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 photorespiration 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 CO₂ 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 reaction taking place in chloroplasts, peroxisomes, mitochondria and the 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 CO₂ with an addendant cost of 4 NAD(P)H and 7 ATP. 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. CO₂ is lost from photorespiration under 25°C at about 25% the rate of net CO₂ 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., submitted for publication). 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., submitted for publication). 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) or easy to transform non-staple crops such as tobacco (Lin et al., 2014a), 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, 1992; 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 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 CO₂ 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 CO₂ can be refixed while passing through the chloroplasts (Sage and R., 2009; Busch et al., 2013). Some plants optimized this mechanism known as photorespiratory CO₂ scavenging by maximizing the likelihood for CO₂ to pass the chloroplasts. Firstly, these plants enhanced the surface of chloroplasts via stromules, connecting them to a net like structure (Sage and Sage R., 2009). Secondly, they associated chloroplasts tightly with mitochondria and peroxisomes (Sage and Sage R., 2009; Busch et al., 2013). Rice has such morphological features and it was shown that its CO₂ compensation point is lower than that of other C₃ crops not showing this morphological adaption (Sage et al., 2009). Similar to rice, the dicot C₃ plants Flaveria pringlei and Flaveria robusta also associated all three organelles and showed a reduced CO₂ compensation point compared to other C₃ 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 C₄ or C₂ photosynthesis plants, it still accounts as a considerable improvement (Sage et al., 2013). Therefore, installing this anatomy in a C₃ 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 cells 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, were 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 CO₂ fixation of these mutants with the wild-type ones.

Introducing C₄ metabolism into C₃ species
C₄ photosynthesis greatly reduces photorespiration by concentrating CO₂ at the active site of Rubisco. With the exception of the so-called single-cell C₄ plants (Sharpe and Offermann, 2014), C₄ plants have adopted different biochemical and anatomical modifications. C₄ leaves have two distinct layers of photosynthetic tissue (the so called “Kranz” leaf anatomy): mesophyll cells that are in contact with atmospheric CO₂ via intercellular air spaces, and bundle sheath cells with cell walls that are less permeable to CO₂. CO₂ is assimilated into oxaloacetate in the mesophyll cells via PEP carboxylase, which is then converted to a more stable 4-carbon organic acid, malate or Asp, which diffuse to the bundle sheath cells (Gowik and Westhoff, 2011). Here the C₄ acid is decarboxylated, releasing CO₂ near the active site of Rubisco, which is located only in this cell type in C₄ plants. Given the higher efficiency of the C₄ photosynthetic mechanism under current atmospheric [CO₂], efforts are underway to install C₄ photosynthesis in C₃ plants such as rice (the International C₄ 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 C₄ photosynthesis is relatively small, the introduction of C₄ photosynthesis into C₃ crops will also require major changes in leaf anatomy (von Caemmerer et al., 2012). Initial progress toward the identification of the genes responsible for C₄ anatomy has been reported (Feldman et al., 2014; Rizal et al., 2015). On the other hand, terrestrial plants capable to carry out C₄ 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 CO₂ at the active site of Rubisco. The genes involved in the development of this peculiar subcellular anatomy are unknown.

Considering the scarcity of sequence information for single cell C₄ species, it is difficult to judge if single cell C₄ metabolism can be bio-engineered into C₃ crops.

Introduction of CO₂-concentrating mechanisms into chloroplasts

Another strategy to reduce oxygenation and thereby photorespiration is to introduce cyanobacterial CO₂-concentrating mechanisms (CCM) into the chloroplasts of land plants (Zarzycki et al., 2013). Cyanobacteria suppress the
oxygenating reaction of Rubisco by concentrating CO₂ 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 releases CO₂ inside the carboxysome. The high [CO₂] obtained near to the active site of 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 but also a lower affinity for CO₂ and specificity factor (meaning that is more prone to oxygenating RuBP) compared to the plant one (Zarzycki et al., 2013). This 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 organized, although empty, microcompartments. The same group was also able to introduce a functional cyanobacterial Rubisco in tobacco chloroplasts together with an internal carboxysomal protein (Lin et al., 2014b). In this instance they replaced the native Nicotiana tabacum gene encoding for the large subunit of Rubisco and replaced it with the large and small subunits of the Synechococcus elongatus Rubisco, an enzyme with lower CO₂ affinity but higher catalytic rate compared to the endogenous one. The transformed lines were photosynthetically competent albeit at very high [CO₂] and the formation of complexes between the cyanobacterial Rubisco and the carboxysomal protein was observed within the chloroplast stroma as occurs during cyanobacterial β-carboxysomes biogenesis, representing an important step toward the introduction of a CCM into C₃ plants. Simpler CCM mechanisms have been also considered for the transformation of C₃ 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 CO₂-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., 2014). 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 chaperonins needed for the correct folding of the plant enzyme in the bacterial host (Saschenbrecker et al., 2007; Liu et al., 2010; Feiz et al., 2012). 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. Even without engineering CCM into chloroplasts, the raise in CO$_2$ levels that is expected by the end of the century will also probably allow for a less specific, and hence faster Rubisco. 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., 2014).

