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REVIEW

Exploring natural variation of photosynthesis in a site-specific manner: evolution, progress, and prospects

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Abstract

Main conclusion **Site-specific changes of photosynthesis, a relatively new concept, can be used to improve the productivity of critical food crops to mitigate the foreseen food crisis.**

Abstract Global food security is threatened by an increasing population and the effects of climate change. Large yield improvements were achieved in major cereal crops between the 1950s and 1980s through the Green Revolution. However, we are currently experiencing a significant decline in yield progress. Of the many approaches to improved cereal yields, exploitation of the mode of photosynthesis has been intensely studied. Even though the C_4 pathway is considered the most efficient, mainly because of the carbon concentrating mechanisms around the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, which minimize photorespiration, much is still unknown about the specific gene regulation of this mode of photosynthesis. Most of the critical cereal crops, including wheat and rice, are categorized as C_3 plants based on the photosynthesis of major photosynthetic organs. However, recent findings raise the possibility of different modes of photosynthesis occurring at different sites in the same plant and/or in plants grown in different habitats. That is, it seems possible that efficient photosynthetic traits may be expressed in specific organs, even though the major photosynthetic pathway is C_3. Knowledge of site-specific differences in photosynthesis, coupled with site-specific regulation of gene expression, may therefore hold a potential to enhance the yields of economically important C_3 crops.

Keywords Crop improvement · Engineering C_4 into C_3 · Photorespiration · Photosynthesis gene regulation

Introduction

Photosynthesis is the biological process that produces organic carbon compounds by using solar energy, water and atmospheric carbon dioxide (CO_2). In this process, atmospheric CO_2, after going through a series of photosynthetic reduction steps, is converted to carbohydrates (Evans 2013). This biological process is used by autotrophic and semi-autotrophic organisms, being the main driver of food production for heterotrophs for billions of years (Wang et al.

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2011). However, the global production of food for humans has been challenged as a result of increasing population and a potential decrease in food productivity driven by factors related to climate change (Berardy and Chester 2017; Crist et al. 2017).

It is anticipated that the global population will have increased to 9.5 billion by 2050 (Alexandratos and Bruinsma 2012). To sustain this increase, food production must increase by at least 60% (Clay 2011; Crist et al. 2017). Towards the end of 2016, Asia accounted for 59.5% of the global population, and it is undergoing continuous growth (Worldmeters 2017). Tilman et al. (2009) and Sheehy et al. (2008) have suggested that a 60% improvement in the yield of rice (*Oryza sativa* L.) might be needed in order to cater for the growing Asian population in the near future. This translates to catering to 43 people from a hectare of rice instead of the present 27. A dramatic improvement in yield of C_3 cereal crops like wheat (*Triticum aestivum* L.) and rice was achieved between the 1950s and 1980s during the Green Revolution, mainly through the introduction of dwarf varieties via molecular breeding (Furbank et al. 2015). This also improved the fertilizer use efficiency by increasing the grain mass of the crop at the expense of the rest of the above ground biomass (increase in harvest index) (Stapper and Fischer 1990; Evans 2013). Outcomes of the Green Revolution have helped to satisfy the global food demand for many years. However, feeding the projected global population seems impossible without another massive breakthrough as the genetic yield potential of current cereal crops, using conventional breeding, has almost plateaued (Kropff et al. 1994; Furbank et al. 2015; Taylor and Long 2017; Wang et al. 2017b). Meeting future food production goals has become even more challenging because of the predicted and even current changes in climate, such as the increase in global temperature and water scarcity (Elliott et al. 2014). Of the few possible solutions for the upcoming food crisis, increasing the biomass of critical food crops is one of the most intensely studied (Evans 2013; Taylor and Long 2017).

The Green Revolution increased the harvest index with little change to the total aboveground biomass (Austin et al. 1989; Evans 2013; Ort et al. 2015). Evans (2013) suggested that further increases in harvest index may come at the expense of leaves, the major source of carbon fixation, and consequently decreased grain yields. As a corollary, it was argued that total biomass may have to be increased to achieve higher yields (Evans 2013), which would require either enrichment of the light interception of crop plants throughout the growing season and/or an increase in the photosynthetic efficiency (Evans 2013; Taylor and Long 2017). Since there appears little possibility of further improvement to light interception (Evans 2013), increasing photosynthetic efficiency merits investigation.

Of the different strategies for improving photosynthetic efficiency (Fig. 1), engineering a more efficient mode of photosynthesis for food crops is being intensively studied, e.g., by engineering C_4 into C_3 (Gao et al. 2014; Wang et al. 2014; Li et al. 2017); mitigating the functional limitations of the major CO_2 fixing enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Cai et al. 2014; Galmes et al. 2014; Sáez et al. 2017); increasing the mesophyll conductance of CO_2 diffusion (Cano et al. 2014; Gu and Sun 2014; Xiong et al. 2017); minimizing the inefficiencies due to dynamic environmental factors (Williams et al. 2014; Retkute et al. 2015; Vialet-Chabrand et al. 2017); and regulating sink–source feedback on photosynthesis (Fatichi et al. 2014; Campany et al. 2017). However, improvement of photosynthetic efficiency is challenging because of the complex nature of photosynthetic mechanisms and inadequate understanding of the genetic processes (Rangan et al. 2016b).

Until now, angiosperms have been divided into functional categories according to the photosynthetic mode of the primary photosynthetic organs, typically the leaves. However, this categorization has been brought into question by the fact that, within the same plant, different organs may perform different modes of photosynthesis (Rangan et al. 2016a, b). We refer to this phenomenon as site-specific or dual biochemistry photosynthesis. The hypothesis of site-specific photosynthesis also includes the possibility that the mode of photosynthesis may change with the environment. Here we mainly focus on the site-specific C_4 photosynthesis since it is the most efficient mode at the current atmospheric conditions. This conceptually novel hypothesis is supported by a growing number of observations, e.g., the cells surrounding the vascular bundles in the stem and petiole of tobacco and celery have C_4-like photosynthetic mechanisms, even though the leaves are C_3, and consequently, that is how the species is categorized (Hibberd and Quick (2002). Furthermore, changes in photosynthesis similar to the C_4 pathway were observed in rice panicles during grain filling (Imaizumi et al. 1990), and evidence for grain-specific C_4 photosynthesis has been reported in the C_3 species, wheat (Rangan et al. 2016b). Further, the existence of different photosynthetic pathways in the husk and foliage leaves of maize (*Zea mays*) is perhaps the most obvious case of site-specific changes in photosynthesis.

The different modes of photosynthesis have been extensively reviewed (Edwards et al. 2004; Kiang et al. 2007; Hibberd and Covshoff 2010), including the possibility of engineering the C_4 pathway into the C_3 (Matsuoka et al. 2001; von Caemmerer 2003; Kajala et al. 2011), and other ways to improve photosynthetic efficiency (Long et al. 2006; Zhu et al. 2010; Blankenship et al. 2011; Ort et al. 2015). However, site-specific photosynthesis and dual-biochemistry photosynthesis have not been sufficiently addressed.

