*: A pathway composed of exogenous Arabidopsis glycolate oxidase (*GOX1*),

increased vegetative biomass and a faster development. Indeed, they produced substantially larger leaves compared to the WT (representing increases of 30% (partial) and 48% (complete)). Shoot DW was also higher in bypass lines compared to the WT (62% and 118% (partial), 65% and 75% (complete)). They flowered, set seed, and reached seed maturity approximatively 1 to 2 weeks earlier than WT plants. The partial bypass increased seed yield by 50–57%, while the full bypass increased seed yield by 57–73% with no loss in seed quality. Bypass plants generated more siliques and more seeds (seed DW was 50–60% higher in GlcDH plants and 60–72% higher in complete bypass plants) compared to the WT. Although seed weight was not altered, more seeds were produced due to the increase in silique number per plant (Dalal et al. 2015).

The bacterial glycolate catabolic pathway was also partially or completely produced in cucumber chloroplasts (Chen et al. 2019). As with Arabidopsis, cucumber lines expressing either GlcDH alone (partial pathway) or the complete pathway had higher day-time photosynthetic activities and biomass yield was improved when compared to WT plants. It should be noted that plants were grown under greenhouse conditions where atmospheric CO_2 levels varied during the day-night cycle (up to 0.06% CO_2 during the night and decreasing to 0.01% CO_2 during the day). Both the partial and complete pathways increased net CO_2 assimilation, plant height, leaf area, above and below ground DW, and cucumber fruit weight. These increases were always higher in complete pathway plants. Transgenic lines also had higher Rubisco and Rubisco activase activities but a reduced GOX activity (Chen et al. 2019).

This bypass has also been introduced into rice chloroplasts either as a partial (GlcDH only) or as a complete pathway (Nayak et al. 2022). Both the full and partial bypass plants showed improved photosynthetic efficiencies, leaf area, total DW, panicle number, and seed yield per plant when compared to WT rice transformed with an empty vector. Perhaps surprisingly, the partial bypass rice showed a higher increase in maximal net CO_2 assimilation rates in normal air when compared to full pathway plants (41% compared to 24%, respectively). Again, surprisingly, all bypass rice lines still exhibited improved CO_2 fixation compared to the WT in a high 0.16% CO_2 atmosphere. It appeared that both bypasses favoured Rubisco carboxylase activity and lowered its oxygenase activity thus improving Calvin cycle functioning and leading to increased C-assimilation (Nayak et al. 2022).

An *E. coli* GlcDH polyprotein (DEFp) was addressed to chloroplasts of potato (*Solanum tuberosum*). This approach was used to overcome the need to carry out

Fig. 3 (continued) cucumber malate synthase (MS), and bacterial CAT, with endogenous NADP-malic enzyme (NADP-ME) and pyruvate dehydrogenase (PDH) was tested in Arabidopsis (Maier et al. 2012) and tobacco (South et al. 2019). In tobacco, a pathway where Chlamydomonas GlcDH replaced GOX1 was also tested (South et al. 2019). *Lower, centre*: The GOC pathway containing plastid-located rice glycolate oxidase (GLO3), oxalate oxidase (OXO), and CAT that was tested in rice (Shen et al. 2019). For simplicity, bypasses with an additional RNAi-induced down-regulation of PLGG1 are not shown (see South et al. 2019). This bypass scheme was inspired by a figure in Maurino (2019)

multiple transformations with different constructs to express each GlcDH subunit and it aimed at improving the stability of GlcDH expression (Nölke et al. 2014). Transgenic potato lines with the highest DEFp levels and GlcDH activities produced plants with significantly higher levels of glucose (5.8-fold), fructose (3.8-fold), sucrose (1.6-fold), and transitory starch (3-fold). Such changes probably reflected the 18–34% increase in the maximal photosynthetic rates of DEFp lines grown under normal air conditions. DEFp plants also exhibited growth and development phenotypes including an increase in shoot and leaf biomass with more leaves, thicker stems, and a 2.3-fold increase in tuber yield. A good correlation was found between GlcDH activity and tuber yield. At the time, this was the first report showing a substantial photorespiratory bypass effect in a crop species (Nölke et al. 2014).

