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C₄ rice: Gateway to the second green revolution

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Abstract

Rice is a staple food crop for more than 50% of world population. The demand for food grain is increasing day by day with the population growth across the globe. Production of cereals and other food grains accelerated with the onset of green revolution. By 2050 there would be a demand of 1310 million tonnes of rice and such a huge target could not be achieved with the first green revolution. So there is a call for increasing the production manifold which can be made possible through installing the C₄ photosynthetic pathway into C₃ crops like rice. C₄ photosynthesis is much more efficient than the C₃ system due to its carbon dioxide tunneling system and negligible photorespiration. Crops with an enhanced photosynthetic mechanism would better utilize the solar radiation that can be translated into yield. This review throws light on the basic biochemistry and physiology for which C₃ differs from C₄ and provides a summary of the several approaches for C₄ rice engineering so that the pathway can be introduced successfully.

Keywords: Green revolution, carbon dioxide tunneling, photorespiration, photosynthesis, C₃ and C₄ system, C₄ rice engineering

Introduction

Rice (*Oryza sativa*) a member of the grass family is the most important crop globally in terms of the number of people who depend upon it as the primary source of nutrition. It serves as the staple food source for people in East and South-East Asia. The green revolution started in 1960s led to rapid increase in rice grain production but it has been realized that the gain from the revolution have already exhausted. Like most crop species rice carries out C₃ photosynthesis but the highest yielding crops are often those which carry out C₄ photosynthesis such as corn, sorghum and sugarcane. A proposal was made to introduce C₄ photosynthetic machinery into C₃ plants such as rice, which could enhance the yield by 50-60%, with a simultaneous increase in nitrogen and water use efficiencies (Langdale, 2011; Hibberd *et al.*, 2008) [6, 8-11, 26, 31, 43-44]. Elevated partial pressure of carbon dioxide (CO₂) at the site of ribulose biphosphate carboxylase oxygenase (RuBisCO) in the bundle sheath cells is reported in the case of plants carrying out C₄ pathway. C₄ photosynthesis has evolved independently more than 60 times, providing one of the most effective solutions for overcoming the catalytic inefficiency of RuBisCO (Sage *et al.*, 2012; Christin and Osborne, 2013) [3, 19, 33-35]. By virtue of their superior nitrogen and water use efficiency, transfer of C₄ photosynthetic traits has been proved to be an efficient strategy for improving C₃ photosynthesis (Sage, 2004; Miyao *et al.*, 2011) [19, 21-22, 33-35]. Use of transformation technology paved the way for introducing C₄ photosynthetic genes into C₃ species (Hausler *et al.*, 2002; Miyao, 2003) [7, 21, 22].

Fundamentals of C₃ and C₄ photosynthesis

In general C₃ cycle the carbon dioxide combines with Ribulose 1, 5-Bisphosphate (RuBP) in the presence of an enzyme RuBisCO and thus fixed into a three-carbon compound known as 3-phosphoglycerate (3PGA) which further yields a molecule of sugar accompanied by regeneration the RuBP (Bassham *et al.*, 1956) [1]. This is accomplished by a battery of enzymes which utilize the ATP and reducing equivalents (NADPH) generated earlier using sunlight. Under high temperature or high light intensity, RuBisCO develops more affinity towards oxygenase activity. This phenomenon of increased respiratory activity called photorespiration leads to loss of carbon which is the fundamental reason behind the lower photosynthetic efficiency in C₃ plants.

