Manipulating photorespiration to increase plant productivity: recent advances and perspectives for crop improvement.

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ABSTRACT

Recycling of the 2-phosphoglycolate generated by the oxygenase reaction of Rubisco requires a complex and energy-consuming set of reactions collectively known as the photorespiratory cycle. Several approaches have been proposed with the aim of producing plants with reduced rates of photorespiratory energy or carbon loss, both by screening for natural variation and by means of genetic engineering. Recent works indicate that plant yield can be substantially improved by the alteration of photorespiratory fluxes or by engineering artificial bypasses to photorespiration. However, there is also evidence indicating that, under certain environmental and/or nutritional conditions, reduced photorespiratory capacity may be detrimental for plant performance. Here, we summarize recent advances obtained in photorespiratory engineering and discuss prospects for these advances to be transferred to major crops to help address the globally increasing demand for food and biomass production.

Keywords

Crops, Food production, Genetic engineering, Photorespiration, Rubisco, Yield improvement

Highlight

Manipulation of the photorespiratory pathway may greatly increase plant productivity. Here we summarize recent advances in the engineering of photorespiration and discuss how to use these approaches for crop improvement.

Introduction

There is an urgent demand for increased crop productivity due to the world's population growth, increasing global affluence, reduction of cultivable soils and higher demand for plant based biofuels. The required increase in agricultural productivity required by 2030 may be in the range of 60 to 120% as compared to the levels of 2005 (Ort *et al*., 2015). A rapid increase in crop yield, especially for cereals, was obtained in the second half of the 20th century during the so-called "Green Revolution". Resulting from breeding strategies, this led to the introduction of new crop strains with a greater proportion of biomass partitioned into grains and greater inputs of fertilizer, pesticides and water. However, increases in yield for several major crops such as rice in recent years have been scarce (Zhu *et al*., 2010), and it is possible that actual crop yield is approaching the ceiling of maximal yield potential (Tilman *et al*., 2002). Further increases in nitrogen and phosphorous fertilization are unlikely to solve this problem and indeed many countries are currently attempting to reduce the levels of fertilization used in intensive agriculture. For these reasons, attention is being paid to the improvement of photosynthesis, a process that is still far from its theoretical maximum efficiency. Several recent reviews summarise the opportunities that have been so far identified to improve photosynthetic efficiency (Zhu *et al*., 2010; Raines, 2011; Maurino and Weber, 2013; Long *et al*., 2015; Ort *et al*., 2015).

Photosynthetic CO_2 fixation starts with the carboxylation of ribulose 1,5-bisphosphate (RuBP), catalysed by ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco), to yield two molecules of 3-phosphoglycerate (3PGA). An unavoidable side reaction of Rubisco is the oxygenation of RuBP to produce one molecule of 3PGA and one molecule of 2-phosphoglycolate (2PG). Photosynthetic organisms evolved a complex pathway to recycle 2PG that involve reactions taking place in chloroplasts, peroxisomes, mitochondria and cytosol, (Bauwe *et al*., 2010). In this photorespiratory cycle, two molecules of 2PG are transformed into one molecule of 3PGA and one carbon atom is lost as CO_2. The cost of the recycling of one molecule of 2PG is high (12.5 ATP per molecule of 2PG produced; Peterhänsel *et al*., 2010), and for this reason photorespiration has long been viewed as a target for crop improvement due to the seemingly wasteful

nature of the cycle and the high energetic cost that it imposes on plant metabolism.

The cost of photorespiration is massive at both the leaf and canopy scale. CO_2 is lost from photorespiration under 25°C at about 25% the rate of net CO_2 fixation (Sharkey, 1985; Sage *et al*., 2012). For example, photorespiration results in the loss of ~322 trillion Calories annually in the US Corn Belt alone. Even a 5% reduction in photorespiration would be worth almost $540 million a year in yield gain in this growing region (Walker *et al*., 2016). This high cost stems in part from the energy used in the reassimilation of the ammonia produced following glycine decarboxylation in the mitochondrion. Moreover, rates of photorespiration increase with temperature and the scarcity of water as these conditions favour increased Rubisco oxygenation (Walker *et al*., 2016). It is thus not surprising that several groups tried to develop plants with reduced rates of photorespiration with the aim of increasing productivity (Peterhänsel *et al*., 2013a). However, the view of photorespiration as a pathway that only aims at recycling the carbon of 2PG may be simplistic. In addition to photosynthesis, photorespiration interacts with several central metabolic pathways (Foyer *et al*., 2009; Bauwe *et al*., 2010; Fernie *et al*., 2013), and both the relevance and the regulatory aspects of these interactions need further investigations. Furthermore, photorespiration may contribute substantially to the production of serine (Benstein *et al*., 2013; Ros *et al*., 2013) and has been implicated in the response to certain biotic (Taler *et al*., 2004) and abiotic stresses (Wingler *et al*., 2000; Voss *et al*., 2013). It was additionally recently demonstrated that there is a positive correlation between photorespiration and productivity (Aliyev, 2012) and between photorespiration and nitrate assimilation (Bloom *et al*., 2010). While most efforts are aimed at generating plants with reduced photorespiratory rates, the eventual performance of these plants in the field and thus under stress conditions needs also to be considered. Tantalizing results have been obtained by re-engineering photorespiratory pathway in model plants (Kebeish *et al*., 2007; Timm *et al*., 2012a), but the transfer of these manipulations to our major crops and demonstration of benefits under field conditions is still lacking. In this article we summarise the different approaches that have been used to manipulate photorespiration and their possible application for crop improvement.

