

Molecular and Genomic Tools for Improving Photosynthetic Efficiency of C_3 Species in the Changing Climates

Summary

There is an urgent need to increase crop productivity beyond the existing yield potentials to tackle the challenge of food security in the near future. One of the areas of plant physiology that promises hope in overcoming this problem is increasing photosynthetic efficiency of C_3 plants. In C_3 plants photosynthesis is limited by features of the carbon-fixing enzyme called Rubisco because it promotes a parallel reaction that captures oxygen, thereby reducing photosynthesis by initiating photorespiration. Here, we review recent information's about the potential blockages of C_3 photosynthesis in a changing climate, with attention to future targets to overcome this problem. Lastly, emerging approaches to improving C_3 photosynthetic efficiency are discussed, and recent advances in molecular and genomic techniques needed to realize these improvements are presented.

Introduction

Today seven crop species in the Poaceae family coupled with other specialty crops feed over 7 billion people in the world. But the current rate of increase in crop yield does not sufficient to feed the ever increasing population in the future. This can be explained by the fact that the actual crop yield in most areas of the globe is approaching the ceiling of maximal yielding potential. For example, yield of rice per hectare in Africa was increased only by 18% between 1979 and 1998 and only by 9% between 2007 and 2013 [1]. Similar yield stagnation is evident in other areas of the globe. Moreover, improvement in some staple food crops in Africa such as cassava and yam are even slower than the major cereal crops. For example, the global average increase in yield per hectare of yam, a major staple for West Africa, between 1979 and 2013 was 41%. This is less than half of the 108% increase for maize over the same period; which became a big worry especially for the poorest countries in the future.

The discrepancy observed especially in the first half of 21st century, makes us to review: why actual yield of a crop reach its maximum yielding potential? In the second half of the 20th century, packages such as high-yielding varieties and management techniques developed by some agricultural scientists brought about an unprecedented boom (more than double) in world grain yields. This is the so-called green revolution, one of the 20th century's greatest technological achievements. The achievement of the first green revolution is great in increasing crop yield. But it seems to be inadequate to feed the exponentially increasing global population, due to the fact that the measures adopted in the first green revolution are now no longer

important in increasing crop yield. Due to this fact one may ask: what are the possible ways of dealing with it? The realization of future global food and energy needs will require improvements in crop productivity and carbon fixation. One fundamental component of plant productivity that has yet to be utilized to potential for increasing yield is photosynthesis. Photosynthesis is the most important metabolic process relative to crop productivity, in which the maximum dry matter yield of crop plants is derived from photosynthetic CO_2 assimilation [2-5].

Improvement in photosynthetic efficiency of a crop can be possible mainly by way of DNA transfers through genetic engineering. Recombinant DNA technique is a recent technological development which provides us with the means to engineer changes to photosynthesis that would not have been possible previously. Gene editing technique as a sharp tool in improving crop photosynthetic efficiency has been reported by several earlier authors [6]. These approaches are valuable, especially as long-term adaptation strategy to alleviate climate change impacts. In this review, it has been examined the potential blockage of C_3 photosynthesis and an important part of future strategy to overcome the obstruction of C_3 photosynthesis. Lastly, the recent genetic engineering tools needed to realize these improvements are overviewed.

What are the Potential Blockages of C_3 Photosynthesis?

Photosynthesis in C_3 plants is limited by features of the carbon-fixing enzyme Rubisco, which exhibits a low turnover rate. Rubisco typically represents 50% of the soluble protein in the leaf and is the most abundant protein on the world [5,7]. It is not only the world's most abundant protein but also the world's most incompetent enzyme. Why such an abundant enzyme is incompetent? This is because Rubisco is an enzyme having two functions, catalyzing both carboxylation and oxygenation. The oxygenation reaction of Rubisco puts an extra cost on the cell in re-assimilating the carbon lost in this process. The process of oxygenation reaction induces photorespiration. Photorespiration imposes a large penalty on net



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photosynthetic efficiency, which increases with temperature [3,5]. In fact temperature has a significant effect on the solubility of both gases but the relative solubility of O₂ is greater than that of CO₂ as the temperature increases. In addition, oxygenation increases relative to carboxylation due to the fact that the reaction between the 2,3-enediol intermediate of Rubisco and O₂ has a higher free energy of activation than its reaction with CO₂. Therefore, loss due to photorespiration rises from up to 30% in cool climates to more than 50% in hot climates. Lowering photorespiration therefore would reduce the inhibition of net photosynthesis of C₃ plants. To avoid such loss, it appears that Rubisco in plants has evolved to become more specific for CO₂.