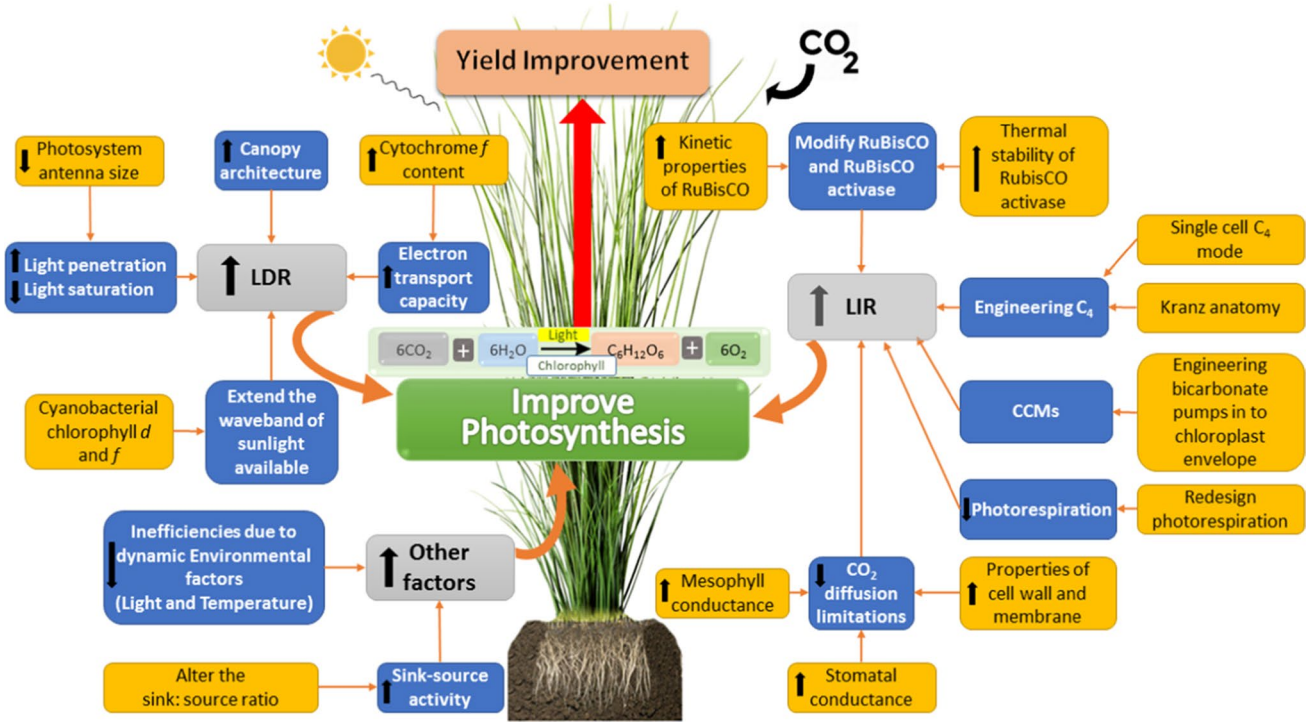

Fig. 1 Possible approaches to improve photosynthesis (upward arrows represent an improvement and downward arrows represent an impairment. LDR and LIR represent light-dependant reactions and light-independent reactions, respectively). Improving the efficiency of the LDR could have an impact on improving the overall efficiency of photosynthesis. Reduction of photosystem antenna size might increase the light penetration and decrease the light saturation of the photosynthetic tissue. In addition, canopy architecture is also important. To expand the available waveband of sunlight, Cyanobacterial chlorophyll d and f can be used and increase the cytochrome f content may lead to increase the electron transport capacity. Moving to the improvement of LIR, modification of RuBisCO is one of the hot research trends. Engineering C_4 mode to C_3, initiate CCMs, redesigning photorespiration and mitigation of the limitations of CO_2 diffusion in photosynthetic tissues are other possible approaches that have been proposed. In addition, alteration of the sink: source ratio and activity; minimize the inefficiencies of photosynthesis due to dynamic environmental factors have also been proposed

In this review, we summarize the literature on site-specific photosynthesis and its importance. We also critically analyze the evolutionary linkage between C_3 and C_4 photosynthesis through photorespiratory modifications, importance of photorespiratory carbon pump as an evolutionary bridge for C_4 pathway, recent approaches that have been taken to improve the efficiency of photosynthesis, photosynthetically efficient physiological traits to understand site-specific photosynthesis, gene-regulatory characteristics of C_4 photosynthesis in a site-specific manner. Also, we discuss the knowledge gaps and the future directions that need to be undertaken to get a better understanding of site-specific photosynthesis. Finally, we summarise the other possible ways of increasing the efficiency of photosynthesis by enhancing the properties of the light-independent and light-dependent reactions.

Natural variation of photosynthesis in angiosperms

Being the most important biochemical light energy using process on earth, photosynthesis converts CO_2 into organic sugars through plants, algae, and photosynthetic bacteria. Photosynthesis consists of two distinct biochemical processes, the light-dependent reaction (LDR) and the light-independent reaction (LIR). The LDR produces ATP (adenosine triphosphate) and NADPH (reduced form of nicotinamide adenine dinucleotide phosphate), which are later utilized by the LIR: the Calvin–Benson cycle. Production of carbohydrates using atmospheric CO_2 and the products of the LDR take place in the Calvin–Benson cycle. Currently, four major photosynthetic mechanisms have been identified in angiosperms: C_3, crassulacean acid metabolism (CAM), C_4, and C_3–C_4 intermediates (Yamori et al. 2014) (Fig. 2). Out of those, the most common photosynthetic mechanism is C_3 where RubisCO fixes atmospheric CO_2 into a three-carbon compound; mainly, phosphoglyceric acid (PGA).

On the other hand, the first stable compound of photosynthesis in the C_4 pathway is the four-carbon molecule, oxaloacetate (OAA). Also, C_4 appears the most efficient form of carbon fixation in the majority of current environmental conditions, i.e., C_4 is theoretically 30% more efficient than C_3 photosynthesis (Zhu et al. 2008). The C_4 grass *Echinochloa polystachya,* growing on the Amazon floodplain,

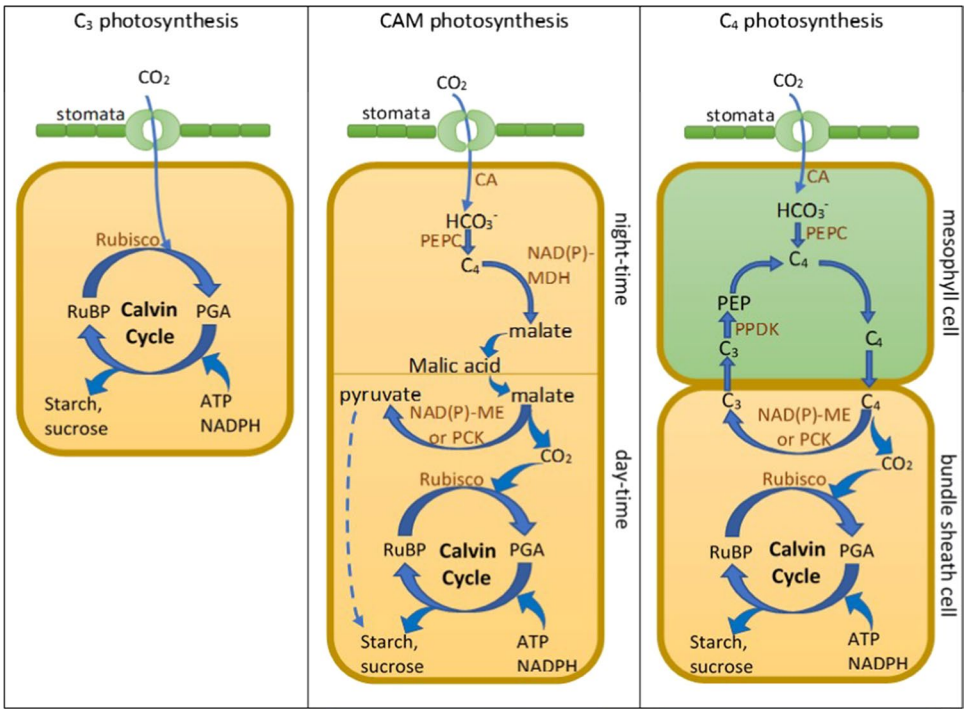

Fig. 2 Schematic diagram of C_3 CAM and C_4 photosynthesis. *Rubisco* ribulose-1,5-bisphosphate carboxylase/oxygenase, *RuBP* ribulose 1,5-bisphosphate, *PGA* phosphoglyceric acid, *ATP* adenosine tri phosphate, *NADPH* nicotinamide adenine dinucleotide phosphate hydrogen, *CA* carbonic anhydrase, *HCO 3* bicarbonate, *NADP* nicotinamide adenine dinucleotide phosphate, *NADP-MDH* NADP-malate dehydrogenase, *NADPME:NADP*-dependent malic enzyme, *PCK* phosphoenolpyruvate carboxykinase, *PEPC* phosphorenolpyruvate carboxylase, *PPDK* pyruvate orthophosphate dikinase, *PEP* phosphorenolpyruvate

holds the record for the highest recorded annual dry-matter productivity, for any terrestrial vegetation, having an average of 100 tonnes per hectare per year (t ha^{-1} $year^{-1}$; productivity is given throughout as mass of oven-dry matter) (Piedade et al. 1991). C_4 forage grass *Pennisetum purpureum* cultivated in El Salvador, recorded the highest annual dry-matter yield for a crop, producing 88 t ha^{-1} $year^{-1}$ (Beadle and Long 1985). Engineering C_4 photosynthesis into critical C_3 food crops has been intensely studied (Kajala et al. 2011; Rangan et al. 2016b; Wang et al. 2017b); however, engineering a complete C_4 pathway into C_3 has proven elusive due to the lack of knowledge of specific gene regulation in both photosynthetic pathways (Wang et al. 2011). Photosynthesis of both C_3 and CAM plants takes place in a single photosynthetic cell. The primary difference between C_4 and CAM is that the CAM pathway expresses a temporal separation of the photosynthetic process (Fig. 2), rather than a spatial separation seen in C_4 Kranz, which will be discussed later.