C_4 plants carry out carbon fixation in two morphologically distinct cell types, namely the mesophyll cells (M cell) and bundle sheath cells (BS cell) (Figure 1). In C_4 plants phosphoenol pyruvate (PEP) is primary acceptor of CO_2 which upon fixation yields in a $4C$ organic acid oxalo acetic acid (OAA) catalyzed by PEP carboxylase (PEPC). OAA is then transferred to BS cell where it is decarboxylated to produce a $3C$ compound (pyruvate) and a molecule of carbon dioxide which is again fixed through the C_3 pathway. This process recurs to build up a high gradient of CO_2 near RuBisCO site in the bundle sheath cell and hence arresting oxygenase activity of the enzyme bringing photorespiration to negligible levels. Though both cell types are arranged in a wreath like structure for better contact with each other, still C_4 system needs a set of complex transporters and high energy to carry out photosynthesis efficiently (Langdale, 2011) [6, 11, 26, 31, 43].

Three different biochemical subtypes of C_4 pathway have been identified based upon differences in subcellular localization, transported acids and C_4 acid decarboxylase used. All the three subtypes of the C_4 pathway yield OAA as the primary fixation product. In NADP-Malic enzyme (ME) type, OAA is converted to malate and the decarboxylase used is NADP-ME. The C_4 acid decarboxylase used in NADP-

Malic enzyme (ME) type is NAD-ME and PEP-CK in the PEP-CK type. This classification is not considered as a robust one as because PEP-CK activity has also been reported in crop like maize which is an example of NADP-ME type plant (Furbank, 2011) [5]. NADP-ME pathway has been considered for engineering C_4 rice (Figure 2).

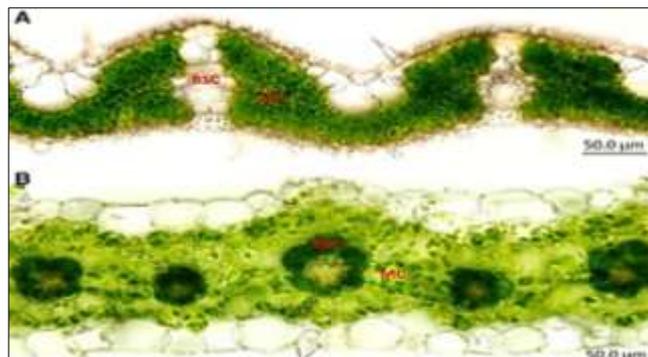


Fig 1: Anatomical differences between C_3 and C_4 leaves. (A) C_3 (*Oryza sativa*) and (B) C_4 (*Setaria viridis*) leaf. MC: Mesophyll cell and BSC: Bundle sheath cell (Source: Karki *et al.*, 2013) [14, 30]

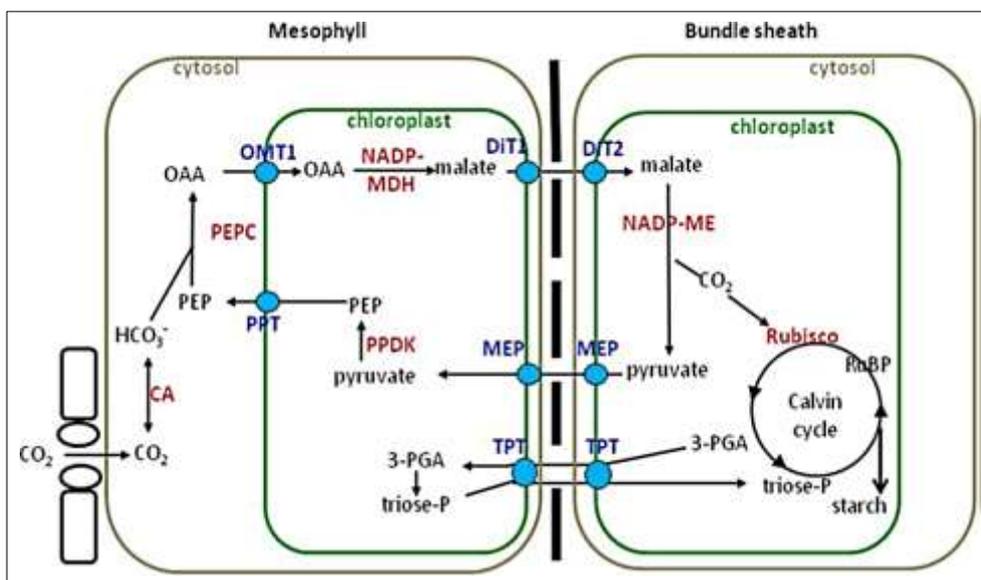


Fig 2: Schematic diagram of NADP-ME C_4 pathway (Source: Rizal *et al.*, 2012) [14, 30]

What makes C_4 plants metabolically superior?