Emerging Strategies to Improving Photosynthetic Efficiency in C₃ Plants

In view of this, more recent researchers have been identified different alternative strategies to increasing photosynthetic efficiency in C₃ plants. Some of the identified targets include; engineering of a full C₄ pathway into C₃ plants, the introduction of improved Rubisco from other species and introducing carbon-concentrating mechanisms

Introducing C₄ metabolism into C₃ species

Carbon dioxide is a competitive inhibitor of the oxygenation reaction of Rubisco. Evolution has exploited this in some photosynthetic organisms by the addition of structures to compartmentalize Rubisco and pathways that concentrate CO₂ in that compartment. C₄ photosynthesis is one solution that has a carbon concentrating mechanism involving two cell types, the mesophyll and bundle sheath cells [5,8]. In C₄ plants, which include the crops maize, sorghum, sugarcane, and grain amaranth, Rubisco is isolated to an inner green bundle sheath surrounding the leaf veins. In these plants, carbon dioxide is first captured by carboxylation of phosphoenolpyruvate (PEP) to form a C₄ dicarboxylate in an outer photosynthetic tissue or mesophyll and is then transferred to the inner tissue that it surrounds the bundle sheath. Here, it is decarboxylated, releasing pyruvate that is then recycled back to the outer tissue, where it is phosphorylated back to PEP to complete the cycle [5].

Indeed, one approach to increasing photosynthetic efficiency in C₃ crops such as wheat and rice is to convert them to C₄ plants. But it needs various structural and functional changes. The first manipulation that is necessary is the structural change. This includes the differentiation of the two cell types, increases in bundle sheath size and in their complement of organelles, such as chloroplasts, mitochondria and peroxisomes. Typically, a C₃ leaf might have 9-10 mesophyll cells between adjacent bundle sheath cells, whereas in C₄ leaves, this reduces to 2-3 mesophyll cells, so that mesophyll and bundle sheath cells are in close proximity [8]. The second manipulation that is necessary is functional change. This involves expressing proteins, whether they are enzymes or transporters, in different cells and organelles, in different amounts and certainly, in some cases, isoforms that are more suited to their role in C₄ rather than C₃ photosynthesis, such as PEP carboxylase.

With genetic engineering, ability to transform plants at the nuclear and plastid (chloroplast) level opened up a new world of possibilities for novel C₄ crops [9,10]. The main method has been to target PEP carboxylase and over-express this enzyme by genetic engineering in

tobacco, potato, rice and *Arabidopsis*. In most of these endeavors, the maize version of PEP carboxylase was used, and its gene was driven with a 35-S promoter, so the enzyme was produced constantly. Effects varied: more efficient use of CO₂, excess malate production, less oxygen inhibition of photosynthesis, improved dark respiration, a neutral state of no effect, negative effects such as chlorosis, less sugars and more acids, reduced phosphorylated metabolites, and even stunted growth. The enzyme NADP-malic enzyme has also been over expressed to remove the malate bottle neck and it did improve efficiency. When both enzymes together have been over expressed in the same cell, results were promising in one species and neutral (no improved photosynthesis) in another. PEP carboxykinase (PCK) from the C₄ *Urochloa* has also been expressed in rice, with and without PEP carboxylase, but overall photosynthesis has not been higher. Most attempts to shift C₄ have used two-cell systems, meaning classical C₄ fixation which is split in space in mesophyll and bundle sheath cells [11]. A single-cell version of C₄ metabolism may produce a functioning C₄ pathway in C₃ plants, but using four enzymes (PEP carboxylase, pyruvate orthophosphate dikinase, NADP-malate dehydrogenase, NADP-malic enzyme) has not yet produced greater plant growth [9,10,12].

Since most of the C₃ plants lack the typical Kranz features, they possess a sub cellular separation that enables a concentrating of CO₂ at the active site of Rubisco. The genes involved in the development of this peculiar sub cellular anatomy are unknown [13]. 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. Today, the coexistence of C₃ and C₄ plants in the same environment brought up a controversial debate among agricultural scientists. For instance, rice is a C₃ plant grown in warm climates where the C₄ pathway should be superior. Using this argument, different researchers challenged the scientific community to consider the consequences of engineering the C₄ pathway into rice.

