

Review Article

<https://doi.org/10.20546/ijcmas.2017.612.131>

C4 Rice - Tweaking Rice Physiology for Second Green Revolution

Sudershan Mishra^{1*}, Mithilesh Kumar Singh², Sikha Snehal³ and Himanshu Pathak⁴

¹M.Sc.Ag. (ICAR-JRF), Department of Plant Physiology, CBSH, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India

²Central Seed Testing Laboratory, National Seed Research and Training Center, Varanasi, India

³M.Sc.Ag. (Biotechnology), Department of Molecular Biology and Genetic Engineering, CBSH, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India

⁴M.Sc. (Biochemistry), Department of Biochemistry, CBSH, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India

**Corresponding author*

ABSTRACT

Rice is a staple food crop for more than 50% of world's population. The success of green revolution that began in 1960's led to a tenfold increase in rice yield but it is clear now, that the gains from the grains of the previous green revolution have exhausted. By 2050 there would be 1309 million tonnes demand of rice and C3 rice even when best managed can yield only 915 million tonnes. Increasing radiation use efficiency (RUE), water use efficiency (WUE) and nutrient use efficiency (NUE) are the contemporary approaches being tested on a wider scale. C4 type photosynthesis due to its carbon dioxide tunneling system and negligible photorespiration is much more efficient than the C3 system even at tropical temperatures where rice is generally grown. Even though engineering C4 rice requires syndromic large scale tweaking in physiology, advances in genome wide deep sequencing and genome editing platforms have brought the possibility of making C4 rice closer than ever before. A selected group of C4 genes have been inserted into rice through mutagenesis and hybridization and their effects recorded in transgenics upto 3 generations. The compartmentalized overexpression of key C4 genes using Rice DNA activation tagging constitutes another approach towards C4 rice. Since C4 plants have evolved independently multiple times from C3 origins, it is being investigated whether the key genes and gene regulatory networks that regulate C4 plants have been recruited from C3 ancestors. To facilitate this, comparative transcriptomes analysis of C3 vs. C4 leaves and other C3 and C4 tissues has been done and thus the exact number of genes differentially expressing between C3 and C4 can now be calculated. The high throughput OMIC data thus generated is cross referenced with whole genome databases and this has yielded sufficient number of candidate genes for bundle sheath specific expression. Fox hunting systems and Tos 17 transposable systems have also yielded a set of interesting mutants in this regard. Identification of mutants through DHPLC and TILLING are used to track down whole genome duplication events in the evolution towards C4 rice. While the discovery of cis acting sequences in C3 to C4 transition is a favorable advance, our further studies are limited by the poor resolution of transcript profiles and epigenetic signatures. Availability of only a few models of in silico studies about performance of C4 rice under dense crop canopies is another limitation.

Keywords

C4 Rice, Second Green Revolution, Gene discovery, Rice yield.

Article Info

Accepted:
10 October 2017
Available Online:
10 December 2017

Introduction

The Twin Tale

The latter part of 1960s marked two very important events that continue to have a recurrent bearing on the global food security. One of the two was characterized by use of short statured high yielding varieties and was popularly summarized as green revolution, as a result of which rice yields enhanced from a mere 1.5 ton ha⁻¹ to the contemporary yield rate of 8-10 ton ha⁻¹(31)(33). Around the same time a whole new field of discovery was initiated by the finding that instead of fixing carbon dioxide into a 3 carbon (3C) compound some plants proximally fix carbon dioxide into a 4 carbon (4C) compound, subsequently decarboxylated and then refixed into a 3C compound⁽²¹⁾⁽²²⁾. In the decades that followed much of the C₄ associated morphology and biochemistry was unraveled. However virtually nothing was known about the genetic patterns that underpinned this biochemistry. Gradually with time, development in both the fields stagnated. While on one hand green revolution cultivars reached a yield barrier, on the other hand in absence of advanced genetic tools most C₄ models started to display recalcitrance towards genetic manipulation.

However over the latter half of the last decade there has been resurgence regarding high yield requirements and hopes are being laid on C₄ systems. By 2050 the global population will reach 9 billion⁽⁴⁹⁾ and yield enhancements of the order of 50-60% are required. Since photosynthesis underpins agricultural productivity one of the alternatives to break the yield barriers could be the introduction of naturally selected better variant of photosynthesis *i.e.* C₄ pathway into contemporary rice cultivars⁽⁶³⁾. C₄ pathway with its enhanced carbon tunneling and concomitant increase in nitrogen and water

use efficiencies may provide the scale of yield enhancement required⁽²⁵⁾.