The higher photosynthetic efficiency of the C_4 plants could be attributed to their carbon dioxide concentrating mechanism which is based on arrangement of the mesophyll and the bundle sheath cells and the compartmentalization of enzymes into these two different cell types. Reduced photorespiration adds to the higher efficiency of these plants by reducing loss of carbon as well as energy. Biochemically this might be achieved either by phosphorylation of conserved serine residue in N-terminal of PEPC or by the PPDK regulatory protein (a kind of phosphatase) catalyzing ADP dependent inactivation of PPDK (Yokota and Shigeoka, 2008; Tiwari *et al.*, 2005) [39, 46]. However it would be wrong if we say C_4 plants entirely eliminate photorespiration. Maize mutants deficient for glycolate oxidase (a key enzyme in photorespiration) are seedling lethal at normal carbon dioxide concentration and are able to survive only at carbon dioxide levels that are inhibitory to photorespiration (Zelitch *et al.*, 2009) [47]. This indicates that early stages of pathway are functional in mutant and buildup of glycolate is toxic.

Biochemical basis of C_4 photosynthesis

The major biochemical difference between C_3 and C_4 photosynthesis is the CO_2 concentrating mechanism found in the C_4 photosynthesis which increases the CO_2 concentration at the RuBisCO site. As a result of the CO_2 concentrating mechanism photorespiration is reduced and net CO_2 fixation rate increases. Photorespiration otherwise known as C_2 cycle is an ATP consuming process which usually operates in the C_3 plants relies upon the collaboration of two specialized cell types, i.e. the bundle sheath (BS) and the mesophyll (M) cells. The concentration of RuBisCO is high in the bundle sheath cells; whereas in mesophyll cells, both the concentration of PEP carboxylase (PEPC) as well as PSII and PSI activities are maintained at high levels (Sage, 2004) [19, 33-35]. C_4 plants have also evolved an efficient metabolite transportation system between the two cell types (Leegood, 1999) [19, 24]. Furthermore, the cell wall of bundle sheath cells are thickened to form a Kranz structure popularly known as "Kranz anatomy" (Sage, 2004) [19, 33-35] (Figure 3).