A major effort is underway to achieve this in rice; however, it requires many changes in both anatomy and inserting and expressing the genes of C₄ photosynthesis. In this regard, most of the earlier endeavors have got some sort of challenges. The first challenge is in choosing which type of C₄ to shift, with its several pathways and enzymes involved so photorespiration is taken into account. The key enzymes like PEP carboxylase and Rubisco are large and complex with numerous subunits, they are repeatedly turned over in the cell, and they require numerous other proteins to assemble them. This means those enzymes plus their assembly mechanisms all need genes encoded into the DNA. And not all the DNA is nuclear; some of this is chloroplastic DNA, although the control of everything is inherited from nuclear DNA. The second difficulty is ensuring sufficient C₄ activity in the absence C₄ anatomical features. The third difficulty is placing the required C₄ enzymes where they should be functioning in a target cell compartment, and with the original C₃ enzymes elsewhere in the cell now switched off. For greatest efficiency, no Rubisco should be present or active in the same cells containing PEP carboxylase. Nevertheless, the fact that this process has successfully and independently achievable, although it will require further understanding of the genetic basis of the dimorphic photosynthetic tissue and localization of components of the C₄ and Calvin cycles. Cognizant of the difficulty described above, recent works are focused

on the following two novel approaches.

Introducing improved Rubisco from other species into plants

One means of alleviating the deficiencies of Rubisco is to introduce improved Rubisco from other species into plants. For example, thermophilic red algae have a Rubisco that has an improved specificity factor [8]. Increasing photosynthetic efficiency in C₃ plants through introduction of improved Rubisco specificity factor from other species was also reviewed by Long et al. and Singh et al. [3,5]. A further opportunity to address the cost of photorespiration is to engineer a more efficient pathway for metabolism of the first product of the oxygenase reaction, phosphoglycolate. Plants and green algae use a single energy-consuming and protracted pathway involving the chloroplast, peroxisome, and mitochondrion, with the release of both carbon dioxide and ammonia in order to recover PGA that is then re-assimilated into the Calvin cycle.

The preceding authors summarized three different approaches for manipulating photorespiration in C₃ plants. The first pathway has been engineered using *Escherichia coli* encoded genes from the glycerate pathway that convert glycolate to glycerate and release CO₂ within the chloroplast. This pathway bypass photorespiration by 32% and plants engineered with this pathway resulted in reduced photorespiration and enhanced photosynthesis [3]. In the second approach, transgenic *Arabidopsis* plants engineered with a pathway to fully oxidise glycolate to CO₂ in the chloroplasts. This pathway is found to be beneficial for plants and can enhance photosynthesis. Both bypasses should avoid ammonia release in the mitochondria, which is quite expensive to re-assimilate in terms of the ATP and reducing equivalents required [13]. 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. While this alternative pathway may potentially reduce the cost of 2PG recycling

In recent reports, the potential of photorespiratory bypasses for the improvement of plants photosynthetic efficiency has been demonstrated. It was shown that introduction of the first bypass in the oilseed crop *Camelina sativa* greatly increased seed yield, which may be used for the production of biofuels [14]. Another photorespiratory bypass in potato was reported by Betti et al. [13]. They reported that potato (*Solanum tuberosum*) plants were transformed with the three genes that encode for *E. coli* glycolate dehydrogenase subunits and the corresponding poly-protein was successfully expressed in the chloroplast, where it was able to catalyze the conversion of glycolate to glyoxylate.

Direct insertion of inorganic carbon transporters into leaf cells

The other alternative approach is direct insertions of bicarbonate and carbon dioxide pumps to the chloroplast membrane and production of carboxysomes and pyrenoids within the chloroplast.

Carboxysomes and pyrenoids are structures within cyanobacteria and algae that provide a compartment that facilitates the concentration of CO₂ around Rubisco, thereby reducing photorespiration [15]. Cyanobacteria suppress the oxygenating reaction of Rubisco by concentrating CO₂ inside a proteinaceous microcompartment

called carboxysomes. The carboxysome is a protein micro body in cyanobacteria, comprising a protein coat, and an interior soluble protein phase containing most, possibly all, of the cellular Rubisco [8]. Cyanobacteria, the ancestors of modern day crop chloroplasts, use a different method of concentrating CO₂ at Rubisco. These prokaryotes are actively up taking bicarbonate into their cells. Within the cells, both Rubisco and carbonic anhydrase are localized within isoanhydral protein shell bodies termed carboxysomes. Here, carbonic anhydrase catalyzes the formation of CO₂, serving to concentrate CO₂ around Rubisco to a sufficient level to minimize oxygenation and photorespiration.