It can be seen that the addition of only a bacterial GlcDH within the chloroplast improved plant performance. Further work is required to understand the mode of action of this partial bypass. It should be noted that it has been reported that glyoxylate inhibits Rubisco activation (Cook et al. 1985; Campbell and Ogren 1990; Lu et al. 2014) while, on the other hand, it has been shown that glyoxylate can increase CO_2 assimilation (Oliver and Zelitch 1977; Oliver 1980). However, most of these experiments were carried out under non-physiological conditions using high glyoxylate levels and isolated leaf disks, leaf mesophyll cells, isolated chloroplasts, and purified Rubisco.

4.6.2 Bypasses Using Plant GOX

Several photorespiratory bypasses have been tested using a plant GOX instead of a bacterial GlcDH. These pathways require the expression of a chloroplast-addressed catalase (CAT) to remove harmful H_2O_2.

GOX, malate synthase, and catalase: The first attempt to replace bacterial GlcDH with a plant GOX was carried out using *Arabidopsis thaliana* and consisted of expressing Arabidopsis GOX1 along with either a pumpkin (*Cucurbita maxima*) malate synthase (MS) without the putative C-terminal peroxisomal targeting sequence or an *E. coli* tartronate-semialdehyde synthase (TSS) (Fahnenstich et al. 2008). Although TSS protein was observed in transformed lines, a measurable TTS activity was absent. Arabidopsis over-expressing GOX in chloroplasts accumulated H_2O_2 and glyoxylate and plants exhibited light-dependent retarded growth producing yellowish rosettes and a reduced photosynthetic activity. However, the chloroplast-located GOX plants grew normally under high (0.3%) atmospheric CO_2 conditions. The transgenic lines expressing either GOX and MS or GOX and TTS also accumulated H_2O_2 and exhibited similar phenotypes to GOX-only lines (Fahnenstich et al. 2008). To overcome the oxidative stress lesions arising from chloroplastic GOX-derived H_20_2 production, an *E. coli* CAT was added to Arabidopsis plants already containing GOX1 and pumpkin MS, with all three proteins addressed to chloroplasts (Maier et al. 2012, see Fig. 3). In these bypass plants, glycolate is converted to glyoxylate by GOX1 and H_20_2 is removed by the bacterial CAT. Glyoxylate is then converted to malate by the action of MS. The malate can be

metabolized to pyruvate by endogenous chloroplastic NADP-ME and the resulting pyruvate can be decarboxylated by endogenous plastidial pyruvate dehydrogenase (PDH) to produce CO_2 and acetyl-CoA (required for MS activity). When compared to WT Arabidopsis, the transgenic line with the highest CAT activity (GMK3) exhibited a DW increase and an altered Gly/Ser ratio but maximal photosynthetic CO_2 assimilation on a leaf area basis was not improved. GMK3 plants showed modified amino acid levels with less Asp and Asn but more Val, Ile, and Phe. They also had altered TCA cycle organic acid amounts with more isocitrate, 2OG and malate but less fumarate (and less photorespiratory glycolate) (Maier et al. 2012). This second photorespiratory bypass confirmed that it was possible to improve C3 plant growth by diverting glycolate away from the endogenous photorespiratory pathway and metabolizing it within the chloroplast.