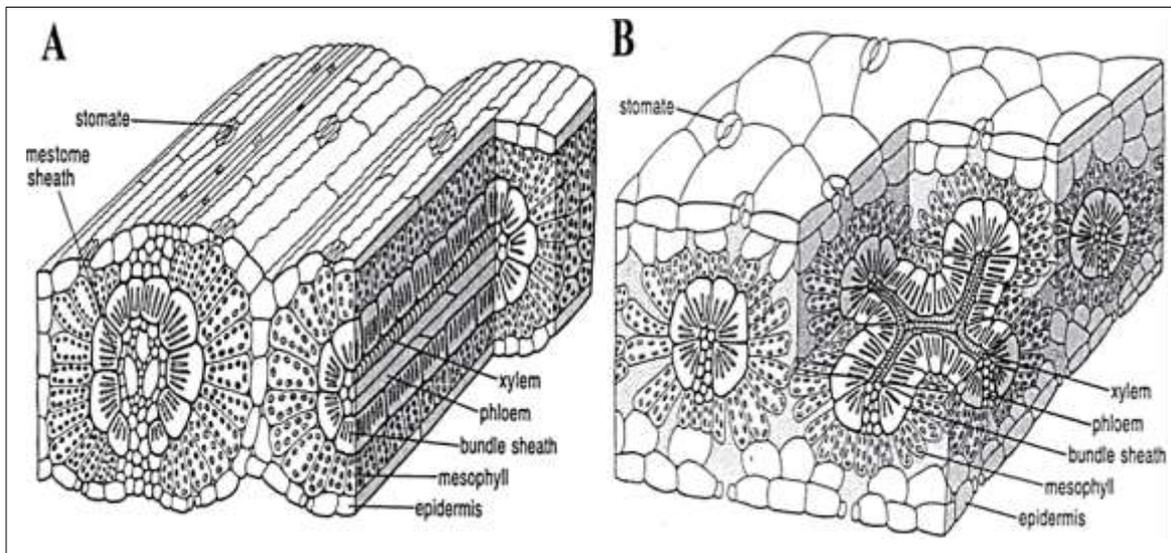


Fig 3: Diagrams of classical forms of C₄ Kranz anatomy drawn from (A) C₄ grass (*Panicum capillare*) and (B) C₄ eudicot *Atriplex rosea* (Source: Sage *et al.*, 2014) [19, 33-35]

The monsoon family of models for C₄ evolution

The evidence from the many C₄ lineages consistently supports gradual models of C₄ origin, with a critical intermediate role for a glycine shuttle that concentrates photorespired CO₂ into a BS-like compartment (Monson and Rawsthorne, 2000; Sage *et al.*, 2012; Williams *et al.*, 2013) [19, 23-25, 28, 29, 33-35]. The glycine shuttle CCM was first proposed by Monson *et al.*, (1984) [19, 23-25] to explain the photosynthetic physiology of the C₃-C₄ intermediate species known at that time (Rawsthorne *et*

al., 1988; Rawsthorne, 1992) [24, 28-29]. For easy understanding, the model is presented as a flow diagram that describes the transition from C₃ to C₄ through a series of intermediate phases which these correspond to known physiological states in existing lineages. Three distinct intermediate phases are delineated named (i) proto-Kranz, (ii) C₂ photosynthesis or the photorespiratory glycine shuttle, and (iii) C₄-like photosynthesis (Figure 4).