To realize the full potential of these bicarbonate pumps, carboxysomes would need to be introduced into chloroplasts to enable CO₂ to be concentrated around Rubisco [15]. The feasibility of introducing carboxysomes in to higher plants was boosted by Betti et al. [13]. They reported that a functional cyanobacterial rubisco was successfully introduced in to tobacco chloroplast together with an internal carboxysomal protein [16]. Similar study was reported by Hay and he reported that soybean transgenic lines expressing *ictB* gene exhibited large increase in photosynthesis and productivity at ambient as well as elevated CO₂ environments under both green house and field agricultural condition [17]. Recent developments in chloroplast transformation technology and the successful assembly of a functional carboxysome in *E. coli* have lined the foundations for synergizing a functional carbon concentrating mechanism in higher plants [18]. Many eukaryotic algae also include inorganic carbon concentrating mechanisms to suppress the oxygenase activity of Rubisco and photorespiration. These also require bicarbonate transporters in the cell and chloroplast membrane. Within the chloroplasts of these algae, Rubisco concentrates in a region, typically surrounded by starch, and is termed the pyrenoid [5]. Pyrenoids play a major role in global aquatic CO₂ fixation. However, their mode of operation is much less well understood than that of carboxysomes.

New Techniques or Tools Used for Genetic Engineering

Tools that would allow insertion of constructs at the same point in the genome, a point that does not interfere with expression of other genes, would decrease variability between events and increase comparability of transformations with different constructs [5]. Genetic engineering involves changing the genetic material of organisms using artificial laboratory techniques. Changing the genes of a plant can change its properties or traits e.g. how it responds to disease, pesticide products or lack of water. More recently, many new molecular and genomic tools have been developed to change the DNA of plants [19]. Collectively many of these new genetic engineering techniques are referred to as “gene editing” techniques. Gene editing technique such as regularly interspaced short palindromic repeats system (CRISPR/Cas), zinc Finger Nucleases (ZFN) types -1, -2 and -3, transcription activator-like effector nucleases (TALENs), Meganucleases (MN) and Oligonucleotide Directed Mutagenesis (ODM) are widely used in engineering of microbial genome [20]. This provides a means to achieve directed insertions in crops either by deleting, substituting or adding DNA sequences in pre-defined locations. But this has only been achieved in a few species such as herbicide tolerant oilseed rape and male sterile trees. Other new genetic engineering techniques: Cisgenesis and intragenesis; RNA-

dependent DNA methylation (RdDM); Grafting of non-GMO graft (scion) on GMO rootstock (and vice versa); Reverse breeding (RB) and Agro-infiltration are also widely applicable. Cisgenesis and intragenesis involve insert of DNA sequences derived from the same or a crossable species. So far it is applicable in disease resistant apple and potato. RNA-dependent DNA methylation (RdDM) provides a change in the properties of plants through silencing specific genes in a way that will usually disappear after several generations. For example it delayed tomato ripening and insecticide production in potatoes. The remaining technological advances would also allow successful reconstruction of parts of the plant genome.

Conclusion

Increasing the efficiency of photosynthesis could increase productivity and this is a key research target in the first half of 21st century. In view of this, improvement of the enzyme Rubisco is the main focus of cutting age technology. Photosynthesis in C₃ plants is limited by features of the carbon-fixing enzyme Rubisco, which exhibits a low turnover rate and can react with O₂ instead of CO₂, leading to photorespiration. However, recent technological advance now allow higher plant Rubisco to be engineered and assembled successfully through different approach. Some of the most novel approaches to improve photosynthetic efficiency include; the introduction of improved Rubisco from other species, introducing carbon-concentrating mechanisms and engineering of a full C₄ pathway into C₃ plants. A simpler approach is to transfer bicarbonate transporters from cyanobacteria into chloroplasts of plants. This is because, in cyanobacteria, the carboxysomes improve the efficiency photosynthesis by concentrating CO₂ near the enzyme Rubisco. It has been realized that gene editing technique is a sharp tool in improving crop photosynthetic efficiency in the changing climate.

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