The evolutionary rise of C_4 photosynthesis through photorespiratory modifications

Current differences in photosynthetic properties of higher plants can be considered as an evolutionary adaptation to diverse ecosystems/niches. The most diverse modifications of photosynthesis occur in the LIRs. However, although there are spatial and temporal differences among photosynthetic modes, the enzyme RuBisCO universally catalyzes the fixation of CO_2 into a stable, three-carbon intermediate. RuBisCO is bifunctional, also binding O_2 in competition with CO_2 and catalyzing a reaction known as photorespiration, which negatively affects the overall efficiency of photosynthesis, and consumes ATP and NADPH (Bowes et al. 1971; Furbank 2011, 2016). At current atmospheric concentrations of CO_2 and O_2, photorespiration can decrease photosynthesis to 30% of its potential (Sharkey 1988; Zhu et al. 2004). This happens mainly by losing assimilated C and N as photorespiratory CO_2 and NH_3 (Fig. 2) (Sharkey 1988; Zhu et al. 2004). However, despite all the inefficiencies that adversely affect photosynthesis, photorespiration is nonetheless the second-most important biochemical reaction on earth, because of its salvage action against 2-phosphoglycolate (2PG): a toxic by-product from oxygenation of RuBisCO (Hagemann et al. 2016). Oxygenation of RuBisCO produces a large amount of 2-PG during the day. Photorespiration is a light-induced biochemical process which converts 2-PG into 3-phosphoglycerate (3-PGA), a Calvin–Benson cycle intermediate (Fig. 2). Therefore, photorespiration is an essential metabolic process for all organisms which perform oxygenic photosynthesis (Somerville 2001). Reactions of photorespiration take place in the chloroplasts, peroxisomes, cytosol and mitochondria. Also, for the main photorespiratory cycle, at least eight enzymes are needed other than a few more auxiliary enzymes (Bauwe et al. 2010).

Photorespiration was not a concern during the early evolutionary stages of photosynthesis when the atmosphere was dominated by CO_2 with only a trace of O_2 (Evans 2013). However, photosynthesis has so successfully reversed that ratio that competition for binding O_2 to RuBisCO has appreciably increased. Hence,

photorespiration may have co-evolved with oxygenic photosynthesis around 3.8–2.5 billion years ago in cyanobacteria that initially lived in O_2-free Precambrian oceans (Canfield 2005; Allen and Martin 2007; Bauwe et al. 2010). The evolution of algae and higher plants may have taken place after the uptake of cyanobacteria into heterotrophs about 1.5 billion years ago (Reyes-Prieto et al. 2007; Bauwe et al. 2010). During this time, C_3 photosynthesis became the dominant carbon assimilation pathway on earth. It is believed that the earth's climate transformed in the past 40 million years from a warm, humid atmosphere having temperate poles, to a less humid atmosphere, with frozen poles, deserts, and grasslands (Zachos et al. 2008). Parallel to this climate shift, the atmospheric CO_2 concentration decreased from over 1000 µmol mol^{-1}, 50 million years ago, to less than 200 µmol CO_2 mol^{-1}, 20,000 years ago (Zachos et al. 2008; Beerling and Royer 2011). In response to this shift, the modern biosphere evolved Bouchenak-khelladi et al. (2009), and two main photosynthetic strategies evolved to deal with the increasing O_2/CO_2. First, carbon concentrating mechanisms (CCMs) evolved in photosynthetic organs to allow RuBisCO to operate in a relatively enriched CO_2/O_2 environment (Hatch and Slack 1966; Sage 2016; von Caemmerer et al. 2017). C_4, CAM, and C_3–C_4 intermediates have CCMs in their photosynthetic pathways. Amongst them, C_4 photosynthesis has the most efficient and complex CCM. Secondly, the kinetic properties of RuBisCO changed to improve its selectivity towards CO_2 relative to O_2 (Evans 2013).

Photorespiratory carbon pump as an evolutionary bridge to the C_4 pathway

There is increasing evidence for the linkage of photorespiration to the evolution of C_4 photosynthesis from its C_3 ancestors (Sage 2004; Hagemann et al. 2016). The development of a photorespiratory carbon pump (Fig. 3), which is commonly seen in C_3–C_4 intermediates, is considered to be an evolutionary bridge for decarboxyacid-based C_4 photosynthesis (Mallmann et al. 2014). The CO_2 released during the decarboxylation in photorespiration may not be a complete loss to the plant since it can be refixed by RuBisCO (Sage and Sage 2009). The photorespiratory carbon pump facilitates efficient refixation of CO_2, by allowing photorespiration to occur in two different types of cells: mesophyll cells (MCs) and bundle sheath cells (BSCs) (Fig. 3). The two-carbon metabolite glycine acts as the CO_2 transporter of this mechanism, which is therefore termed the C_2 pathway. To perform the C_2 pathway more efficiently, a large number of cell-type-specific physiological, biochemical and gene regulatory preconditioning is needed. Localization of mitochondria, which contain glycine decarboxylase (an essential enzyme in photorespiration) into BSCs is one such modification, which forces photorespiratory glycine to migrate from MCs to BSCs (Fig. 3). The CO_2 released by the decarboxylation of glycine at least doubles the CO_2 concentration at BSCs, which creates a favorable condition for BSC RuBisCO to perform at a higher catalytic efficiency and increase photosynthesis. The arrangement of cell organelles linked with photorespiration is also essential for an

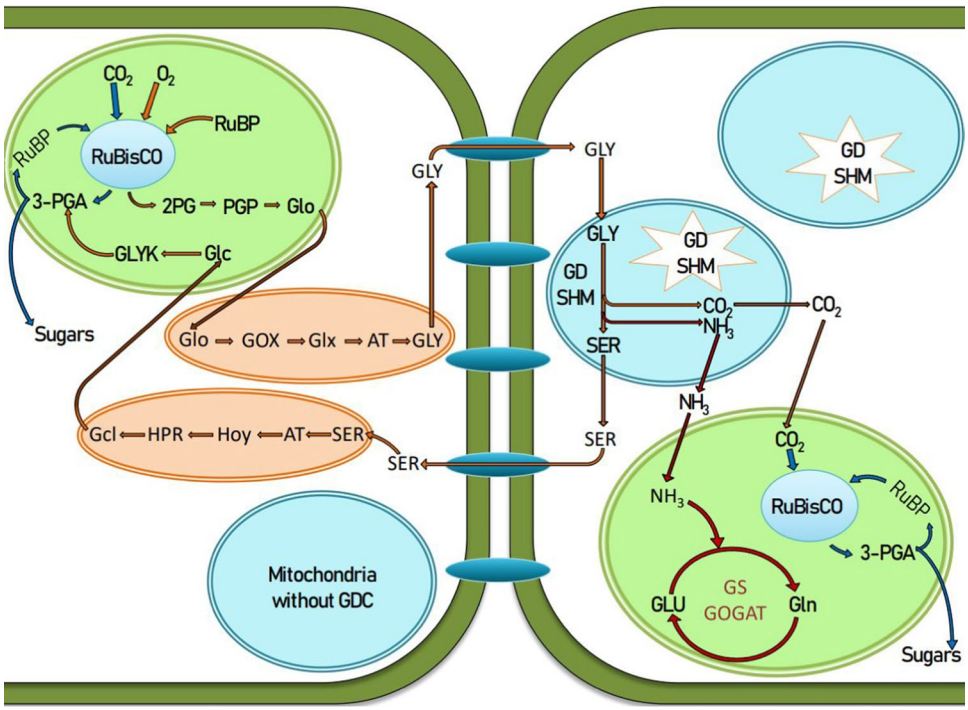

Fig. 3 Schematic diagram of the photorespiratory carbon pump. Green colored circles represent chloroplasts, orange circles represent peroxisomes and blue circles represents mitochondria. *AT* aminotransferase, *GD* glycine decarboxylase, *Glc* glycerate, *Gln* glutamine, *Glo* glycolate, *GLU* glutamate, *GLY* glycine, *GOX* glycolate oxidase, *HPR* Hyp reductase, *Hoy* hydroxypyruvate, *PGP* phosphoglycolate phosphatase, *Pyr* pyruvate, *RuBP* ribulose-1,5-bisphosphate, *SER* serine, *SHM* serine hydroxymethyltransferase, *2-PG* 2-phosphoglycolate, *3-PGA* 3-phospoglycerate

efficient C_2 pathway. The tight arrangement of chloroplasts and mitochondria on the inner cell-walls of BSCs, modifications of chloroplasts to increase their surface area, reduced MC:BSC through increased venation, higher plasmodesmata density connecting adjacent MCs and BSCs, less permeable cell walls of BSCs, and development of mechanisms for photorespiratory NH_4^+ metabolism are some other favorable factors to minimize photorespiratory CO_2 leakage and improve C_2 pathway efficiency (Fig. 3) (Sage and Sage 2009; Busch et al. 2013).