Screening for plants with naturally reduced rates of photorespiration

Screenings of mutagenized plants that showed an altered phenotype under normal air conditions but not under conditions in which photorespiration is suppressed (CO_2-enriched atmosphere) were carried in several C_3 species, notably barley and Arabidopsis (Sommerville and Ogren, 1982; Blackwell *et al*., 1988; Foyer *et al*., 2009; Peterhänsel *et al*., 2010). This approach permitted the identification of the genes that encode for the core enzymes of the photorespiratory cycle. However, the mutants obtained generally show poor performance under normal air conditions associated with different stress symptoms (Timm and Bauwe, 2013). In another approach, natural variants with reduced rates of photorespiration associated with higher yields were screened across broad populations. While preliminary trials carried out with tobacco gave promising results (Zelitch and Day, 1973), subsequent studies failed to identify plants with low levels of photorespiration paralleled by high productivity. Zelitch (1989) successfully isolated plants resistant to high levels of O_2 but the trait seemed more related to increased levels of catalase than to reduced rates of photorespiration. Other works of the same author identified tobacco plants with low photorespiratory rates and high catalase activity associated to higher yield, but this increase in yield was not robust across harvests (Brisson *et al*., 1998; Zelitch, 1992). Similarly, screening of mutagenized tobacco plants identified genotypes with higher yield at low CO_2 concentrations but the high yield trait could not be related to reduced photorespiration (Medrano *et al*., 1995). A more recent study that summarized the data obtained over 40 years of field trials using two major crop species, wheat and soybean, concluded that attempts to find highly productive genotypes with high photosynthetic but low photorespiratory rates are inconsistent instead showing that the highly productive cultivars have high rates of photosynthesis accompanied by high rates of photorespiration (Aliyev, 2012). These results, argue against the use of natural variation as a strategy to alleviate the yield penalty of photorespiration suggesting that genetic engineering might be the only viable route.

Enhancing the amount of photorespiratory CO_2 scavenging

The CO_2 released during the decarboxylation step of photorespiration in mitochondria is not completely lost for the plant. On its way out of the cell, the released CO_2 can be refixed while passing through the chloroplasts (Sage and Sage, 2009; Busch *et al*., 2013). Some plants optimize this mechanism known as photorespiratory CO_2 scavenging by maximizing the likelihood for CO_2 to pass the chloroplasts. Chloroplasts can form a barrier that covers the cell wall space in order to trap photorespiratory CO_2 (Figure 1). A tight association between mitochondria and chloroplasts can enhance this effect (Figure 1, Sage and Sage, 2009; Busch *et al*., 2013).Some plants also enhance the surface of chloroplasts via stromules, connecting them to a net like structure (Sage and Sage, 2009). Rice has such morphological features and it was shown that its CO_2 compensation point is lower than that of other C_3 crops not showing this morphological adaption (Sage and Sage, 2009). Similar to rice, the dicot C_3 plants *Flaveria pringlei* and *Flaveria robusta* also associate these organelles and show a reduced CO_2 compensation point compared to other C_3 *Flaveria* species (Sage *et al*., 2013; Sage *et al*., 2014). Although the effect of this anatomical adaption is not as big as the one found in C_4 or C_2 photosynthesis plants, it still accounts as a considerable improvement (Sage *et al*., 2013). Therefore, installing this anatomy in a C_3 crop plant might be an alternative approach to optimize the yield. Compared to other approaches, a modification of cell anatomy should have little impact on cell metabolism. To install this anatomy in a plant, a better understanding of organelle movement and partitioning is needed. Natural varieties of rice and other plants showing an enhanced chloroplast surface and tight connecting of the three organelles should be analysed. Additionally a mutant screen of these varieties combined with RNA sequencing might reveal major regulators for the anatomy of cell organelles. Interestingly, in *Arabidopsis thaliana*, it was shown that stromules, which are used to enlarge the chloroplast surface, are established when plants were stressed with heat (Holzinger *et al*., 2007). It would therefore be of interest to study mutant lines affected in stromule formation such as *arc(s)* (Holzinger *et al*., 2008), or even lines affected in chloroplast movement such as *chup1* (Oikawa *et al*., 2008) and compare the rates of CO_2 fixation of these mutants with the wild-type ones.