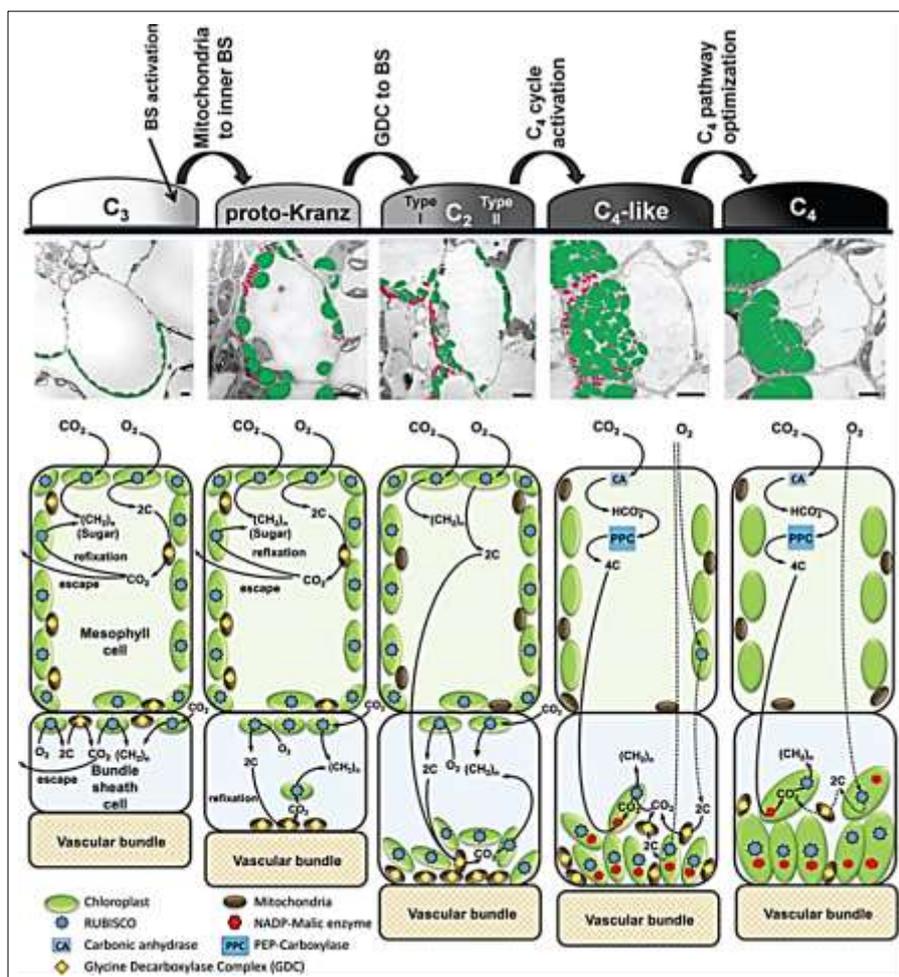


Fig 4: A diagram illustrating the evolutionary progression from C₃ to C₄ photosynthesis via three distinct phases termed proto-Kranz, C₂ photosynthesis (C₂), and C₄-like photosynthesis (Source: Sage *et al.*, 2014) [19, 33-35]

Engineering C₄ machinery into C₃ crops is feasible

C₄ enzymes have their counterparts in C₃ plants. Roots of C₃ plants show significant thickening and suberisation of cell walls in the endodermis which resembles the cell wall in bundle sheath cells (Hibberd, 2007) [6, 8-11, 26, 31, 43-44]. Production of four carbon compounds and allied carbon fixation reactions observed in C₄ plants are already used within guard cells (Tallman, 2004) [37]. Thus practically C₃ plants possess the basic biochemical and anatomical elements required to construct the C₄ photosynthetic machinery. Characteristics of C₄ photosynthesis was reported in cells around the vascular tissues in tobacco plant (Brown *et al.*, 2010; Hibberd and Quick, 2002) [6, 8-11, 26, 31, 43-44, 47]. The most convincing evidence supporting feasibility of C₄ rice engineering is the transition between C₃ and C₄ photosynthesis reported in *Eleocharis vivipara* in different environmental conditions (Ueno, 1996; Ueno *et al.*, 1988) [40, 41]. *Eleocharis vivipara* develops C₃ anatomy in submerged leaves and C₄ anatomy in aerial leaves. Transition of *Eleocharis vivipara* into C₄ can be induced by the use of ABA. *Flavaria brownii* plants grown in higher light intensities are more C₄ like than those grown in lower light intensities (Monson *et al.*, 1987) [19, 23-25]. Vicentini *et al.*, (2008) [42] found a cluster of C₄ origin in the Mid-Miocene was associated with an increase in temperature, suggesting the relative ease of the C₃ to C₄ transition once this type of photosynthesis conferred competitive advantage over the C₃ type. C₃ system of photosynthesis operates as a default system prior to appearance of mature Kranz anatomy (Miranda *et al.*, 1981; Dengler and Dengler, 1990) [4, 20]. All these above cited evidences suggest that understanding basic mechanisms regulating expression of key genes involved in C₄ photosynthesis is a critical step towards C₄ rice engineering. By linking biological processes at different scales, facilitating the study of development of C₄ photosynthesis, and helping design the future C₄ rice ideal-type, systems biology will play a critical role to bridge scientists from diverse disciplines (Zhu *et al.*, 2010) [38, 48].