Even though photorespiration has been traditionally regarded as energy wasteful, a process that needs to be redesigned in order to improve photosynthesis, a growing body of evidence suggests otherwise. Biotic and abiotic stresses commonly limit crop productivity throughout the world (Mittler 2006; Kangasjärvi et al. 2012) and it has been reported that photorespiration mitigates their adverse effects. The H_2O_2 produced by the reaction of glycolate oxidase in photorespiration is a useful defense against a wide range of pathogens (Taler et al. 2004; Rojas et al. 2012). Strong correlations between photorespiration and tolerance of abiotic stresses, such as drought (Li and Hu 2015), salt (Hoshida et al. 2000) and exposure to heavy metals (Voss et al. 2013), have also been reported. In addition, recent studies have provided evidence for the interaction of photorespiration with other metabolic activities such as nitrogen assimilation (Rachmilevitch et al. 2004; Linka and Weber 2005; Keys 2006), respiration (Bykova et al. 2005; Tcherkez et al. 2008), one-carbon metabolism and associated purine biosynthesis (Rontein et al. 2003), and redox signaling (Foyer et al. 2009).

Past studies, combined with modern technological advancements, have answered some important questions regarding the photorespiratory pathway, its evolutionary linkage to C_4 photosynthesis and some of its interactions with other metabolic pathways (Bauwe et al. 2010). Yet there are several key areas that still need investigation, e.g., the changes in photorespiration in dynamic environments, how photorespiration interacts with other metabolic pathways (Bauwe et al. 2010) and, most importantly, how photorespiration may change in different photosynthetic organs of the same plant (site-specific changes of photorespiration). In most angiosperms, tissue/organ-specific anatomical, biochemical and gene regulatory differences are prominent. Therefore, expression of differences in metabolic pathways, including site-specific photorespiration, may be possible. For example, the efficiency of photorespiration could be high/low in some photosynthetic organs compared to the leaves of the same plant based on structural and biochemical differences. Understanding photorespiration in a site-specific manner generally is a better approach to address the existing knowledge gap: specifically the transcriptional, post-transcriptional and post-translational regulation of the pathway. This understanding could lead to redesigning of photorespiration in a more energy efficient way to increase the overall photosynthetic productivity of critical C_3 food crops. Knowledge of site-specific photorespiratory changes may also facilitate bio-engineering a photorespiratory carbon pump as in C_3-C_4 intermediates into C_3 plants. This would be an important intermediate step towards bio-engineering a complete CCM as in C_4 plants into critical C_3 food crops.

C_4: the ultimate result of the photosynthetic evolution

The C_4 photosynthetic mechanism was elaborated from the C_3 form simply by the coordination of existing anatomical and biochemical traits that were not efficiently utilized in C_3 plants (Christin and Osborne 2013). The C_4 carbon cycle is the most efficient mechanism for atmospheric CO_2 fixation in higher plants with respect to the use of nitrogen, water and light (Christin and Osborne 2013; Long and Spence 2013). The C_4 pathway may have originated independently up to 60 times during angiosperm evolution (Sage 2004). Most C_4 species are grasses that evolved 30 million years ago, and a lesser number of C_4 dicots that evolved some 20 million years ago (Sage 2004, 2016).

To acquire CCMs in C_4 plants, RuBisCO has to be isolated from the intercellular air spaces. If not, photorespiration takes place because of the high O_2 concentrations in inter-cellular spaces. Although C_4 photosynthesis may take place within a single cell, e.g., in the *Chenopodiaceae* (Voznesenskaya et al. 2002; Chuong et al. 2006), in most cases CCMs of C_4 photosynthesis are a result of distinct metabolic activities of two types of cells, BSCs and MCs, arranged around the vascular tissue (Kranz anatomy), which is supported by a specialized enzymatic activity through gene regulation (Voznesenskaya et al. 2001a). Most of the Calvin cycle enzymes including RuBisCO are localized in BSCs and the arrangement of BSCs around the vascular tissue is known as the photosynthetic carbon reduction (PCR) tissue (Dengler and Nelson 1999). In the majority of C_4 plants, initial CO_2 fixation takes place in MCs, and this carboxylation is catalyzed by phosphoenolpyruvate carboxylase (PEPC). RuBisCO is hardly seen in MCs in C_4, and for this reason, oxygenation activity leading to photorespiration is retarded. The four-carbon acid so produced by PEP is then translocated to the BSCs. This process tends to initiate the CCMs near RuBP by releasing CO_2 from the decarboxylation of the four-carbon acid. The CCM of C_4 increases the CO_2 concentration at the site of RuBisCO about tenfold (Furbank 2011). For example, under high irradiance, C_4 leaves operate with a ratio of the intercellular-to-ambient CO_2 concentration of around 0.3 compared with around 0.7 for C_3 leaves (Wong et al. 1985; Evans 2013). This also

allows the RuBisCO to perform closer to its catalytic maximum (V_{max}) (von Caemmerer and Furbank 2003).

Four subtypes of C_4 photosynthesis have been identified based on the differences in decarboxylation reactions. They are NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), PEP carboxykinase (PEP-CK), and combination of these enzymes (Prendergast et al. 1987). Although each subgroup shows both morphological and biochemical differences, production of OAA, a four-carbon compound from the initial fixation of HCO_3^- by PEPC in MC cytoplasm, is common to all C_4 plants (Prendergast et al. 1987).

Modifications for spatial compartmentation of the C_4 pathway could have indirect impacts on other metabolic processes. For example, large-scale transcriptomic and proteomic studies of some C_4 plants have revealed that metabolic processes other than photosynthesis, such as nitrogen or sulfur assimilation, protein synthesis, and lipid metabolism are also compartmentalized between BSCs and MCs (Majeran and van Wijk 2009). Such modifications of C_4 photosynthesis could perhaps improve overall plant performance. In addition, the Kranz anatomy may also impact on plant defense mechanisms to biotic and abiotic stresses. An experiment by Ku et al. (2000)using transgenic rice plants that overexpressed maize C_4 specific PEPC and pyruvate orthophosphate dikinase (PPDK), highlighted this phenomenon. The importance of the C_4 pathway, and its future potential for mitigating the upcoming food crisis are illustrated by the fact that even though only 3% of all plant species perform C_4 photosynthesis, they contribute to 25% of global photosynthesis (Sage et al. 2012). Because of its higher efficiency, engineering C_4 into the critical C_3 food crops has been studied profoundly during the last two decades (Burgess and Hibberd 2015).

Recent approaches for engineering the C_4 pathway into major C_3 plants and possible insights for success

Changing the mode of photosynthesis of classical C_3 food crops into a more efficient photosynthetic pathway could improve global food production. At least a 30% yield improvement is expected from engineering the C_4 pathway into C_3 food crops (Wang et al. 2017b). However, knowledge about the gene regulation of the complete C_4 photosynthesis is inadequate to achieve this ambitious and challenging goal (Schuler et al. 2016).

Recent advances in techniques of molecular biology, like Next Generation Sequencing, have provided new insights into engineering C_4 into C_3 plants. Complete genome sequencing of critical C_4 crop plants such as: *Zea mays*, *Sorghum bicolor, Setaria italica*, *Amaranthus hypochondriacus and Panicum virgatum*, can be considered as an important step in detailed understanding of C_4 photosynthesis (Paterson et al. 2009; Schnable et al. 2009; Casler et al. 2011; Zhang et al. 2012a; Sunil et al. 2014). Also, new evidence for gene regulation of C_4 biochemistry was revealed by a systems biology approach utilizing transcriptomic data. These transcriptomic data also provided a platform for the computational modeling that facilitated the creation of a metabolic map of the C_4 pathway (Burgess and Hibberd 2015). The first molecular evidence of C_4 Kranz anatomy is one such finding that can be further developed for engineering the C_4 pathway into C_3 plants (John et al. 2014; Li et al. 2015).

Significant earlier approaches to engineering the C_4 pathway into the C_3 focused on the submerged aquatic plant *Hydrilla verticilliata*, which performs a single-cell CCM, C_4-like pathway (Caemmerer et al. 2014). This approach may have been taken as it was thought that engineering a single-cell C_4 pathway into C_3 would be easier than engineering a complete Kranz anatomy. This has, however, been questioned as it requires a large number of structural modifications of the internal organelles, such as chloroplasts and mitochondria, in addition to the necessary biochemistry to minimize carbon leakage (Price et al. 2011).

The requirement of the internal organelles and structural modifications to engineer C_4 photosynthesis into a C_3 pathway was highlighted by Wang et al. (2017a), indicating the evolutionary importance of transitioning from C_3 to "proto-Kranz" anatomy in C_4 precursors (Wang et al. 2017a). Increased organelle volume (chloroplasts and mitochondria) in sheath cells surrounding leaf veins is the crucial characteristic of proto-Kranz anatomy. From the constitutive expression of maize *Golden*2 like genes in C_3 rice, Wang et al. (2017a) were able to induce chloroplast and mitochondrial development in rice vascular sheath cells. Further, increased organelle volume with the accumulation of photosynthetic enzymes and increased intercellular connections were also observed. This can be considered as a critical intermediate step towards engineering a complete C_4 pathway into C_3.