Introducing C_4 metabolism into C_3 species

C_4 photosynthesis greatly reduces photorespiration by concentrating CO_2 near Rubisco. With the exception of the so-called single-cell C_4 plants (Sharpe and Offermann, 2014), C_4 plants have adopted different biochemical and anatomical modifications. C_4 leaves have two distinct layers of photosynthetic tissue (the so called "Kranz" leaf anatomy): mesophyll cells that are in contact with atmospheric CO_2 via intercellular air spaces, and bundle sheath cells with cell walls that are less permeable to CO_2. HCO_3^- is assimilated into oxaloacetate in the mesophyll cells via phosphoenolpyruvate carboxylase, which is then converted to a more stable 4-carbon organic acid, malate or aspartic acid, which diffuse to the bundle sheath cells (Gowik and Westhoff, 2011). Here the C_4 acid is decarboxylated, releasing CO_2 near Rubisco, which is located mainly in this cell type in C_4 plants. Given the higher efficiency of the C_4 photosynthetic mechanism under current atmospheric [CO_2], efforts are underway to install C_4 photosynthesis in C_3 plants such as rice (the International C_4 rice consortium, http://c4rice.irri.org/) and other crops (www.3to4.org). While the number of genes necessary for the main enzymatic reactions and transporters involved in C_4 photosynthesis is relatively small, the introduction of C_4 photosynthesis into C_3 crops will also require major changes in leaf anatomy (von Caemmerer *et al*., 2012). Initial progress toward the identification of the genes responsible for C_4 anatomy has been reported (Feldman *et al*., 2014; Rizal *et al.*, 2015). On the other hand, terrestrial plants capable of carrying out C_4 photosynthesis within a single cell were discovered about 10 years ago (Sharpe and Offermann, 2014). While these plants lack the typical Kranz features, they possess a subcellular separation that enables a concentrating of CO_2 near Rubisco. The genes involved in the development of this peculiar subcellular anatomy are unknown. Considering the scarcity of sequence information for single cell C_4 species, it is difficult to judge if single cell C_4 metabolism can be bio-engineered into C_3 crops.

Introduction of CO_2-concentrating mechanisms into chloroplasts

Another strategy to reduce oxygenation and thereby photorespiration is to introduce cyanobacterial CO_2-concentrating mechanisms (CCM) into the chloroplasts of land plants (Price *et al*., 2013). Cyanobacteria suppress the oxygenating reaction of Rubisco by concentrating CO_2 inside a proteinaceous

microcompartment called carboxysome. The β-carboxysome is constituted by an outer shell composed of several different proteins that enclose Rubisco and carbonic anhydrase, which maintains high CO_2 inside the microcompartment. The high [CO_2] obtained near the cyanobacterial Rubisco suppresses oxygenation thereby increasing the catalytic efficiency of the carboxylation reaction of the enzyme. Furthermore, the use of CCM paves the way to potentially replace the native Rubisco with the cyanobacterial enzyme that has higher catalytic rate albeit at the expense of a lower affinity for CO_2 and specificity factor (meaning that is more prone to oxygenating RuBP) compared to plant Rubisco (Price and Howitt, 2014). A completed cyanobacteria CCM in plants would reduce the amount of Rubisco needed to sustain photosynthesis and permit the allocation of nitrogen for other purposes, thus increasing nitrogen use efficiency (Zhu *et al*., 2004). The feasibility of introducing carboxysomes into higher plants was boosted by Lin *et al*., (2014a) demonstration that the shell proteins of the β-carboxysome could be assembled in *Nicotiana benthamiana* chloroplasts producing structures suggestive of carboxysome self-assembly. An exciting step towards the engineering of a CCM into chloroplast was made by the same group, which transformed tobacco plants to express a functional cyanobacterial form of Rubisco together with proteins involved in the enzyme's assembly (Lin *et al*., 2014b). However, the engineered plants were able to survive only at high CO_2 concentration. This indicates that a stand-alone substitution of the endogenous Rubisco with a faster one does not provide advantages without the co-engineering of a CCM (Price and Howitt, 2014). Simpler CCM mechanisms have been also considered for the transformation of C_3 plants. For example, a recent work described the introduction of a cyanobacterial bicarbonate transporter into tobacco chloroplasts (Pengelly *et al*., 2014). The transformed plants expressed ample amount of the foreign transporter but displayed the same CO_2-assimilation rates than the WT, implying that the transporter had little or no *in vivo* activity.

Rubisco engineering and screening for natural variation

Despite its central role in plant metabolism, Rubisco is a relatively inefficient enzyme (Carmo-Silva *et al*., 2015). In addition to its oxygenase activity, Rubisco also shows a relatively low k_{cat} value for CO_2 that obliges plants to produce very