GOX, GCL, TSR, and CAT (the GCGT pathway): Another synthetic photorespiratory shortcut using a plant GOX addressed to the chloroplast was designed to boost photosynthesis, biomass, and grain yield in rice (Wang et al. 2020, see Fig. 3). In this case, the photorespiratory shortcut (named the GCGT bypass) consisted of genes encoding rice GOX (*GLO1*) and *E. coli* CAT, GCL, and TSR. Therefore, this GCGT pathway is a modified version of the original bacterial glycolate catabolism pathway (Kebeish et al. 2007) with bacterial GlcDH replaced by a plant GOX thus requiring the addition of a plastidial CAT (as seen in Maier et al. 2012). GCGT rice had a 6–16% increase in maximal photosynthetic CO_2 assimilation rate, a 16–28% increase in above-ground DW, a 9–21% increase in seed per panicle, a 14–42% increase in tiller number, and a 5–9% increase in panicle length. Overall, this led to up to a 27% increase in seed yield (g/plant). However, GCGT rice plants showed a reduction in seed setting rate (number of seeds per pollinated flower). This was explained by the inefficient transport to seeds of the additional leaf starch and sucrose produced by the GCGT lines (Wang et al. 2020).

GOX, oxalate oxidase, and CAT (the GOC bypass): Rice has also been engineered with an alternative photorespiratory route named the GOC bypass, where a complete oxidation of glycolate to CO_2 is catalysed by the combination of three rice-originating enzymes: GOX (*GLO3*), oxalate oxidase (OXO), and CAT (Shen et al. 2019, see Fig. 3). Here photorespiratory glycolate is transformed to glyoxylate and then to oxalate by the double activity of rice GLO3. The oxalate is then converted to CO_2 and H_2O_2 by the chloroplast-located rice OXO. Toxic H_2O_2 is removed by the rice CAT addressed to plastids. Compared to WT rice, GOC-containing plants had increased photosynthetic efficiencies (by 15–23%), N-content (by 10%), biomass yield, and productivity. Under field grown conditions, GOC rice consistently appeared larger and greener than the WT. Spring seeding led to GOC plants with 15%–27% more tillers and 6%–10% wider flag leaves than WT rice but only minor increases were seen in plant height and flag leaf length. With autumn seeding, plant height (9–17%), tiller number (14–20%), length (9–28%), and width of flag leaves (6–13%) were improved in GOC rice. Above-ground DW (14%–35%), main panicle length (3–14%), and seed number per main panicle (10–30%) were also higher for GOC plants regardless of the seeding season.

However, no differences in 1000-grain weight were observed. As for GCGT rice, the seed setting rate of GOC plants was decreased compared to WT plants and this was more pronounced in the autumn seeding season (by 30%–43%) compared to spring seeding (with only a 13%–18% reduction). This led to a seed yield increase of 7%–27% in the spring seeding season but a 13%–16% decrease was observed in the autumn seeding season. It was reported that GOC rice plants had advantages under high light conditions and the improved traits of GOC plants resulted mainly from a photosynthetic CO_2-concentrating effect rather than from an improved energy balance (Shen et al. 2019).

Algal GOX, MS, and RNAi PLGG1: Transgenic tobacco (*Nicotiana tabacum*) was used to compare different chloroplast-targeted photorespiratory bypasses (South et al. 2019). AP1 lines contained the *E. coli* glycolate catabolism pathway of Kebeish et al. 2007. AP2 lines contained the bypass described by Maier et al. (2012). AP3 was a modified GOX-MS pathway where Arabidopsis GOX1 was replaced by a *Chlamydomonas reinhardtii* GlcDH (encoded by a single gene and not three genes as for the *E. coli* enzyme) and therefore an additional chloroplastic CAT was not required. Each bypass was also tested with the additional down-regulation of PLGG1 using an RNAi strategy to maximize flux through the bypass. Overall, 17 different constructs were tested. AP1 and AP3 designs significantly increased DW biomass relative to WT plants whereas the introduction of AP2 did not significantly change DW. The 13% increase in DW of AP1 plants was lost when PLGG1 was down-regulated. Three greenhouse-grown AP3 lines showed the largest increases in biomass with a 24% increase when PLGG1 RNAi was included and an 18% increase without PLGG1 RNAi when compared to WT tobacco. A number of homozygous AP3 transgenic lines exhibited a >40% increase of total biomass in field trials. AP3 either with or without the PLGG1 RNAi module increased leaf glyoxylate (6 to 7-fold) and pyruvate (around 4-fold) levels, and reduced Ser and glycerate amounts compared to WT plants, suggesting an altered endogenous photorespiratory cycle due to bypass pathway flux. AP3 plant lines also exhibited improved maximal rates of photosynthetic CO_2 assimilation with increased maximum Rubisco carboxylation rates without alterations in photosynthetic electron transfer rates (South et al. 2019). AP3 (with RNAi PLGG1) tobacco lines were used to test their resilience to high (+5°C) temperatures in the field (Cavanagh et al. 2021). They produced 26% more total biomass than WT plants under hot conditions thus sustaining lower yield losses under the high temperature condition compared to WT tobacco.