The C_4 rice consortium has initiated many projects to engineer the C_4 mode of photosynthesis into rice (von Caemmerer et al. 2012). The NADP-ME subtype of C_4 in maize was selected as the model pathway, as it was the best characterized of the C_4 subtypes (Kajala et al. 2011; von Caemmerer et al. 2012). Also, of the three biochemical C_4 subtypes, NADP-ME requires the minimum number of enzymes and transporters (Weber and von Caemmerer 2010). To investigate the differences between C_3 and C_4 photosynthetic pathways, Wang et al. (2014) profiled metabolites and transcript abundance of leaves of C_4 maize (*Zea mays*) and leaves of C_3 rice (*Oryza sativa*) using a statistical methodology named the unified developmental model (UDM). Interestingly, the UDM identified possible candidate cis-regulatory elements and transcription factors of

photosynthesis together with the differences between C_3 and C_4 carbon and nitrogen metabolism. One of the crucial aspects of UDM algorithms is the possibility of comparing development in other species. These approaches are useful to close the knowledge gap concerning gene regulation which leads to transforming C_3 into C_4 photosynthesis. Nonetheless, the horizon for releasing a commercial C_4 rice variety is unclear.

Rather than focusing on engineering the entire mode of C_4 photosynthesis into C_3, identification of site-specific changes in photosynthesis and photorespiration, and the induction of efficient C_4-like traits in the specific sites of the plant could improve its photosynthetic efficiency. Also, the site-specific changes in photosynthesis might be used in conventional breeding, especially for crop plants which have just a few C_4-like traits, if only at specific sites. If nothing else, more research on site-specific changes of photosynthesis may not only speed up the process of improving the efficiency of photosynthesis, but also be an intermediate step towards engineering a complete C_4 plant from the C_3.

Evidence for site-specific variation in photosynthesis

Even though the coordination of various anatomical and biochemical C_3 traits was required to evolve a functioning C_4 carbon cycle, all of the traits were present in the ancestral C_3 plants (Christin and Osborne 2013). The evolution of C_4 photosynthesis is a result of transcriptional, post-transcriptional, post-translational and epigenetic regulation. Evolution of Kranz anatomy generally seems to be one of the initial steps towards C_4 photosynthesis (McKown and Dengler 2007). Kranz anatomy facilitates CO_2 concentration at the site of RuBisCO. However, *Bienertia cycloptera* (Chenopodiaceae), which grows under salinity stress in Asian drylands, performs the C_4 mode of photosynthesis in a single chlorenchyma cell, without having Kranz anatomy (Voznesenskaya et al. 2002). This single-cell C_4 mode of photosynthesis is achieved by positioning dimorphic chloroplasts, photosynthetic enzymes, and other cell organelles, like mitochondria and peroxisomes, in distinct places within the cell (Sage and Monson 1998; Voznesenskaya et al. 2002). Voznesenskaya et al. (2002) also showed that the division of labour between the two types of photosynthetic cells (MCs and BSCs) in Kranz anatomy can be achieved by compartmentation within a single chlorenchyma cell. Similar evidence for the single-cell C_4 pathway was shown using *Borszczowia aralocaspica* (subfamily Salsoloideae, family Chenopodiaceae) (Voznesenskaya et al. 2001b; Sage 2002). This takes us to a novel understanding of C_4 photosynthesis, which is that a C_4-like mode of photosynthesis might be engineered in a plant, or at a specific site within it, in the absence of Kranz anatomy.

Possible evidence for site-specific C_4-like photosynthesis was observed by Brown et al. (2010). According to these authors, activation of the C_4 decarboxylation enzymes required for C_4 photosynthesis occurs in the mid-vein region of the classical C_3 plant, *Arabidopsis,* and plays an important role in amino acid and sugar metabolism (Brown et al. 2010). The maximum catalytic activity in isolated mid-veins of *Arabidopsis* leaves, for NADP-ME activity, is six times higher than in the entire leaf, and ten times higher in both NAD-ME and PEPCK on a chlorophyll basis (Brown et al. 2010). The catalytic activities of these decarboxylation enzymes are significantly higher than the values obtained from C_3 plants, and are similar to the catalytic activities in the BSCs of C_4 (Brown et al. 2010). Two transcripts derived from NADP-ME genes of C_4 (*NADP-ME2*, *NADP-ME4*) and transcripts derived from *PCK1 C*$_4$ were identified in mid-vein regions of Arabidopsis; nonetheless, the full-length protein of *NADP-ME4* was not detected (Brown et al. 2010). From work on the mid-vein region of *Arabidopsis*, Brown et al. (2010) further suggested that the high activities of NADP-ME, NAD-ME, and PEPC in veinal cells may provide CO_2 for an efficient photosynthesis. It is also possible that differences in photorespiration may exist at different photosynthetic sites within a plant. Therefore, it is worthwhile to study photorespiration along with photosynthesis in a site-specific manner. Wang et al. (2014) demonstrated the differences in gene expression and metabolites in different sites along the developing leaves of C_4 *Z. mays* (maize) and C_3 *O. sativa* (rice). This would again seem to be an indication of site-specific photosynthesis of plants.

Environmental conditions are also capable of causing both morphological and biochemical changes in photosynthesis. Evolution of C_4 photosynthesis is considered as an adaptation to dry, humid and hot environments, in conjunction with low atmospheric CO_2 levels (Sage 2004; Furbank 2011). Thus environmental extremes may be a way of initiating C_4-like photosynthetic traits (Ueno 1998), e.g., transformation from the C_3 mode to the CAM mode of photosynthesis occurs in some succulent plants (Edwards et al. 1985), while a transformation from C_3 to C_4, again without Kranz anatomy, has been detected in *H. verticillata* (Bowes and Salvucci 1989). The amphibious, leafless sedge, *Eleocharis vivipara*, has a Kranz anatomy and expresses a C_4-like mode of photosynthesis under terrestrial conditions, even though it expresses C_3-like photosynthesis under submerged conditions (Ueno 1998). These findings provide support for the possibility of dual photosynthetic biochemistry and evidence for the impact of environmental factors on the mode of photosynthesis.

Ueno (1998) modified the experiment mentioned in the previous paragraph to analyze the hormonal regulation of the structural and biochemical differentiation of photosynthesis in *E. vivipara*. For this study, submerged *E. vivipara* was

stressed using 5 µM abscisic acid (ABA), which induced the initiation of photosynthetic organs having C_4-like Kranz anatomy, and also, Kranz cells with many organelles (Ueno 1998). Accumulation of large quantities of PEP carboxylase, pyruvate orthophosphate dikinase, and NAD-malic enzyme was also observed at the appropriate sites of the cells, which provided rich evidence for the induced C_4 mode of photosynthesis from ABA exposure. Such results on induced C_4 photosynthesis are also supported by the findings in carbon-14 pulse and carbon-12 chase experiments (Ueno 1998). Solar radiation and temperature are another two factors governing photosynthesis, and it is therefore worthwhile to analyze their impact.

Casati et al. (2000) analyzed the impact of temperature and light on the induction of C_4 photosynthesis in *Egeria densaleaves,* a submerged plant, and observed the expression of PEPC and NADP-malic enzyme (NADP-ME) under two environmental conditions, namely low temperature and low light (LTL) and high temperature and high light (HTL). Plants grown in HTL showed higher expression and activity of both C_4 enzymes than in the plants grown in LTL (Casati et al. 2000).

Rice and wheat are critical to global food security and their leaves photosynthesize using the C_3 pathway (Still et al. 2003; Yuan 2012). It is estimated that in wheat, 10–44% of the photosynthate in grains may arise from photosynthesis in the ear, and about 33–44% from photosynthesis in the grain (Kriedemann 1966; Evans and Rawson 1970; Maydup et al. 2010). The photosynthetic pathway in cereal grains is not well defined; therefore, a fuller understanding of site-specific photosynthesis in immature grains might provide a target for enhanced grain filling.

Data indicative of site-specific differences of photosynthesis in major food crops has been observed for several decades (Nutbeam and Duffus 1976). The evidence of a mode of photosynthesis other than C_3 was observed in wheat, despite the fact that it is categorized as a typical C_3 plant (Nutbeam and Duffus 1976; Rangan et al. 2016b). Duffus and Rosie (1973) reported that there was a 50–100 times higher activity of carbon fixation in PEPC from the barley pericarp organs of developing grains, than in RuBisCO. The possibility of C_4 photosynthesis by the pericarp organs of developing grains was also suggested by Duffus and Rosie (1973), from evidence of the enzyme activity of malate dehydrogenase, malic enzyme, and pyruvate-orthophosphate dikinase. A few years later, clues for the site-specific photosynthesis in wheat were observed by Duffus and Rosie (1973). In their study, the flag leaf and developing grain of wheat showed significantly different carbon isotope discrimination values and had a strong correlation with water usage and transpiration efficiency, even though the values were not comparable with classical C_4 photosynthesis (Merah et al. 2001; Monneveux et al. 2004). However, until the findings of Rangan et al. (2016b) the concept of a C_4 mode of photosynthesis in different wheat organs was not accepted and until now remains contested.