high amounts of the enzyme in order to sustain adequate photosynthesis, representing a large nitrogen investment (Zhu *et al*., 2007). Understandably, considerable effort has been made to address these inefficiencies by trying to engineer a more efficient Rubisco. One first challenge for replacing the plant endogenous Rubisco with a more efficient one is that the large subunit of the enzyme is encoded by a single chloroplastic gene and the small one by several nuclear genes. Transformation of both the nuclear and chloroplast genomes of the same plant is thus required in order to substitute the endogenous enzyme with a more efficient one. Given that the active sites of Rubisco are on the chloroplast-encoded large subunit (Andersson, 2008), it may be possible that changing only the large subunit will improve enzyme efficiency, but this would require the transformation of the chloroplast genome, a technique that is currently available only for a small number of species. High-resolution crystallographic structural data are available for several plant Rubiscos and were used in site-directed mutagenesis approaches in order to try to improve Rubisco efficiency. However, this effort was hindered by the propensity of plant Rubisco to form insoluble aggregates when expressed in *E. coli*, probably caused by the lack of the complex network of chaperones needed for the correct folding of the plant enzyme in the bacterial host (Hauser *et al*., 2015). For this reason, structure-function studies were carried out mainly with the enzymes from cyanobacteria and from the alga *Chlamydomonas reinhardtii* (Whitney *et al*., 2011a; Parry *et al.*, 2013 and references therein). Another limitation to rational Rubisco engineering is our poor knowledge of the mechanism of Rubisco-catalysed oxygenation (Tcherkez, 2015). To overcome these technical difficulties, Whitney *et al*. (2011b) used transplastomic tobacco lines that expressed WT and mutated genes encoding the large Rubisco subunit from either C_3 or C_4 plants as well as from C_3-C_4 intermediate species. Using this approach, the investigators were able to identify a single amino acid residue responsible for the different catalytic properties of the Rubiscos from C_3 and C_4 plants (low k_{cat} combined with low K_m for CO_2 and high k_{cat} combined with high K_m for CO_2, respectively). Together, these results have opened the door to further possibilities for crop improvement. In fact, the co-engineering of a C_4-type Rubisco with high k_{cat} for CO_2 together with the engineering of a CCM in the chloroplast to compensate for its low affinity for CO_2 may in theory be able to greatly enhance C_3 plant yield. More complex

approaches for the optimization of Rubisco via the manipulation of the activation state of the enzyme and its interaction with the various effectors that modulate its activity can also be envisaged (see the review of Carmo-Silva *et al*., 2015).

The enormous natural variability that exists between terrestrial plants can be exploited in order to develop new strategies for reducing photorespiratory losses. Plants have developed several strategies, both anatomical and metabolic, to reduce photorespiration and compensate for its inhibitory effects (Sage, 2013). However, several of these mechanisms such as the regulation of leaf temperature, regulation of stomatal opening, establishment of CCM etc. are generally controlled by large sets of genes, some of which are unknown. On the other hand, Rubisco is encoded by a small set of known genes and the natural variability of this enzyme among different plant species has been taken into consideration in order to look for more efficient forms of the enzyme. The Rubisco specificity factor (i.e. the ratio of carboxylation to oxygenation at any given ratio of [CO_2] and [O_2]) displays some variation among the different C_3 species. For example, species growing in hot and dry environments seem to have Rubiscos with higher specificity factor (Galmés *et al*., 2005), which may be taken in consideration as a criteria for selection of candidates to use in the substitution of the less efficient endogenous enzymes of different C_3 crops. While the potential of more efficient forms of Rubisco has yet to be exploited, several theoretical models suggest that changing the endogenous Rubisco with an enzyme with a more favourable specificity factor may improve crop yields (Zhu *et al*., 2004; Parry *et al*., 2011). It should be also taken into consideration that the Rubisco specificity factor may not necessarily reflect the effectiveness of the enzyme depending on the mechanism of the oxygenation reaction, which is still not completely known (Tcherkez, 2015).

The natural variability of photorespiration is not only limited to the variation in the characteristics of Rubisco. Species-specific changes in the route are also possible, which implies that the pathway may be different from the basic "textbook" version. For example, it was demonstrated that the conversion of hydroxypyruvate to glycerate can also occur in the cytosol (Timm *et al*., 2008). Arabidopsis may also show peculiar characteristics in the reassimilation of photorespiratory NH_3. Mutants of plastidic glutamine synthetase (GS_2), the enzyme in charge of the reassimilation of photorespiratory ammonium, have been

306 isolated in barley (Blackwell *et al*., 1988) and in the model legume *Lotus*
307 *japonicus* (Pérez-Delgado *et al*., 2013) by screening EMS populations for the
308 typical "photorespiratory" phenotype. However, no GS_2 mutants have been found
309 in Arabidopsis. Given that the mutagenesis screen that was carried out in
310 Arabidopsis was probably saturating (for example, 58 mutants were found
311 affecting Fd-GOGAT, the other plastidic enzyme involved in NH_3 reassimilation)
312 and that Arabidopsis GS_2 is encoded, as in most plants, by a single gene
313 (At5g35630), it is puzzling why GS_2 mutants were not been isolated either in the
314 original screening or by means of transposon insertion. Another example of
315 variation in photorespiratory metabolism related to ammonia reassimilation can be
316 found in conifers, where the plastidic isoform of GS is not present but, unlike
317 other higher plants, a cytosolic GS isoform is expressed in photosynthetic cells,
318 and photorespiratory ammonia is probably reassimilated through a cytosolic
319 GS/GOGAT cycle (Avila *et al*., 2001).
320
321 *Photorespiratory bypasses*
322
323 Instead of trying to reduce the photorespiratory rates, a different approach is to
324 install alternative and less energetically expensive routes for the recycling of
325 2PG. Three bypasses to the reactions of the photorespiratory pathway were
326 successfully engineered in model plants (Figure 2). In the first approach,
327 glycolate was converted to glycerate directly in the chloroplast by introducing the
328 *Escherichia coli* glycolate catabolic pathway, thus avoiding or at least competing
329 with the peroxisomal and mitochondrial reactions of photorespiration (Kebeish *et*
330 *al*., 2007). The second approach was to introduce a complete glycolate catabolic
331 cycle that oxidized 2PG to CO_2 in the chloroplast (Maier *et al.*, 2012). However,
332 while the "Kebeish" bypass resulted in an improved energy balance, the "Maier"
333 bypass had higher energetic costs compared to the standard photorespiratory
334 cycle (Peterhänsel *et al*., 2013b). Moreover, kinetic models of C_3 photosynthesis
335 indicated that the installation of the Maier bypass should theoretically reduce the
336 photosynthetic rate due to the decreased re-supply of RuBP (Xin *et al*., 2015).
337 Despite this, both bypasses were reported to enhance biomass production by up to
338 30% although only under short-day conditions. In the case of the "Maier" bypass
339 it is speculated that this benefit may be due to the release of CO_2 from 2PG