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 into 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 NH₃. In fact, mutants of plastidic GS₂, the enzyme in charge of the reassimilation of photorespiratory ammonium, have been isolated in barley (Blackwell et al., 1988) and in the model legume Lotus japonicus (Pérez-Delgado et al., 2013) by screening an EMS population for the typical “photorespiratory” phenotype. However, no plastidic GS₂ mutants have been found in Arabidopsis. Given that the mutagenesis screen that was carried out with these plants was probably saturating (for example, 58 mutants were found affecting Fd-GOGAT, the other plastidic enzyme involved in NH₃ reassimilation) and that Arabidopsis GS₂ is encoded, as in most plants, by a single gene (At5g35630), it is puzzling why GS₂ mutants were not been isolated either in the original screening or by means of transposon insertion. Another example of variation in photorespiratory metabolism related to ammonia reassimilation can be found in conifers, where the plastidic isoform of GS is not present but, unlike other higher plants, a cytosolic GS isoform is expressed in photosynthetic cells, and photorespiratory ammonia is probably reassimilated through a cytosolic GS/GOGAT cycle (Ávila et al., 2001).

**Photorespiratory bypasses**

Instead of trying to reduce the photorespiratory rates, a different approach is to install alternative and less energetically expensive routes for the recycling of 2PG. Three bypasses to the reactions of the photorespiratory pathway were successfully engineered in Arabidopsis. In the first approach, glycolate was converted to glycerate directly in the chloroplast by introducing the Escherichia coli glycolate catabolic pathway, thus avoiding or at least competing with the peroxisomal and mitochondrial reactions of photorespiration (Kebeish et al., 2007). The second approach was to introduce a complete glycolate catabolic cycle that oxidized 2PG to CO₂ in the chloroplast (Maier et al., 2012). Both bypasses should avoid ammonia release in the mitochondria, which is quite
expensive to reassimilate in terms of the ATP and reducing equivalents required. However, while the “Kebeish” bypass resulted in an improved energy balance, the “Maier” bypass was costlier compared to the standard photorespiratory cycle (Peterhänsel et al., 2013b). Despite this, both bypasses were report to enhance biomass production by up to 30%. In the case of the “Maier” bypass it is speculated that this benefit may be due to the release of CO₂ from 2PG oxidation directly in the chloroplast, this might increase the chloroplastic CO₂ concentration and reduce the probability of further oxygenating reactions. Interestingly, both bypasses resulted in increased biomass production only under short-day conditions but not in long days. 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, thus once again avoiding ammonia release in the mitochondria (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 recent reports, the potential of photorespiratory bypasses for the improvement of plants of agronomical importance has been demonstrated. It was shown that 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). Also, in another study, potato (*Solanum tuberosum*) plants were transformed with the three genes that encode for *E. coli* glycolate dehydrogenase subunits and the corresponding polyprotein was successfully expressed in the chloroplast, where it was able to catalyze the conversion of glycolate to glyoxylate (Nölke et al., 2014). The enhancement in assimilation rate led to an increase in shoot biomass and subsequently to a greater tuber yield in the transgenic lines. This suggested that part of the glyoxylate produced in the chloroplast by the bacterial enzyme may be completely oxidized *in situ* to CO₂ that would be released near the Rubisco active site and would thereby reduce the rate of Rubisco oxygenation. Recent evidences support the idea that glyoxylate can be decarboxylated in the chloroplast by the action of the endogenous pyruvate dehydrogenase (Blume et al., 2013). However, in order to try to establish a highly efficient partial or complete ‘Kebeish’ bypass, it should be taken into consideration that plastids
contain a highly active NADPH-dependent glyoxylate dehydrogenase, which is able to reduce this molecule back to glycolate (Allan et al., 2009) and should be probably silenced in order to avoid a futile cycle in the chloroplast.

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 CO₂ directly in the chloroplast. In a recent report, a synthetic pathway that worked both as a photorespiratory bypass and as an additional CO₂-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 be taken into 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 CO₂ probably by a non-enzymatic reaction that takes place in the peroxisomes of higher plants in the presence of H₂O₂ (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 NH₃, 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. Therefore, a current challenge resides in finding
better tools to challenge these alternative pathways and assess their natural occurrence under both normal and stress conditions.