Rangan et al. (2016b) observed the presence and expression of all the genes specific to NAD-ME subtype of C_4 in developing wheat grains, and consequently argued the possibility of a complete C_4 pathway in classical C_3 plants. Subsequent to this, Rangan et al. (2016b) reported the presence of a complete C_4 mode of photosynthesis in the developing wheat grain. This was further supported by evidence of the compartmentalization of C_4 gene expression in the pericarp, which is anatomically fitted to perform C_4 photosynthesis. The C_4 photosynthesis in the wheat pericarp may be an adaptation to heat and water stress in wheat (Rangan et al. 2016a).

One of the most significant outcomes of the study by Rangan et al. (2016b) was the optimal expression of specific C_4 genes in the pericarp during the early- to mid- period of grain filling. Despite the apparent strength of this study, Busch and Farquhar (2016) argued that in the absence of biochemical and physiological data, the evidence was insufficient to support such a conclusion. This opens an avenue for future research, specifically for a biochemical and physiological exploration of the dual biochemistry of photosynthesis in parallel with a molecular analysis.

Interestingly, site-specific photosynthesis occurs in maize (*Z. Mays*), where the foliage uses the C_4 photosynthetic pathway, while the husk surrounding the ear operates through a partial C_3 pathway (Pengelly et al. 2011; Wang et al. 2013). Although high vein density is a prominent feature of C_4 photosynthesis to facilitate efficient transport of C_4 acids from MCs to BSCs (McKown and Dengler 2007), photosynthetically active husk leaves shows a very low vein density as in C_3 plants (Langdale et al. 1989, Wang et al. 2013). Based on the presensce of Rubisco mRNA transcripts, Langdale et al. (1989) inferred that MCs distant from the vascular bundles may perform C_3 photosynthesis. Further, carbon isotope discrimination (^{12}C and ^{13}C) of foliage and husk leaves of maize showed that there was a significant CO_2 fixation in husks through C_3 photosynthesis (Yakir et al. 1991). These findings further highlight the importance of vein density and spacing for the modes of photosynthesis within the same plant.

Identification of C_4-like photosynthesis in C_3 plants and elucidation of the mechanism might be used to induce a similar photosynthetic pathway in other plant organs such as leaves (Fig. 4). This may prove simpler than engineering a complete C_4 pathway into C_3, and improve the yield of critical food crops. Understanding the impact of environmental parameters on the site-specific changes in photosynthesis may also prove to be important. Finally, whether a thorough understanding of site-specific photosynthesis has the potential to mitigate the looming food

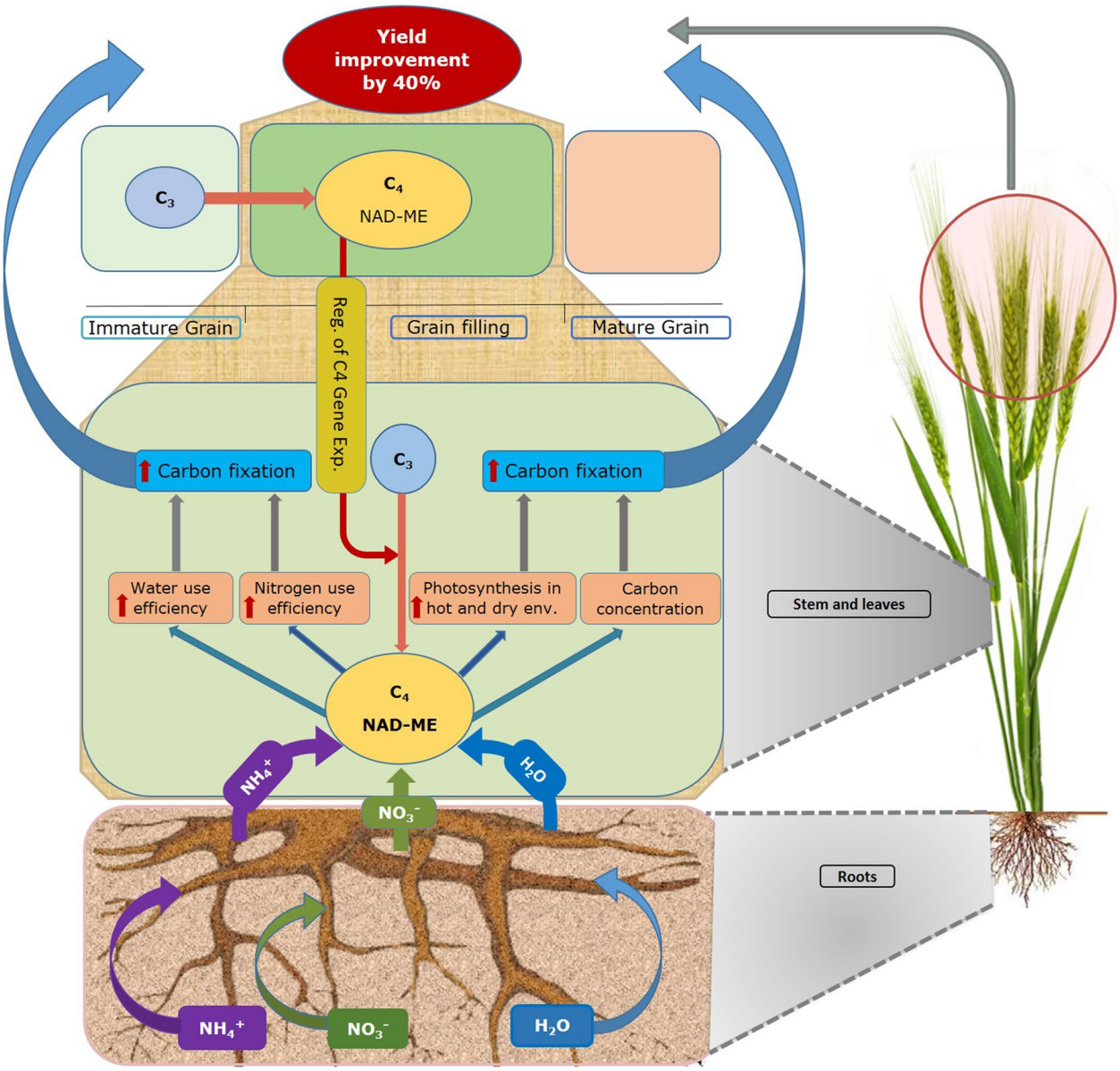

Fig. 4 Utilization of the site-specific C_4 photosynthesis in wheat to increase crop productivity. *Reg.of C_4 Gene Exp* regulation of C_4 gene expression, *env* environment. It has been hypothesized that the developing wheat grain performs NAD-ME subtype C_4 like photosynthesis during mid-grain-filling although it performs C_3 like photosynthesis during its early stages. Understanding this temporal variation of photosynthesis of wheat grain could be highly important to understand the site-specific photosynthesis in wheat. Knowledge of site-specific photosynthesis in wheat can be utilized for an expression of C_4 photosynthesis in major photosynthetic tissues such as leaves. This may facilitate increased efficiency of carbon fixation from the improvement of efficiency in the use of water and nitrogen, and by initiating CCMs. The ultimate outcome would be a yield improvement of ~ 40%

crisis is an open question because the subject has not been intensely studied, the literature is diffuse, and the practical challenges remain unknown. The findings and evidence of the dual biochemistry of photosynthesis/site-specific photosynthesis may provide a novel way of improving the crop yield of critical C_3 food crops such as rice and wheat by increasing the efficiency of photosynthesis by inducing C_4 pathway into specific organs rather than changing the entire mode of photosynthesis in the plant.

Identification of the specific photosynthetic organs which contribute appreciably to crop/grain yield, and the induction of C_4-like traits in those specific organs, could be highly important to yield improvement. Understanding the dual biochemistry of photosynthesis, or site-specific photosynthesis, may be as central to yield improvement as better understanding the contribution of the different anatomical elements of the C_4 pathway to photosynthetically efficient carbon fixation.

Importance of identifying photosynthetically efficient physiological traits to understand site-specific photosynthesis

McKown and Dengler (2007) state that Kranz anatomy had to be fully evolved before a complete C_4 carbon shuttle was initiated in most plants. However, it is possible that the efficiency of photosynthesis might be increased, albeit not to the full potential, without the complete complement of traits. For example, a correlation between higher vein density of leaves and grain yield of rice has been proposed (Feldman et al. 2017; Nawarathna et al. 2017). Below we summarize the literature on photosynthetically efficient physiological traits of C_4 that might improve the efficiency of nett carbon fixation in C_3 crop plants. By altering site-specific gene expression, there is also a possibility of transforming these traits into other photosynthetic organs of the same plant.