4.6.3 A Cyanobacterial Glycolate Decarboxylation Pathway

When individual cyanobacterial glycolate decarboxylation pathway proteins were addressed to chloroplasts there was an improved biomass production in *Arabidopsis thaliana* (Bilal et al. 2019; Abbasi et al. 2021). A complete bypass would transform glycolate to glyoxylate to oxalate to formate and to CO_2; however, this has not yet been tested. To date, transgenic *Arabidopsis* plants expressing either cyanobacterial

GLCD1 (GlcDH) (GD), *HDH* (hydroxyacid dehydrogenase) (HD), or *ODC* (oxalate decarboxylase) (OX) alone, and a double transformed *HDH::ODC* (HX) line have been studied. Phenotypic characterization showed that the different transgenic lines showed similar improvements in developmental parameters. Rosette diameter of GD, HD, OX, and HX was 20%, 22%, 17%, and 16% higher and total leaf number was 32%, 35%, 37%, and 34% higher than WT plants after 32 days of sowing. They produced more cauline branches, and plants were higher when compared to the WT after 42 days of growth except for HX transgenic plants. Vegetative DW biomass was 43% (GD), 35% (HD), 42% (OX), and 36% (HX) higher than in the WT (Bilal et al. 2019). GD, HD, and OX lines were further analysed by Abbasi et al. (2021) and observed changes were more variable. Only GD plants exhibited a significant 16% increase in net photosynthesis rate compared to WT controls. Stomatal conductance and soluble sugars were found to be higher in GD and HD plants while starch levels were higher in all transgenic plants. GD, HD, and OX plants produced approximately 35% more biomass and a two-fold higher seed weight. Based on these promising results, it would be worthwhile to generate plants containing the complete cyanobacterial glycolate decarboxylation pathway.

4.6.4 An Alternative Peroxisomal Glyoxylate Metabolism Pathway

Not all attempts to generate photorespiratory bypasses have been success stories. To avoid photorespiratory ammonium release, an alternative peroxisomal glyoxylate metabolism pathway was designed and tested in tobacco (Carvalho et al. 2011). The idea was to generate plants containing *E. coli* genes encoding GCL and hydroxypyruvate isomerase targeted to peroxisomes. It was presumed that these enzymes would compete with photorespiratory aminotransferases that convert glyoxylate to Gly and in doing so reduce the production of GDC-derived ammonium. However, transgenic lines only contained GCL and they exhibited distinctive atmospheric CO_2-dependent necrotic lesions close to their leaf veins that were only present in normal air. Peroxisomal GCL expression led to higher leaf Gln and Asn levels but less soluble sugars when compared to WT tobacco. To explain these observations, it was proposed that a diversion of glyoxylate away from Gly conversion produced a deleterious short-circuit of the photorespiratory N cycle (Carvalho et al. 2011).