**Optimization of the levels of photorespiratory enzymes**

Analysis of dynamic metabolic models of photosynthetic carbon metabolism suggested that 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). Interestingly, 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. By contrast, a few studies have reported an improved performance of plants with increased levels of photorespiratory enzymes. Overexpression of GDC-H protein or 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 CO₂ 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 CO₂ 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 the plastidic isoform of glutamine synthetase (GS$_2$), and it has been suggested
that this reaction must be the rate-limiting step of the pathway (Wallsgrove et al.,
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 is 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., submitted for publication). 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 and in Table 1, several approaches have been used in order to manipulate photorespiration in attempt to increase 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 into a C₄ 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 if is getting easier and more successful every year. 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). As a first step, 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). Even after careful experimental design and test in intermediate plant models, several challenges would need to be
overcome before new genes and pathways can be introduced into crops. As mentioned before, nuclear and especially plastid transformation techniques are still inefficient or unavailable for most staple crops. In addition to that, promoters and vectors that can permit high expression of transgenes and a correct subcellular localization of the protein product should be available, 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). Furthermore, due to social and political rejection of GMPs, even those transgenic plants that have been approved are not cultivated in most EU countries. 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, like TALENS (transcription activator-like effector nuclease(s)) or the CRISPR/Cas9 system, have been developed in the recent years. 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 these techniques together with cisgenesis and intragenesis, RNA-dependent DNA methylation, grafting (production of chimeric plant with a wild-type scion inserted on a genetically modified rootstock), reverse breeding and agro-infiltration in order to determine the extent to which they should lead to genetically modified organisms (Lusser et al., 2012). Promising steps towards the regulation of these techniques are being given, for example mutant plants obtained with the CRISPR/Cas9 system have not been considered as GMPs in a recent decision of the Swedish Board of Agriculture (http://www.upsc.se/about-upsc/news/4815-green-light-in-the-tunnel-swedish-board-of-agriculture-a-crispr-cas9-mutant-but-not-a-gmo.html).

Should we really look for plants with lower rates of photorespiration?
Regardless of the difficulties that we may face to obtain plants with modified photorespiratory rates, some changes in photorespiration in the field will happen anyway because of the rise in atmospheric [CO$_2$], which is predicted to double by 2100 (Intergovernmental Panel on Climate Change, 2014). On one hand, this increase in [CO$_2$] will reduce photorespiration by increasing CO$_2$ fixation by Rubisco. On the other hand, photorespiration should be stimulated by the predicted increase of the average atmosphere temperature, and subsequently of leaf canopy. Moreover, the expected increased stomatal closure caused by elevated CO$_2$ will contribute to further increase in leaf temperature. Thus, photorespiratory losses are still expected to be high even in a high CO$_2$ world. 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 but also by an increased capacity for photorespiratory flux. The introduction of bypasses to photorespiration can lead to up to 30% of increase in plant biomass (Kebeish et al., 2007; Maier et al., 2012; Nölke et al., 2014). However, these beneficial effects were observed only under short day conditions and/or controlled temperature and humidity, which may not always reflect the conditions that crops will face in the field. Further testing of these GMPs under different conditions would be needed in order to determine if photorespiratory bypasses may be beneficial also under field conditions. By contrast, several studies indicated that a higher capacity for photorespiratory flux is paralleled by increased plant yield (see the 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. 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.
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). 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. 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). Heat also leads to increased photorespiration of decreased Rubisco specificity and secondarily due to the changes in the relative solubility of CO$_2$ and O$_2$. 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). Barley mutants with reduced levels of different photorespiratory enzymes as well as Arabidopsis mutants of the peroxisomal hydroxypyruvate reductase (HPR1) enzyme were more sensitive to drought (Wingler et al., 1999b; Li and Hu, 2015). On the other hand, rice plants with increased photorespiratory capacity showed enhanced tolerance to salt stress (Hoshida et al., 2000). A protective role of photorespiration in the dissipation of excess energy has been already hypothesized long time ago (Heber and Krause, 1980) and a demonstration to this hypothesis was provided later by Kozaki and Takeba (1996), who showed that photorespiration protects against photoinhibition caused by high light. A more recent work demonstrated that when the photorespiratory cycle is impaired, the excess of reducing power and the consequent over-production of ROS prevent the repair of photosystem II, thus leading to accelerated photoinhibition (Takahashi et al., 2007). A role for photorespiration in the response to other kinds of stress such as chilling or exposure to heavy metals has also been proposed (Voss et al., 2013 and references therein). Interestingly, several photorespiratory genes are co-expressed with genes involved in the resistance to Al, that although not technically a heavy metal is also a stressor that constrains plant productivity (Nunes-Nesi et al., 2014a). Since abiotic stress is one of the factors that most frequently limits crop productivity worldwide (Mittler, 2006), the performance of plants with reduced rates of photorespiration should be tested carefully under different stress conditions. This should be carried out also for plants expressing bypasses to photorespiration, since
the sink effect for excess reducing power exerted by photorespiration under stress conditions may be lost in such organisms. 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. 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.