oxidation directly in the chloroplast that might increase the chloroplastic CO_2 concentration and reduce the probability of further oxygenating reactions (Peterhänsel *et al*., 2013b). A third bypass to photorespiration has been engineered by introducing the *E. coli* enzymes glyoxylate carboligase and hydroxypyruvate isomerase into tobacco for the conversion of glyoxylate into hydroxypyruvate directly in the peroxisome (Carvalho *et al*., 2011). While this alternative pathway may potentially reduce the cost of 2PG recycling (Peterhänsel *et al*., 2013b), hydroxypyruvate isomerase protein was not detectable in these tobacco lines, so its impact on plant yield remains to be proven. In a recent report the introduction of the "Kebeish" bypass in the oilseed crop *Camelina sativa* greatly increased seed yield, which may be used for the production of biofuels (Dalal *et al*., 2015). A partial Kebeish bypass was established in potato (*Solanum tuberosum*) by expressing the *E. coli* glycolate dehydrogenase polyprotein, resulting in an increase in shoot biomass and tuber yield (Nölke *et al*., 2014) These results suggested that part of the glyoxylate produced in the chloroplast by the bacterial enzyme may be completely oxidized *in situ* to CO_2, probably by the action of the endogenous pyruvate dehydrogenase (Blume *et al.*, 2013). It is interesting to notice that the beneficial effects of the Maier and Kebeish bypasses were observed only under short day conditions and optimal water and nitrogen supply (Kebeish *et al*., 2007; Maier *et al*., 2012), which may may not necessarilyreflect the conditions that crops will face in the field. Further testing of these genetically modified plants (GMPs) under different conditions would be needed in order to determine if photorespiratory bypasses may be beneficial also under field conditions.

Completely new bypasses can be also designed by taking advantage of the enormous amount of different enzyme activities that can be found in bacteria, algae and Archeae (see Ort *et al*., 2015 for some examples). More ambitious approaches would be to design bypasses that involve intermediates that are not present in the plant or to genetically engineer a single enzyme able to degrade 2PG to CO_2 directly in the chloroplast. In a recent report, a synthetic pathway that worked both as a photorespiratory bypass and as an additional CO_2-fixing pathway, the hydroxypropionate bi-cycle was successfully engineered in a cyanobacterium (Shih *et al*., 2014). Simulated energy balance analyses can be

performed in order to predict the potential benefits of a bypass to photorespiration (Xin *et al*., 2015).

When designing synthetic routes for the recycling of 2PG, it has to take consideration that alternative routes to the core photorespiratory pathway are already present in nature, although their physiological meaning and the flux that may pass through them is not known. For example, glyoxylate can be oxidatively decarboxylated to formate and CO_2 probably by a non-enzymatic reaction that takes place in the peroxisomes of higher plants in the presence of H_2O_2 (Igamberdiev *et al*., 1999). Cyanobacteria on the other hand are able to enzymatically decarboxylate glyoxylate via oxalate by using an alternative pathway for the recycling of 2PG (Eisenhut *et al*., 2008). In barley mutants with reduced glycine decarboxylase (GDC) activity, this formate may be used to support the synthesis of serine through a GDC-independent pathway that does not release NH_3, thus greatly reducing the energy cost of the photorespiratory cycle (Wingler *et al.* 1999a). As aforementioned, glyoxylate can be decarboxylated in the chloroplast by the action of the endogenous pyruvate dehydrogenase (Blume *et al.*, 2013), and a cytosolic hydroxypyruvate reductase provides an alternative route to the peroxisomal conversion of hydroxypyruvate to glycerate (Timm *et al*., 2008). Several other possibilities for peroxide-mediated decarboxylations have also been proposed (Grodzinski and Butt 1977; Cousins *et al.* 2008; Keech *et al.* 2012), but the extent to which these reactions would happen under natural conditions still remains unclear. Further work should be carried out in order to assess the impact of these alternative pathways in plant photorespiratory metabolism and their possible interactions with synthetic 2PG recycling routes.

Optimization of the levels of photorespiratory enzymes

While the overexpression of Rubisco protein in rice does not improve photosynthesis (Suzuki et al., 2007), the analysis of dynamic metabolic models of photosynthetic carbon metabolism suggested that in some plants there may be an underinvestment of resources in the biosynthesis of Rubisco and of the enzymes of the Calvin-Benson cycle, and concomitantly an overinvestment in photorespiratory enzymes. This scenario may be responsible of a less than optimal photosynthetic efficiency leading to reduced crop yields (Zhu *et al*., 2007).