Estimating the effects of installing C₄ chassis for C₃ rice engine

Rice has mainly two phases of logistic growth, vegetative and reproductive wherein most of the grain yield is a function of weather during the latter phase⁽⁵⁹⁾⁽⁶⁰⁾⁽⁶¹⁾ and it has been reported that enhanced carbon dioxide in atmosphere within temperature range of 22⁰C-32⁰ C can enhance grain yield by about 0.5 t ha⁻¹ per 75 ppm enhancement in carbon dioxide⁽³⁾. A simplified grain equation for rice may be written as

$$Y_g = H \epsilon \int_{t_i}^{t_f} I_{int}(t) dt$$

Where in Y_g is grain yield, H is harvest index (HI), t_i is the date of transplantation, t_f is the date of harvesting and I_{int} is the total amount of PAR (photosynthetically active radiation *i.e.* 400-700 nm of the spectrum) intercepted by the crop. The yield of crops is hence proportional to their RUE (radiation use efficiency). When crop duration and HI are constant⁽⁴²⁾⁽⁴⁴⁾. Since C₄ plants have an RUE greater than 40-50 % they can out yield rice by 40-50% proving the fact that rice can yield better with a C₄ chassis.

The sink in rice is big enough to accommodate C₄ productivity

Enhanced photosynthesis for 33 days just prior to heading brought about by carbon dioxide enrichment causes upto 30% increase in yield⁽⁵⁸⁾ due to enhanced grain filling percentage and weight of individual grain. If we compare the number of juvenile spikelets 10-15 days before panicle initiation, number of spikelets at maturity and number of filled

spikelets (grains) averaged for IR72 in 2 dry seasons (Table 1) we find that juvenile spikelet production (113448 on average) is more than twice the final number of filled spikelets at maturity (38793 on average). Thus it is clear that the capacity of rice crop for spikelet production is more than double of what is filled in the form of grain. Hence the sink is much larger than required for C₃ rice and above observations suggest that another half of unfilled grains stand a chance to be filled in C₄ rice⁽⁵⁸⁾.

Basic metabolics

Tit bits of supercharged photosynthesis

In the normal C₃ cycle (elucidated by Calvin and Benson⁽⁴⁾) the Carbon dioxide that has been taken by plants combines with Ribulose 1,5Bisphosphate (RUBP) in presence of enzyme ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) to fix the carbon dioxide into a three-carbon compound 3-phosphoglycerate (3PGA) which is then cycled to yield a molecule of sugar, at the same time regenerating the RUBP initially used. This is accomplished by a battery of enzymes which utilize the ATP and reducing equivalents (NADPH) generated earlier using sunlight. But all is not so sunny. This C₃ system loses 25% of its fixation ability when the operating conditions have high light intensity or temperature or both. Under high temperature or high light intensity, Rubisco instead of showing carboxylase activity starts showing oxygenase activity due to adjacency in active site of the enzyme for carboxylase and oxygenase activity. This phenomenon of increased respiratory activity by plants upon induced by high temperature or light or both is called photorespiration. The loss of carbon that is increased via this additional respiration is called “photorespiratory carbon loss” and is the fundamental reason behind C₃ plants being less efficient than C₄ plants. C₄ plants carry

out the whole carbon fixation process in two morphologically different cell types, the mesophyll cells (M cell) and Bundle sheath cells (BS cell). In M cells carbon dioxide is primarily accepted by phosphoenol pyruvate (PEP) and instead of a 3C 3PGA a 4C organic acid (oxalo acetic acid OAA) is formed. The reaction is catalyzed by PEP carboxylase (PEPC) enzyme. This 4C organic acid is then transferred to BS cell where most of the C₃ photosynthetic machinery is present. The OAA is decarboxylated in BS cell to give a 3C compound (pyruvate) and a molecule of carbon dioxide (which is then refixed via the C₃ pathway). The repetition of this process builds a high gradient of carbon dioxide near Rubisco present in BS cell and hence oxygenase activity of Rubisco is never activated irrespective of the light intensity or temperature, rounding off photorespiration to negligible levels. Although the 2 types of cells (BS and M) are arranged in form of concentric rings forming wreath like structure (Kranz Anatomy) to maximize contact, still C₄ pathway requires a complex battery of transporters and a relatively higher amount of energy. However its mechanisms to prevent loss of photosynthetically fixed carbon offsets the energy cost⁽³⁷⁾.