Engineering C₄ rice

Several approaches such as mutagenesis, OMICs approach and epigenetic approaches are employed for engineering C₄ rice. Mutagenesis and rice DNA activation tagging together account for both loss of function and gain of function mutants. Loss of function mutations are induced in order to randomly manipulate some of the C₄ traits and then the underlying genetic factors are analyzed. C₄ plants like *Sorghum bicolor* and *Setaria viridis* are the plants of choice for such studies. Gain of function mutants for C₄ rice is being created through DNA activation tagging which facilitates identification of gene location and the phenotype of the mutant reveals function of gene. Retrotransposons Tos 17 (Hirochika, 2001) [12], maize Ac/Ds elements (Kolesnik *et al.*, 2004; Qu *et al.*, 2008) [16] and full length cDNA over expressor gene (FOX) hunting systems have been exploited to generate a number of rice mutant resources. Gene expression profiling is required to unravel the next level of correlation between the gene and associated phenotype. Comparative transcriptome analysis can reveal the exact number of genes differentially expressing between C₃ and C₄ plants. Putative regulatory genes and conserved cis elements that cause cell specific expression of C₄ proteins may be identified by overlaying metabolic profiles with gene expression pattern along developing leaf gradient (Wang *et al.*, 2016) [43, 48]. Along with cis acting DNA elements, chromatin configuration also has command over C₄ differentiation and associated gene

regulation. In maize, expression of PEP carboxylase has been linked to histone modification, epigenetic control and chromatin remodeling. miRNAs (micro RNAs) also reported to play an important role in vascular differentiation (Janes-Rhoades *et al.*, 2006; Rubio-Somoza and Weigel, 2011) [13, 32]. On this basis potential role of miRNAs in Kranz anatomy development is being explored (Thakur *et al.*, 2011) [30, 38].

Systems biology research for C₄ rice engineering

Identifying the key regulatory elements such as transcription factors and mi RNAs, controlling C₄ photosynthesis is one of the challenging tasks in C₄ rice research. Though many cis-regulatory elements in the promoter regions of C₄ photosynthesis genes have been identified (Sheen, 1999; Hibberd and Covshoff, 2010) [6, 8-11, 26, 31, 43-44], but only a limited number of the transcription factors binding to these cis-regulatory elements were identified. Few identified transcription factors are GLK1 and GLK2 genes in the MYB family (Hall *et al.*, 1998; Rossini *et al.*, 2001) [6, 31], HY5 (Lee *et al.*, 2007) [18], and DOF family proteins (Yanagisawa and Sheen, 1998) [45]. All the newly identified transcription factors or mi RNAs need to be tested through transgenic experiment to finally confirm their biological function. Developing and using system models of photosynthesis can help identify desired designs for a C₄ rice. From the success of the photo-respiratory bypass in enhancing photosynthetic efficiency it was concluded that higher rate of photosynthesis can be gained by implementation of new pathways into existing metabolism (Kebeish *et al.*, 2007) [15]. Following Nelson and Langdale (1992) [6, 11, 26, 31, 43], potential mechanisms controlling C₄ development are shown in a diagram as shown in Figure 5B. In brief, under light, an unidentified signal molecule diffuses from veins into BS and M cells creating a concentration gradient. Some unknown mechanisms are possibly behind the scene which lead to the differential expression of genes controlling C₄ photosynthesis (Figure 5A). In the present scenario of C₄ rice research, there is an urgent demand for elucidation of the molecular mechanisms causing the cascade of events leading to differential expression of C₄ genes.