However, this appears rather contradictory to recent studies in which the amount of photorespiratory enzymes has been modulated. For instance, different studies carried out in crops species indicate that antisense reduction of individual photorespiratory enzymes is associated with lower productivity. Potato plants with reduced levels of the GDC-P protein (Heineke *et al*., 2001) or of serine hydroxymethyltransferase (Schjoerring *et al*., 2006) as well as rice plants with lower levels of glycolate oxidase (Xu *et al*., 2009) showed reduced photosynthetic and growth rates. Moreover, a few studies have reported an improved performance of plants with increased levels of photorespiratory enzymes. Overexpression of the GDC-H protein or of the GDC-L protein in Arabidopsis resulted in enhanced net-photosynthesis and plant growth (Timm *et al*., 2012a; Timm *et al*., 2015). Increased yields were not observed under elevated CO_2 atmosphere, indicating that they were due to a facilitated carbon flow through GDC and the photorespiratory pathway as a whole. It is assumed that increased photorespiratory capacity may reduce negative feedback exerted by photorespiratory metabolites on the Calvin-Benson cycle thus enhancing CO_2 assimilation. Recent data suggest that 2PG levels could be of key importance in this coordinated control of photosynthesis and photorespiration (Timm *et al*., 2012b; Haimovich-Dayan *et al.*, 2015). Overexpression of serine hydroxymethyltransferase, the enzyme that acts in conjunction with glycine decarboxylase to produce serine in the mitochondrion, was also able to improve photosynthetic efficiency and plant productivity in rice (Wu *et al*., 2015). Taken together, these results clearly indicate that the mitochondrial conversion of glycine to serine is a bottleneck of the photorespiratory pathway or is somehow otherwise involved in the regulation of photosynthetic activity. The recent discovery that serine may act as a metabolic signal for the transcriptional regulation of photorespiration (Timm *et al.*, 2013) further supports this idea. In addition to the reactions involved in the glycine to serine conversion, the reassimilation of photorespiratory NH_4^+ is probably another bottleneck of the photorespiratory pathway. Photorespiratory NH_4^+ is reassimilated by the action of GS_2, and it has been suggested that this reaction may be the rate-limiting step of the pathway (Wallsgrove *et al*., 1987, Häusler *et al.*, 1994; Kozaki and Takeba, 1996; Hoshida *et al*., 2000). Plants that overexpress GS_2 showed enhanced growth rate under active photorespiratory conditions (Migge *et al*., 2000; Zhu *et al*.,

2014). Unfortunately, the growth of these GS_2 overexpressors was compared to WT plants under normal air conditions but not under CO_2-enriched atmosphere, so it cannot be ruled out if the increased yield was due to improved nitrogen assimilation rather than to an increased capacity for photorespiration (Migge *et al*., 2000; Zhu *et al*., 2014). However, the fact that mutants lacking GS_2 show a similar growth rate compared to wild-type plants under photorespiratory-suppressed conditions (Wallsgrove *et al*., 1987; Betti *et al*., 2014) indicates that GS_2 is not probably playing an important role in primary nitrogen assimilation. Moreover, overexpression of GS_2 confers resistance under stress conditions like salinity or high light (Kozaki and Takeba, 1996; Hoshida *et al*., 2000). Taking into consideration the promising results obtained with these overexpressors, it would be also worth to exploit natural variability and look for cultivars that already have higher or lower levels of photorespiratory enzymes.

Another important and often neglected parameter lies in the transcriptional and post-translational modifications of photorespiratory genes and enzymes. Different reports suggest that at the transcriptional level photorespiratory genes are regulated in a similar way to the photosynthetic ones (Foyer *et al*., 2009; Pérez-Delgado *et al*., 2013). On the other hand, metabolic data analysis of WT and photorespiratory mutants under different CO_2 and O_2 conditions suggest a fine tuning of photorespiratory metabolism (Timm *et al*., 2012b). Regarding post-translational modifications, it was recently shown that seven enzymes of the photorespiratory cycle could be phosphorylated (Hodges *et al.*, 2013). Furthermore, looking to redox proteome data, it appeared that almost all photorespiratory enzymes could undergo oxidative modifications for some of their cysteine residues, and were therefore identified as potential targets for redox regulations (Keech *et al*., 2016). Undoubtedly, the next step will be to determine primarily the extent to and the conditions for which the proteins or cysteines are modified, the type of modifications that occur, and secondly whether these modifications positively or negatively regulate enzyme activities, and how they are controlled at the cellular level. Altogether, this clearly indicates that a rational bio-engineering of plants with modified levels of photorespiratory enzymes would also benefit from an increased knowledge of the biochemical regulations inherent to this cycle.

Perspectives for crop improvement

As summarized in the above sections, several approaches have been used in order to manipulate photorespiration with the aim of increasing plant yield. However, most of these efforts have been carried out using model plants (with some notable exceptions like the consortia working on the transformation of rice in a C_4 plant, see http://c4rice.irri.org/). In the light of the results obtained by recent field trials (Aliyev, 2012), it would appear unlikely that crops with improved photorespiratory performance can be obtained by screening for natural genetic variation, but they should be rather generated by means of genetic engineering. Unfortunately, transformation of our major crops is still a difficult and time-consuming process, even though is getting easier and more successful every year (Scharff and Bock, 2014). Moreover, some promising approaches such as the engineering of the large subunit of Rubisco require the transformation of chloroplast DNA, a technique that is available only for a few crop species: notably tobacco, potato, tomato and perhaps soybean, but as yet not cereal species (Scharff and Bock, 2014).