5 The Future: Alternative Theoretical and On-Going Photorespiratory Bypasses

In 2015, systems-modelling of three published photorespiratory bypass strategies (Kebeish et al. 2007; Maier et al. 2012; Carvalho et al. 2011) suggested that photosynthesis could be enhanced by lowering photorespiratory energy demands

and by relocating photorespiratory CO_2 release to chloroplasts (Xin et al. 2015). Models indicated that photorespiratory bypass benefits would be improved by increasing SBPase activity and/or increasing the flux through the alternative bypass. However, the effectiveness of such approaches would depend on the complex interactions between photorespiration and other metabolic pathways (Xin et al. 2015). So far, photorespiratory bypass strategies have been restricted to relatively simple modifications of plant metabolism that have taken into consideration some factors predicted to be beneficial from systems-modelling. Next generation solutions will probably require a major rewiring of plant central metabolism. The visionary Arren Bar-Even wrote that this could include shared-enzyme Rubisco catalysis, replacing Rubisco by alternative carboxylation reactions and/or the Calvin Cycle with alternative pathways, and engineering photorespiration bypass routes that no longer release carbon. Innovative engineering strategies will be required to achieve such metabolic routes (Bar-Even 2018). With this in mind, several on-going strategies and hypothetical solutions will now be briefly described.

A synthetic photorespiratory bypass based on the prokaryotic *3-hydroxypropionate bi-cycle* has been engineered into the model cyanobacterium, *Synechococcus elongatus* sp. PCC 7942, and tested (Shih et al. 2014). This cycle was designed to function both as a photorespiratory bypass and an additional CO_2 fixing pathway. Bicarbonate is fixed to acetyl-CoA by a biotin-dependent acetyl-CoA carboxylase to form malonyl-CoA which is converted to propionyl-CoA by two further enzymatic steps. Propionyl-CoA and photorespiratory glyoxylate are then converted to β-methylmalonyl-CoA which is then converted to pyruvate and acetyl-CoA via four more enzymatic steps. In transformed cyanobacterial cells, the six introduced enzymes were shown to be active but no cell growth improvement was observed in normal air. However, bottlenecks were identified as targets for future bioengineering (Shih et al. 2014). Since pyruvate cannot be easily re-assimilated by the Calvin cycle, the utility of such a bypass has been questioned (Weber and Bar-Even 2019).

The β-hydroxyaspartate cycle (BHAC) is a primary glycolate assimilation pathway in marine proteobacteria (Schada von Borzyskowski et al. 2019). Glycolate is first oxidized to glyoxylate which is further converted into OAA via the activity of four enzymes: aspartate glyoxylate aminotransferase, β-hydroxyaspartate aldolase, β-hydroxyaspartate dehydratase, and iminosuccinate reductase. This cycle allows the direct formation of a C4 compound (OAA) from glycolate without any C and N losses. This makes it more efficient than photorespiration and all of the *in planta* photorespiratory bypasses tested so far. A functional BHAC has been engineered into *Arabidopsis thaliana* peroxisomes of WT and *ggt1–1* mutant plants to create a photorespiratory bypass independent of both 3PGA regeneration and photorespiratory decarboxylations (Roell et al. 2021). *In planta* BHAC activity was demonstrated by β-hydroxyaspartate formation under photorespiratory conditions. Ambient air-grown BHAC plants showed a 20% reduction of ammonium and they accumulated soluble amino acids involved in the urea cycle (Glu and ornithine) or dependent on OAA (Lys and Met). However, BHAC plants had reduced growth compared to WT plants in normal air with decreased rosette diameter (50%) and leaf

area (70%) that probably reflected the 25–30% reduction of their maximal CO_2 assimilation capacity. Inefficient OAA conversion appeared to limit the full potential of the BHAC plants. The next step is to design strategies that use BHAC-derived OAA to create a synthetic C4 cycle CCM in C3 plants (Roell et al. 2021).

By developing kinetic-stoichiometric models, promising routes to assimilate photorespiratory 2PG into the Calvin Cycle without C-losses have been identified (Trudeau et al. 2018). *Glycolate reduction to glycolaldehyde* appeared an interesting route but it did not occur in nature. Using computational design and directed evolution, the required activity was achieved by two sequential reactions. An acetyl-CoA synthetase was engineered for better stability and glycolate-use and a propionyl-CoA reductase was engineered for improved glycolyl-CoA selectivity and NADPH-use. This glycolate reduction module was then combined with three existing downstream enzymes (an aldolase, an isomerase, and a kinase) to convert glycolaldehyde to RuBP via an arabinose-5-phosphate shunt (see also Weber and Bar-Even 2019). Conversion of glycolate to RuBP using the glycolate-to-glycolaldehyde module was successfully shown to take place in vitro using recombinant proteins (Trudeau et al. 2018). In this way it should be possible to bypass natural photorespiration without producing CO_2 and ammonium.