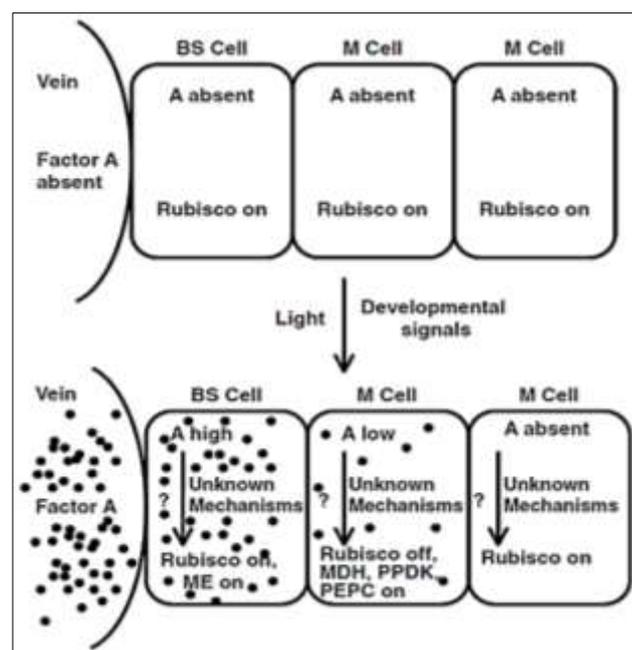


Fig 5a: A theoretical framework of the potential mechanisms underlying development of C₄ photosynthesis. BS, bundle sheath cell; M, mesophyll cell; A, an unidentified signal. (Source: Zhu *et al.*, 2010) [38, 48]

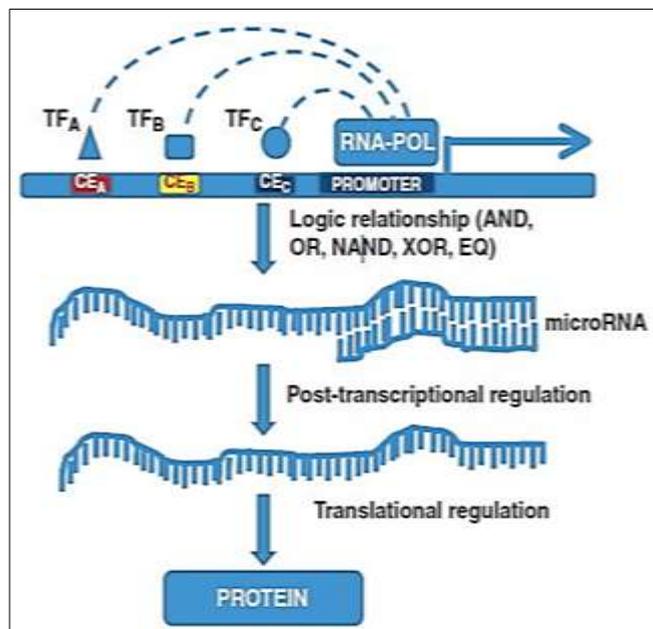


Fig 5b: Potential mechanisms controlling differential expression of photosynthetic genes. Various transcription factors (TFA, TFB, TFC) can potentially interact with various cis-elements (CEA, CEB, CEC) and RNA polymerase to determine the on or off status of gene expression following logic rules (AND, OR, NAND, XOR and EQ).

The mRNA abundance is further regulated through post-transcriptional regulatory processes including regulation through micro RNAs. Different photosynthetic genes or the same gene in different species might use different regulatory mechanisms to achieve differential expression. (Source: Zhu *et al.*, 2010) ^[38, 48]

Conclusion

Engineering the C₄ photosynthetic pathway into C₃ plants has the potential to dramatically increase the yields of major C₃ crops and thus plays a pivotal role in ensuring global food security in future. The C₄ photosynthetic pathway has evolved more than 66 times in several species, indicating that C₃ plants may be in some way preconditioned to C₄ photosynthesis. With a detailed genome sequencing data and large amounts of physiological, genetic and genomic knowledge, rice may be considered as an ideal crop to practice C₄ engineering. C₄ rice research will not only enrich us with basic understanding of molecular mechanisms of C₄ development but also develop many biotechnological approaches like engineering multiple genes into acceptor plants. Therefore, the C₄ rice project will have far-reaching impact on the twenty first century agriculture.

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