Before tackling the genetic engineering of crop species, organisms for which transformation is more tractable such as algae and cyanobacteria can be used in order to obtain clues on the metabolic and physiological consequences of a targeted genetic manipulation. A second step may be the use of tobacco; a plant that is especially easy to transform both in the nuclear and plastid genomes and forms canopies in the field that are similar to those of food crops (Long *et al*., 2015). Moreover, promoters and vectors that can permit high expression of transgenes and a correct subcellular localization of the protein product should be available for these species, together with strategies to avoid gene silencing and random insertion in the genome (see Ort *et al*., 2015 for a more detailed discussion on this topic).

It should also be taken into consideration that crops with engineered photorespiratory pathways will be considered as genetically modified plants (GMP), and the potential use of such GMPs will remain limited under the current legislation, which furthermore can vary greatly between countries. For example in the European Union the authorization procedure for placing a GMP on the market is a long, complex and expensive procedure regulated by directives that were

approved more than 10 years ago (more details in Hartung and Schiemann, 2014). On the other hand, several millions of hectares of GMPs are growing in countries with less restrictive regulations such as the United States, Canada, Brazil, India and China. That said, several new molecular techniques based on the use of site-directed nucleases like TALENS (transcription activator-like effector nuclease(s)) or the CRISPR/Cas9 system, have been developed in the recent years (Araki and Ishii, 2015). The use of these genome editing techniques can lead to the production of plants which cannot be classified as GMPs under current legislations. The European Commission is currently evaluating the use of site-directed nucleases as well as other new breeding techniques in order to determine the extent to which they should lead to genetically modified organisms (Lusser *et al.*, 2012).

Should we really look for plants with lower rates of photorespiration?

Photorespiration has been traditionally considered as a wasteful and unavoidable process that needs to be minimized in order to improve plant yield. However, different lines of evidence suggest that reducing photorespiration may not necessarily always have beneficial effects.

1) Plant productivity may be improved by engineering more efficient ways to recycle 2PG (i.e. photorespiratory bypasses) but also by an increased capacity for photorespiratory flux (see section "optimization of the levels of photorespiratory enzymes). A higher photorespiratory capacity would reduce the levels of photorespiratory metabolites that may inhibit the Calvin-Benson cycle as well as increase the rate at which photorespiratory carbon is returned to the chloroplast in form of 3-PGA, thus facilitating CO_2 assimilation (Timm *et al.*, 2012b). Therefore, CO_2 assimilation may be improved either by bypassing photorespiration or by the overexpression of bottleneck enzymes of the cycle. The best engineering strategy to use will depend on the crop considered and the environmental conditions at the field level.

2) Energetically wasteful and useful are not necessarily antithetic to one another. As mentioned before, under stress conditions such as drought, salinity, cold, high light, heat or a combination of them, an excess of NADPH may be produced that could lead to an increase of reactive oxygen species (ROS)

(Peterhänsel *et al*., 2010). Photorespiration can act as a sink for this excess of reducing power, and this welcome effect can be even more important considering that different stress conditions can increase photorespiratory rates (Kangasjärvi *et al*., 2012). Drought and salinity for example trigger a decrease in stomatal conductance, thus decreasing the CO_2:O_2 ratio and increasing photorespiration (Kangasjärvi *et al*., 2012). High temperatures also favour Rubisco oxygenation by decreasing Rubisco specificity factor as well as the stromal concentration of CO_2 relative to O_2 (von Caemmerer et al., 2000; Kangasjärvi *et al*., 2012). It is not surprising then that attention has been paid to the role of photorespiration in the response to stress (Wingler *et al*., 2000; Voss *et al*., 2013). A direct relationship between the capacity for photorespiratory flux and the tolerance to abiotic stress has been described for different plant species under drought conditions (Wingler *et al*., 1999b; Li and Hu, 2015), salt stress (Hoshida *et al*., 2000), photoinhibition caused by high light (Heber and Krause, 1980; Kozaki and Takeba, 1996; Takahashi *et al*., 2007), chilling and exposure to heavy metals (Voss *et al*., 2013 and references therein). Moreover, several photorespiratory genes are co-expressed with genes involved in the resistance to Al, a stressor that can seriously constrains plant productivity, suggesting a link between Al resistance and photorespiration (Nunes-Nesi *et al*., 2014).

Since abiotic stress is one of the factors that most limits crop productivity worldwide (Mittler, 2006),. the performance of plants with reduced capacity for photorespiration should be tested carefully under different stress conditions. Moreover, since most of the high quality soils available are already farmed, the rising demand for food would probably lead to farm crops in marginal lands with poorer soil and adverse climatic conditions (Long *et al*., 2015). In such a scenario, the use of crops with high resistance to abiotic stress, and not only high yield under optimal conditions, would seem to be desirable.