Increased vein density as a factor for efficient photosynthesis

To establish an efficient CCM in BSC, the distance between MCs and adjacent BSCs should be a minimum, and placement of each MC directly adjacent to at least one BSC is also important (Sage 2004). High vein density of the photosynthetic organ has a vital role in achieving this unique cell arrangement in C_4 plants (McKown and Dengler 2007). It also increases the mechanical integrity of leaves, which is a favorable trait in windy habitats, and improves the water supply of leaves in dry and hot conditions (Sage 2004). Higher vein density increases the efficiency of C_4 photosynthesis by rapid intercellular diffusion of photosynthetic metabolites between MSs and BSCs (McKown and Dengler 2009). Higher vein density has been hypothesized as an initial step in the evolution of C_4 photosynthesis (McKown and Dengler 2009). Although dense venation is observed in most C_4 leaves, studies using C_3 (*Flaveria robusta*) and C_4 species (*Flaveria bidentis*), showed no difference in major vein density, but a higher density of minor veins in the C_4 plants due to a higher initiation frequency and different patterning (McKown and Dengler 2009). According to McKown and Dengler (2009), earlier termination of MC division and reduced enlargement also cause an increase in the vein density in C_4 plants. Although the gene regulation of vein initiation is not well understood, studies on *A. thaliana* show that vein initiation from ground tissues is induced by the polar auxin flow, mediated by auxin efflux carriers (Scarpella et al. 2006; McKown and Dengler 2009). Conversion of the cells along the auxin transport pathway into procambial cells, followed by development into vascular bundles, has been observed in Arabidopsis (Scarpella et al. 2006). Even though a theoretical optimum for vein density has been suggested (Noblin et al. 2008), vein density varies widely, especially in C_3 plants, in response to the environment (Roth-Nebelsick et al. 2001). Langdale and Nelson (1991) suggested that the veins in C_4 leaves play a vital role in determining cell differentiation, especially for MCs and BSCs. Nonetheless, higher vein density is not invariably a significant character of C_4. For example, Muhaidat et al. (2007) showed that vein density does not differ between some closely related C_3 and C_4 grasses. In this study, these authors also suggested that the vein density might not be a reliable indicator of the C_3 or C_4 photosynthetic pathways in the eudicots as a whole. This is exemplified by higher vein densities in the same species grown in dry environments with low humidity and high solar irradiance, compared to wet environments with high humidity and low solar irradiance (Roth-Nebelsick et al. 2001; Zhang et al. 2012b). Nonetheless, with few exceptions, higher vein density of the photosynthetic organ appears to be an important C_4-like trait that could be the first step in the evolution of Kranz anatomy.

The potential role of MCs and BSCs arrangement in efficient photosynthesis

The increased vein density in C_4 plants is associated with an increase in the ratio between MCs and BSCs (Ku et al. 1974; Gowik and Westhoff 2011). In most C_3 photosynthetic cells, a larger number of chloroplasts is concentrated in the MCs, and MCs of the leaves contribute the most carbon assimilation (Gowik and Westhoff 2011). In contrast, MCs of the C_4 photosynthesis have fewer chloroplasts than in closely related C_3 species (Dengler and Nelson 1999; Stata et al. 2014). The combination of a higher ratio of MCs and BSCs in C_4, with lower chloroplast numbers in MCs, would appear to decrease the potential for photosynthesis in a given area. However, this is not the case, because the photosynthetically active BSCs in C_4 have more, larger chloroplasts (Black and Mollenhauer 1971; Muhaidat et al. 2011; Sage et al. 2011). Differences in the chloroplast distribution of C_3 and C_4 leaves can be seen roughly with the naked eye: the veins are colourless in C_3 and dark green in C_4 (Dengler and Nelson 1999; Maai et al. 2011). The reason for having fewer chloroplasts in MCs of C_4 plants could be that the initial site of carbon fixation is not the chloroplast, but instead, the cytosol of the MC. In contrast, since the chloroplast stroma is the site of carboxylation in C_3 species, a large number of chloroplasts is needed for efficient carboxylation in the MCs (Evans and Loreto 2000; Tholen et al. 2008). In BSCs of C_4 a larger number of mitochondria and peroxisomes are also present (Hylton et al. 1988; Gowik and Westhoff 2011). As a result of the higher number of organelles in BSCs and also because of the higher metabolic activity, the BSCs of C_4 are relatively larger than in closely related C_3 species (Hylton et al. 1988; Stata et al. 2014).

Anatomical and structural changes of MCs and BSCs in different C_4 subtypes

Anatomical and structural differences of MCs and BSCs between the three C_4 subtypes (NADP-ME, NAD-ME, PEP-CK) have also been identified (Kanai and Edwards 1999). Changes in chloroplast placement in the BSCs, grana development of the chloroplasts of BSCs and MCs, the number and the sizes of mitochondria of BSCs, and the initiation of suberin lamella within the BSCs are some of the features that differentiate the three subtypes of C_4 (Hatch et al. 1975; Yoshimura et al. 2004; Ueno et al. 2005). For example, C_4 grasses of the NADP-ME subtype show significantly higher vein density and reduced granal development of chloroplasts in BSCs, with fewer in bundle sheaths, than in the NAD-ME and PEP-CK subtypes. Most of the grasses of NADP-ME subtype also have centrifugally placed chloroplasts in BSCs (Hatch et al. 1975).

Importance of the placement and the relative volume of MCs and BSCs for efficient photosynthesis

In C_4 plants, BSCs are positioned to decrease contact with intercellular air spaces to minimize oxygenation of RuBisCo, which leads to photorespiration. According to Dengler et al. (1994), not only the BSCs in C_4 carbon shuttle, but also MCs have a lesser surface area exposed to intercellular spaces. Also, in both MCs and BSCs in C_4, the ratio between surface area and cell volume is lower than in the closely related C_3 species. The proportions of the MCs and BSCs in C_4 eudicots is also appreciably less than in their closely related C_3 counterparts (Muhaidat et al. 2007). This is partly due to thinner leaves and/or a decreased number of MC layers in the leaf (Muhaidat et al. 2007). The relative volumes of the MCs and BSCs may be significant (Hattersley 1984; Soros and Dengler 1998), because it is important to understand the role of major C_4 characteristics, such as enlarged BSCs and the short diffusion distances for C_4 metabolites between MCs and BSCs (Muhaidat et al. 2007).

Importance of plasmodesmata and their density to efficient photosynthesis

An effective connection between MCs and BSCs is needed for an efficient CCM in photosynthesis, and this connection relies on the plasmodesmata. There are extensive metabolic fluxes between MCs and BSCs through interconnecting plasmodesmata (Danila et al. 2018). Danila et al. (2018) combined scanning electron microscopy and three-dimensional immunolocalization to understand the density of plasmodesmata in C_3 *O. sativa* and C_4 *Z. mays*, revealing that the C_4 species had almost twice the number of plasmodesmata per unit area as the C_3 species. Identification of genetic components responsible for high plasmodesmatal density and engineering them into C_3 crop plants could improve their photosynthetic productivity. Moreover, understanding the differences between MCs and BSCs of both C_3 and C_4 species would seem to have important implications when studying the evolution of the C_4 carbon shuttle.

Site-specific gene regulation of C_4 photosynthesis

Site-specific changes of photosynthesis within plant species have not been sufficiently investigated (Rangan et al. 2016a, b). There may be potential to select crop plants for higher yield based on the occurrence of photosynthetically efficient physiological traits at different sites. Further, it is worth studying the possibility of transferring such traits to major photosynthetic sites such as leaf blades. For this task, a sound knowledge of the genetic regulation of photosynthesis is essential. Since C_4 is the most efficient mode, we summarize the current limited knowledge on C_4 gene regulation, because narrowing the knowledge gap about gene regulation of photosynthesis is another step in understanding site-specific photosynthesis.

The evolution of C_4 photosynthesis from C_3 precursors was a result of large-scale quantitative and spatial modifications in gene expression (Bräutigam et al. 2010). Although understanding the specific gene regulation of C_4 photosynthesis is inadequate, it is believed that it is a result of a multi-level regulation of transcriptional, post-transcriptional, post-translational and epigenetic factors (Sheen 1999; Wang et al. 2011; Fankhauser and Aubry 2016; Lovell et al. 2016; Reeves et al. 2016). Amongst these factors, transcriptional regulation of C_4 is the most extensively studied (Hibberd and Covshoff 2010; Gowik et al. 2016).