In a similar strategy to that used for the glycolate-glycolaldehyde module, another C-conserving photorespiratory bypass was designed using *the tartronyl-CoA (or TaCo) pathway* (Scheffen et al. 2021). This hypothetical pathway starts with glycolate that is converted by the activity of glycolyl-CoA synthetase (GCS) to glycolyl-CoA that is then converted to tartronyl-CoA by glycolyl-CoA carboxylase (GCC) and this is finally made into glycerate by the action of tartronyl-CoA reductase (TCR). By applying rational design and high-throughput evolution, GCS was made from a mutated *Erythrobacter* acetyl-CoA synthetase to improve glycolate-dependent activity and GCC was created by selected mutations and directed evolution from a propionyl-CoA carboxylase from *Methylorubrum extorquens*. TCR was a naturally occurring bi-functional malonyl-CoA reductase from *Chloroflexus aurantiacus*. The reconstitution of a functional TaCo pathway interfaced with photorespiration was successfully achieved in vitro with the addition of PGLP and GLYK leading to the conversion of 2PG into 3PGA (Scheffen et al. 2021).

This chapter will close with a few words about a very hypothetical alternative CO_2-assimilatory pathway (the crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle) that fixes CO_2 more efficiently than the native Calvin cycle. It is composed of 17 enzymes (16 are naturally occurring and one is synthetically designed) from nine different organisms. It has been coupled with the AP3-*plgg1*RNAi photorespiratory bypass (South et al. 2019) in an in silico metabolic model to predict the eventual beneficial outcome for CO_2 fixation and energy use (Osmanoglu et al. 2021). The two pathways are coupled by CETCH cycle produced glyoxylate that is metabolized by the photorespiratory bypass to produce CO_2 that can be re-assimilated by the CETCH pathway. Estimated calculated fluxes showed that the combined CETCH and AP3-*plgg1*RNAi pathways gave a higher CO_2-harvesting potential when compared to other tested pathways (Osmanoglu et al.

2021). This alternative strategy to improve plant CO_2 assimilation is still hypothetical and it will be very difficult to instore and test in a plant system due to the number of genes that must be incorporated and the fact that the Calvin cycle (if removed) is central to cell metabolism.

6 Conclusions

Many strategies have been proposed to improve photosynthesis, some appear quite simple to carry out and to test while others appear complex and excessively challenging (for instance, installing a CMM in C3 plants, or producing smart canopies). To date, an improvement of photosynthetic performance that favourably impacts plant biomass and yield has been achieved by improving NPQ relaxation kinetics, improving RuBP regeneration, and implementing chloroplast photorespiratory bypasses. The potential of rerouting photorespiratory glycolate catabolism to reduce energy costs and to increase CO_2 concentration at the site of Rubisco has been clearly shown. These success stories must now be transferred to crop species and tested under current field conditions and climate change-predicted situations. Pyramiding successful strategies should also be tested to see whether yield can be further increased. At the same time, new future-proofed plants should be designed, created, and tested. Perhaps they will contain one of the mentioned theoretical or on-going photorespiratory bypasses or a C4 or algal CCM. With respect to photorespiration as an important interconnected metabolic process, future efforts should be focused on understanding its regulation. From proteomics studies, all photorespiratory enzymes appear as targets for multiple PTMs including protein phosphorylation and redox-controls. There is now a need to validate such modifications by other methods, to understand their physiological functions and to investigate if and when they are occurring *in planta*. Such PTMs might become future targets to modulate photorespiration and to thus impact plant metabolism to favour plant performance under future climate change conditions.

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