Photorespiration has also been shown to play a significant role in the response to biotic stress, where the H_2O_2 produced by the reaction of glycolate oxidase in the peroxisome plays a central role in the defence from pathogen attack (Taler *et al*., 2004; Rojas *et al*., 2012) and is part of the signalling route that leads to programmed cell death (Mateo *et al*., 2004). Plants with reduced rates of photorespiration or engineered with alternative routes that bypass the peroxisomal

part of the pathway may show increased sensitivity to pathogen attacks and should also be tested carefully.

3) Conditions that inhibit photorespiration such as elevated atmospheric CO_2 strongly reduce nitrate assimilation in hydroponically grown Arabidopsis and wheat (Rachmilevitch *et al*., 2004; Bloom *et al.*, 2010). This relationship has even been proposed to explain the lower-than-expected growth increases in plants grown under elevated CO_2 and explain why many C_3 crops and trees grow more slowly when fed with nitrate as a sole nitrogen source (Bloom *et al*., 2011). Recent evidence suggests that these hydroponic-based observations may occur at larger scales when it was shown that wheat grown under free-air CO_2 enrichment had higher nitrate pools and a greater ^{15}N enrichment of both total nitrogen and nitrate, observations consistent with a decrease in nitrate assimilation (Bloom *et al*., 2014). While different physiological mechanisms may explain the inhibitory effect of elevated CO_2 on NO_3^- assimilation, multiple lines of evidence suggest that this may be due to the reduction of photorespiratory rates under elevated CO_2 conditions (Bloom, 2015a). In fact, photorespiration stimulates the export of malate from the chloroplast (Bloom, 2015a); this malate generates NADH in the cytosol and this is probably necessary for the reduction of NO_3^- to NO_2^- by the action of nitrate reductase. C_4 plants on the other hand assimilate NO_3^- independently of atmospheric CO_2 concentration (Bloom, 2015b). Considering the low photorespiratory flux observed in this kind of plants, the supply of reducing power for nitrate reduction in C_4 plants should probably come from sources other than photorespiration.

Nitrate is the most abundant form of N in agricultural soils and is the major N source for most higher plants. This is despite the higher amount of energy that is needed for the assimilation of NO_3^- into organic compounds compared to other N sources such as NH_4^+ or organic forms of nitrogen. Taking this into consideration, it is possible that a reduction of the photorespiratory rates in crops that use mainly NO_3^- may lead to nitrogen deprivation. Reliance on NH_4^+ fertilizers may not always be possible in order to circumvent this since many plants show symptoms of toxicity when grown on NH_4^+ as the sole N source (Britto and Kronzucker, 2002).

In conclusion, different lines of evidence have shown that engineering of photorespiration may greatly improve plant CO_2-assimilation and growth. Several

recent advances have been made in reducing photorespiratory losses in model organisms as well as in some plants of agricultural relevance. A great challenge will be the transfer of these advances to our major food crops, which are generally more recalcitrant to genetic manipulation. Nonetheless, a rational bio-engineering of plants with altered photorespiration should also take into consideration that this pathway is tightly connected with several other aspects of plant metabolism and a reduction of photorespiration may not always be beneficial, especially for plants growing under adverse environmental conditions. Finally, taking into consideration that NO_3^- assimilation depends on photorespiration, the manipulation of the photorespiratory pathway may also affect the rates of N assimilation and may favour the use of one N source over another.

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FIGURE LEGENDS.

Figure 1. The effect of cover and positioning on photorespiratory CO_2 scavenging. (A) When chloroplasts (c) cover a large portion of the cell wall space adjacent to the intercellular air space (IAS) they provide a barrier for the photorespiratory CO_2 released by the mitochondria (m), which can then be reassimilated in the chloroplast. Tight associations between mitochondria and chloroplasts add to this effect. In addition, a high chloroplast cover reduces the resistance for CO_2 entering the chloroplast from the outside of the cell. Both processes increase the CO_2 concentration in the chloroplast and thereby reduce photorespiration. (B) Conversely, low chloroplast cover and/or mitochondria that are not in close contact with the chloroplasts result in a lower capacity to scavenge photorespiratory CO_2.

Figure 2. Reported engineering strategies for the introduction of bypasses into the photorespiratory pathway. Pathways for the native photorespiratory cycle and for the photorespiratory bypasses are indicated. In black an abbreviated summary of the photorespiratory cycle and the Calvin-Benson cycle (dashed lines, shaded green, see Raines 2011 for more details). Shown in blue is the Kebeish bypass (Kebeish *et al*., 2007), in orange the Carvalho bypass (Carvalho *et al*., 2011) and in red the Maier bypass (Maier *et al*., 2012). The abbreviations used for the metabolites are: 2PG, 2-phosphoglycolate; 3PGA, 3-phosphoglycerate; Ac-CoA, acetyl coenzyme A; GA, glycerate; GL, glycolate; GX, glyoxylate; HP, hydroxypyruvate; MAL, malate; PYR, pyruvate; RuBP, ribulose 1,5-bisphosphate; TSA, tartronic semialdehyde. Abbreviations for the enzymes as follows: CAT, catalase; GCL, glyoxylate carboligase; GDH, glycolate dehydrogenase; GOX, glycolate oxidase; HYI, hydroxypyruvate isomerase; ME, malic enzyme; MS, malate synthase; PDH, pyruvate dehydrogenase; TSR, tartronic semialdehyde reductase.