Localization of PEPC in MCs exemplifies the transcriptional regulation of C_4 photosynthesis (Taniguchi et al. 2000; Li et al. 2010). It has not yet fully confirmed whether the complete C_4 mechanism is regulated through transcriptional or post-transcriptional controls; however, expression of NADP-ME in BSCs in maize is considered the result of transcriptional regulation (Sheen and Bogorad 1987; Wang et al. 2011). Regulation of pyruvate orthophosphate dikinase, a C_4 enzyme which is required for PEP regeneration in MCs, is another example of the transcriptional regulation of C_4 photosynthesis (Hibberd and Covshoff 2010). In addition, the maize *Golden2* (*Bsd1*) gene is one of the earliest transcription factors identified through genetic studies which could play a role in the C_4 pathway (Langdale and Kidner 1994). Importance of *Golden2* like genes to transform C_3 into proto-Kranz have been reported from the experiments conducted using rice plants (Wang et al. 2017a). Proto-Kranz may have existed even before the C_2 pathway in C_4 evolution.

The C_4 mechanism with Kranz anatomy is the most efficient mode of carbon fixation. Of about 7500 C_4 species, maize is one of the more intensely studied regarding Kranz development (Wang et al. 2013). From histological and cell lineage analysis, the morphological manifestation of Kranz anatomy of maize has been well documented (Langdale et al. 1989). However, much is still unknown about the genetic regulation of Kranz development despite maize *scarecrow* gene (Wang et al. 2013). To provide a much broader insight into the gene regulation of Kranz anatomy, Wang et al. (2013) conducted a genome-wide comparative analysis using Kranz and non-Kranz organs (leaves and husk sheaths, respectively) of maize at different development stages. The analysis revealed cohorts of genes which showed much higher activity during early leaf development. Furthermore, a group of transcription factors linked with the Kranz patterning was identified. This seems to be an important finding since Kranz anatomy may be fundamental to efficient CCM in C_4, and knowledge of its genetic regulation may provide a doorway to transitioning critical C_3 food crops into C_4 (Sedelnikova et al. 2018). From an experiment conducted by Li et al. (2010) used Illumina sequencing throughout a developmental gradient and in mature MSs and BSCs to analyze the transcriptome of the maize leaf. That study identified about 180 transcription factors which were differentially expressed in MCs and BSCs. These transcriptional factors may be highly useful for future studies in functional genomics to dissect the photosynthetic pathways in leaves.

Within the constraints of the limited literature, it is assumed that the post-translational control of C_4 photosynthesis is highly interconnected with protein and enzyme modification, and that it also applies beyond the phosphorylation of PEPC and pyruvate orthophosphate dikinase (Wang et al. 2011). In addition, the results from a study conducted by Naidu et al. (2003) using *Miscanthus giganteus,* suggest that the abundance of pyruvate orthophosphate dikinase may also be regulated through protein turnover. Moving to the epigenetic control of C_4 photosynthesis, it is believed that the expression of PEPC is predominantly epigenetically regulated (Taniguchi et al. 2000; Kausch et al. 2001). Since the knowledge of specific gene regulation in both the C_3 and C_4 pathways is poor, and the rate of accumulation of knowledge in this area is not high, a novel way of addressing this problem might be needed.

The establishment of the C_4 pathway in maize takes place along the developmental axis of the leaf blade which has undifferentiated cells at the leaf base and highly specialized MCs and BSCs at the leaf tip (Majeran et al. 2010) To obtain a system level understanding of the kinetics of maize leaf development and C_4 differentiation, Majeran et al. (2010) measured the accumulation proteome profiles of the leaf along the developmental gradients using mass spectrometry. The results were supported by analyzing structural features, e.g., Kranz anatomy, cell wall, plasmodesmata, and organelles through microscopy. This study identified and functionally annotated more than 4300 proteins. These results can be considered as a well-defined molecular template that demonstrates the metabolic and structural transitions which occur during C_4 differentiation. One year later, Pick et al. (2011) reported that the C_4 pathway in maize leaves is established from sink tissues without having an intermediate phase of C_2 or C_3 channels. Further, Pick et al. (2011) concluded that the evolutionary linkages of C_4 photosynthesis were not recapitulated during C_4 differentiation in maize. Such findings indicate that development of the C_4 pathway may be more complicated than initially thought and that deeper investigation of the processes may be needed to understand C_4 gene regulation.

Understanding the concept of the site-specific photosynthesis appears to be an ideal way to fill the knowledge gap on gene regulation, both in C_3 and C_4 photosynthesis, because there is a possibility of using different organs of the same plant and/or plants grown in different environmental conditions to analyze both the C_3 and the C_4 photosynthetic models. This might complement the model plant approach, which instead uses different organs of the same plant to analyze C_3 and C_4 pathways.

Other possible approaches to improving photosynthesis

Parallel to the concept of site-specific photosynthesis, it is important to know about other possible approaches that might be utilized to improve photosynthesis. Among these are mitigation of the inefficiencies of LDR and LIR (Evans 2013; Sharwood et al. 2016; Niinemets et al. 2017).

Modifying the LDRs is less-well studied compared with the LIRs (Evans 2013). From the limited research, improvement of photosynthetic efficiency by accelerating recovery from photoprotection through genetic manipulation seems to be important as it increased both CO_2 uptake and dry matter productivity by 15% in *Nicotiana* (tobacco) (Kromdijk et al. 2016). Some other approaches that have been studied include (Fig. 1): reduction of light saturation of leaves by improving light penetration by reducing the antenna size of the photosystem (Ort et al. 2011); engineering the pigment-protein complex of higher plants to extend the waveband of sunlight that can be used for photosynthesis using cyanobacterial chlorophyll *d* and *f* (Chen and Blankenship 2011); and improvement of the electron transport capacity by increasing cytochrome *f* content (von Caemmerer and Evans 2010). Improving kinetic properties of RuBisCo (Sharwood et al. 2016) and mitigating limitations to CO_2 diffusion from the atmosphere to the sites of fixation (Tosens et al. 2016) are some of the major challenges of improving the LIR of photosynthesis and these areas also need intensive study.

Photorespiration can reduce photosynthetic productivity by 20–50% depending on the growth temperature. Hence re-engineering photorespiration seems to be a way of improving the efficiency of photosynthesis in C_3 plants and the crop yield. This was simulated by South et al. (2019) altering the photorespiratory pathway in tobacco. Interestingly, after introducing synthetic glycolate metabolic pathways, 20% quantum yield improvement and 40% more biomass productivity were observed in field-grown tobacco. On the other hand, the ratio between the consumption of carbohydrates from the whole plant (sink) and the supply of carbohydrates from leaves (source); sink–source activity has also been considered as a factor that can regulate photosynthesis (Sugiura et al. 2017). Therefore, manipulation of sink–source balance could also be used as a tool to improve the efficiency of photosynthesis at the whole plant level. In addition to these strategies of improving photosynthesis to achieve higher crop yield, it is noteworthy that recent research has highlighted the use of chemical intervention strategies to increase crop yield and resilience. For example, Griffiths et al. (2016) have demonstrated the use of chemical substances to modulate trehalose 6 phosphate (T6P) levels which play a crucial role in sucrose metabolism and sugar sensing pathways of plants. Results of this study revealed that modulation of T6P levels through chemical interventions altered crop growth and development, and thereby the potential yield.

Conclusions

A significant yield improvement of critical food crops is crucial to assure the global food security because of the increasing population and the factors related to climate change. The yield improvement of most food crops by conventional methods has plateaued, thus feeding the future world seems to be impossible without a significant leap in science. Of the many possible approaches, improving the efficiency of photosynthesis of critical food crops is seen as an essential step in addressing the foreseen risk.

Although the hypothesis of site-specific photosynthesis is novel, evidence of the phenomenon has been accumulating for decades. Direction of more attention to site-specific photosynthesis/dual biochemistry of photosynthesis stands to improve the efficiency of photosynthesis per unit area of critical food crops, thereby increasing the crop yield.

The C_4 pathway is considered the most efficient photosynthetic system; consequently, identification and/or induction of C_4 like photosynthesis in specific sites of classical C_3 plants (site-specific C_4 photosynthesis of classical C_3 plants) promises to increase the yield of classical C_3 crops, with no increase in the existing photosynthetic biomass (increase in harvest index). Understanding and optimization of site-specific photosynthesis could also be a useful intermediate step in engineering a complete C_4 pathway into C_3 plants. In addition, investigation of site-specific photosynthesis will help to fill the knowledge gap of the gene regulation of C_3 and C_4 photosynthesis. Lastly, engineering C_4-like photosynthetic pathways into the classical C_3 food crops using genome editing tools like CRISPR/Cas9 would be a novel way of addressing the ambitious goal of increasing the yield of critical C_3 food crops. This venture will probably be the foundation of the next green revolution.

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Compliance with ethical standards

Conflict of interest The authors declare that this study was conducted in the absence of any commercial relationships that could lead to any potential conflict of interest.

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