Based upon differences in subcellular localization, transported acids and C₄ acid decarboxylase used, 3 different biochemical subtypes of C₄ pathway have been identified. In all the 3 subtypes primary carboxylation yields OAA. In NADP-Malic enzyme (ME) type, OAA is converted to malate and the decarboxylase used is NADP-ME. Both NAD-Malic enzyme (ME) and PEP-carboxykinase (CK) type transport OAA after converting it to aspartate. The C₄ acid decarboxylase used is NAD-ME in former and PEP-CK in latter. However this classification is not robust. For example PEP-CK activity has also been noted in maize which is classically known to be a NADP-ME

plant⁽¹³⁾. Since the NAD-ME and PEP-CK subtypes have more intracellular steps and energy requirement, the NADP-ME pathway is being considered to engineer C₄ rice. Figure 1 represents the schematics of NADP-ME C₄ pathway while Table 2 summarizes the genes being transferred into rice to build NADP-ME type of photosynthesis⁽⁴⁹⁾.

However it would be wrong to say that C₄ plants entirely eliminate photorespiration. Glycolate oxidase (a key enzyme in photorespiration) deficient maize mutants are seedling lethal at normal carbon dioxide concentration and are able to survive only at carbon dioxide levels that are inhibitory to photorespiration⁽⁷⁹⁾. This indicates that early stages of pathway are functional in mutant and buildup of glycolate is toxic. Also it has been proposed that localization of Glycine decarboxylase to BS cells would have been one of the proximal steps of C₄ evolution⁽¹⁶⁾⁽⁵¹⁾.

Why are C₄ plants at a metabolic advantage?

The superior photosynthetic capacity of C₄ plants could be attributed to their exclusive mode of carbon dioxide incorporation, the basis of which, is stringent compartmentalization of photosynthetic enzymes into 2 distinctive cell types BS and M cells. This results in reduction of photorespiration to negligible levels. Biochemically this is achieved by 2 processes. Firstly phosphorylation of a conserved serine residue close to N-terminal end of PEPC polypeptide reduces the sensitivity to feedback inhibitor malate and the enzyme PEPC. Also PPDK regulatory protein (PPDKRP) a bifunctional serine/threonine kinase phosphatase catalyzes both ADP-dependent inactivation and Pi dependent activation of PPDK⁽²⁶⁾⁽⁷¹⁾⁽⁷⁸⁾. Due to both these processes coupled with oxygen

insensitive carboxylase (PEPC), oxygenase activity of Rubisco is reduced to negligible levels and Rubisco protein can be more efficiently used, thus resulting in better Nitrogen use efficiency. Water equilibrated at normal atmospheric pressure dissolves 11mM carbon dioxide which forms 110mM bicarbonate ions at 25⁰C and pH 7.2, while Rubisco fixes carbon dioxide, PEPC fixes bicarbonate ions and this distinguishing feature bestows a remarkable benefit to C₄ plants⁽⁵⁶⁾. A typical C₄ plant requires only 250-300g of water to produce 1g of dry matter while a C₃ plant requires 650-800g water for the same⁽⁴⁹⁾. Based upon the studies on photosynthetic photon flux density (PPFD) using infra-red gas analyzer (IRGA), it was found that C₄ plants have a radiation use efficiency (RUE) that is 50% higher as compared to C₃ plants⁽⁴⁹⁾. This is because a relatively lesser number of photons are required to fix each mole of carbon dioxide in case of C₄ plants⁽⁶²⁾. Thus the active C₄ operation as an auxiliary metabolic carbon dioxide pumping system confers significantly better nitrogen investment, WUE and RUE to C₄ plants as compared to C₃ plants.

Converting C₃ to C₄ – proof of concept

Proofs from phylogeny

Angiosperms are the only land plants exhibiting C₄ photosynthesis where in it is found in 36 eudicots, 6 sedges, 18 grasses and 2 aquatic genera *Hydrilla* and *Egeria* i.e. a total of 62 C₄ taxa⁽⁵⁴⁾. There is a lack of clarity about the evolutionary independence of these 62 taxa but it is a fact that C₄ pathway arose multiple times from ancestral C₃ pathway. It is interesting to note that except 4 lineages (the 2 aquatic and 2 chenopod genera *Binertia* and *Suaeda*) rest 58 times the C₄ pathway evolved in association with Kranz anatomy. Out of 7500 C₄ species 4600 (more than 60%) are grasses⁽⁵⁵⁾ and what is even more interesting

is the fact that all the grasses occur in the same PACMAD clade (PACMAD for six families Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and Danthonioideae) with 17 independent origins of C_4 pathway proposed in this clade only and none of the other 7 grass clades. Another coincidence is the presence of low levels of CA (the first enzyme of C_4 shuttle) in the entire clade. It can thus be inferred that a single mutation in the last common PACMAD ancestor may have facilitated this transition⁽³⁷⁾. In addition to this there are certain genera like *Flavaria* and *Cleome* where all the three evolutionary types C_3 , C_4 , and $C_3 - C_4$ intermediates are found. Intermediate species thus represent a transitional phase in C_4 evolution. At the same time certain intermediates such as *Moricandia arvensis* occur in families with no known C_4 species⁽⁹⁾⁽²⁹⁾.