Interestingly, photorespiration has also been shown to play a significant role in biotic stress responses, where the H$_2$O$_2$ 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. In a recent report it was also showed that some photorespiratory enzymes are highly expressed in plant roots (Nunes-Nesi et al., 2014b), so it is possible that changes in the levels of photorespiratory enzymes may also affect the physiology of heterotrophic tissues.

3) Rates of photorespiration correlate with 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). The exact mechanism that underpins this co-dependency is still unknown but it may be related to the photosynthesis-dependent export of malate from the chloroplast (the ‘malate valve’), which increases the levels of cytosolic NADH thus providing reducing equivalents for nitrate reduction (Bloom et al., 2010). Additionally, increased rates of photorespiration further result in excess NAD(P)H since photorespiration consumes more ATP relative to NAD(P)H than CO$_2$ fixation (Kramer and Evans,
This results in excess NAD(P)H that must be consumed to balance the energy demands of central metabolism with energy production from the light reactions. C4 plants on the other hand assimilate NO3\(^-\) independently of atmospheric CO2 concentration since the cytoplasmic NADH for nitrate reduction can be produced by the same C4 pathway instead of by photorespiration (Bloom, 2015).

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 NO3\(^-\) into organic compounds compared to other N sources such as NH4\(^+\) or organic forms of nitrogen. Taking this into consideration, it is possible that a reduction of the photorespiratory rates in crops that use mainly NO3\(^-\) may lead to nitrogen deprivation. Reliance on NH4\(^+\) fertilizers may not always be possible in order to circumvent this since many plants show symptoms of toxicity when grown on NH4\(^+\) 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 CO2-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 NO3\(^-\) 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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References


and might have been conveyed endosymbiontically to plants. Proceedings of the National Academy of Sciences of the United States of America 105, 17199-17204.


Table 1. Summary of the approaches that can be used to improve crop yield through manipulation of photorespiration.

<table>
<thead>
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<th>Strategy</th>
<th>Advantages (A) / Disadvantages (D)</th>
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| Screening for plants with reduced rates of photorespiration             | A: -No need for genetic manipulation.  
D: -Highly improbable to find plants with high levels of PS and low of photorespiration in field trials. |
| Enhancing the amount of photorespiratory CO\textsubscript{2} scavenging   | A: -Does not imply changes in cellular metabolism.  
D: -Genetic determinants of organelle partitioning and connection are not completely understood. |
| Introduce C\textsubscript{4} photosynthesis into C\textsubscript{3} plants | A: -Great theoretical potential for increase in crop yield.  
D: -Major changes in leaf anatomy are required. -The genes responsible for C\textsubscript{4} anatomy not completely identified. |
| Introduction of CCM into chloroplasts                                   | A: -Should greatly reduce the rates of photorespiration.  
-Should allow replacing endogenous Rubisco with enzymes with higher catalytic rates and lower CO\textsubscript{2} affinity.  
D: -Requires transformation of the chloroplast genome.  
-Complex CCM requires the transformation of multiple genes and the correct assembly of multiprotein complexes. |
| Rubisco engineering and screening for naturally occurring more efficient Rubisco | A: -Rubisco has several catalytic inefficiencies. This implies several opportunities for engineering.  
-Naturally occurring more efficient Rubiscos have been found in some species.  
D: -Structure-function studies with Rubisco are hampered by different technical difficulties. -The exact mechanism of the oxygenating reaction is still not completely understood. |
| Photorespiratory bypasses                                                | A: -Successfully engineered in both model and crop species.  
-Can increase yield up to 30%. -Possibility of complete oxidation of 2PG in the chloroplast, thus raising the [CO\textsubscript{2}] near Rubisco active site.  
D: -Need transfer of multiple genes. -Increased yield is seen only under short day in some bypasses. -The lower energy cost of some bypasses may prevent the protective role of PR under stress conditions. |
| Optimization of the levels of photorespiratory enzymes                  | A: -Relatively easy genetic manipulation.  
D: -Transcriptional and post-translational regulation of photorespiratory genes and enzymes is still poorly characterised. |