Proofs from biochemistry

Most of the enzymes of C_4 pathway have their counterparts in C_3 plants where they play house-keeping roles. In fact production of 4 Carbon compound and related PEPC catalyzed carbon fixation reactions of C_4 plants are already used within guard cells of C_3 plants⁽⁶⁷⁾. Table 3 enlists the role of various C_4 enzymes in C_3 plants. Thus it can be concluded that C_4 biochemistry evolved through modification of existing function rather than de novo. Also biochemical characteristics of C_4 photosynthesis are found around vascular bundles of C_3 plant stems⁽⁷⁾⁽²⁷⁾.

Facultative intermediates

Existence of facultative intermediates is possibly the most convincing evidence which demonstrates the feasibility of C_4 rice engineering. These intermediates can provide us a plethora of information regarding the

developmental transition from C_3 to C_4 type at individual plant level. *Elocharis vivipara* for example develops C_3 anatomy in submerged leaves and C_4 anatomy in aerial leaves⁽⁷²⁾⁽⁷⁴⁾. What is more interesting is that *E.vivipara* can be induced to transform into C_4 by using Abscicic acid (ABA)⁽⁷³⁾. Light intensity is also an important variable affecting the transition. For example in *Flavaria brownii*, plants grown in higher light intensities are more C_4 like than those grown in lower light intensities⁽⁹⁾⁽⁴³⁾.

C_3 is the master plan

Many evidences suggest that C_3 is the master plan or the default developmental state in C_4 plants and C_4 system develops in due course of development. One of the characteristics examples is the single cell C_4 system found in *Binertia* and *Suaeda*. These show remarkable dimorphism intracellularly i.e. chlorenchyma cells in the above two are organized into two distinct cytoplasmic compartments (Proximal and distal in *Suaeda* and central and peripheral in *Binertia*) by means of actin filaments and microtubules.

The two compartments have distinct set of photosynthetic enzymes with the peripheral/distal chloroplast analogous to M cell and central/proximal chloroplast analogous to BS cell.

It is remarkable to note that initially a monomorphic C_3 state develops and this dimorphic C_4 pattern is later on induced by developmental cues⁽³⁷⁾. However this development of C_3 state as default in C_4 plants is not limited to single cell systems only. This has also been reported in maize (a monocot) and amaranth (an eudicot), where Rubisco accumulates in both BS and M cells until light, hormone and/or other developmental hints cause BS cell specific accumulation of Rubisco⁽³⁸⁾⁽⁷⁷⁾.

C₄ leaf anatomy and role of veins

A cross section of a typical C₃ leaf reveals only one major cell type that has chloroplasts, the M cells. In contrast a typical C₄ leaf (Fig. 2) has two distinct chloroplast containing cell types M cells and BS cells⁽⁶⁶⁾. Since the functioning of C₄ pathway requires cooperation between both cell types, the M and BS cells are arranged in form of concentric rings (wreath, Kranz anatomy) to maximize contact. For the same purpose BS cells have centrifugally arranged chloroplasts towards M cells and an extensive plasmodesmal network connects the two cell types to facilitate easy flow of metabolites. BS cells are present in C₃ plants also but there they are involved in non-photosynthetic functions like solute transport and carbohydrate metabolism.

Another peculiar feature is that veins act as organizing centers of the C₄ system. Difference in venation pattern is a characteristic variable between C₃ and C₄ leaf

anatomy. Quantitative measurements of BS to M cell ratios in C₃ and C₄ leaves result in 1:1 ratio of the two cell types in case of C₄ plants⁽¹¹⁾⁽²³⁾⁽⁴⁵⁾. This ratio translates into veins (V) being separated by only four photosynthetic cells in C₄ as compared to upto 20 cells in C₃ leaves. Thus the characteristic C₄ repeating unit of V-BS-M-M-BS-V is formed⁽³⁷⁾. BS cells are very large sized to make this improvisation more efficient.

It can also be proposed that this mechanism to induce procambium at more regular intervals across the leaf (that results in larger number of veins) may be the first step in the evolution of Kranz anatomy and may have preceded the biochemical changes. On comparing similar stages of vascular development in C₃ and C₄ *Flaveria* species, it was found that relatively greater number of veins were initiated in C₄ species⁽⁴¹⁾. In maize it has been observed that C₄ inducing signals are only perceived in cells that are within two cell radius of a vein⁽³⁹⁾. This further establishes the role of veins as C₄ organizing centers.

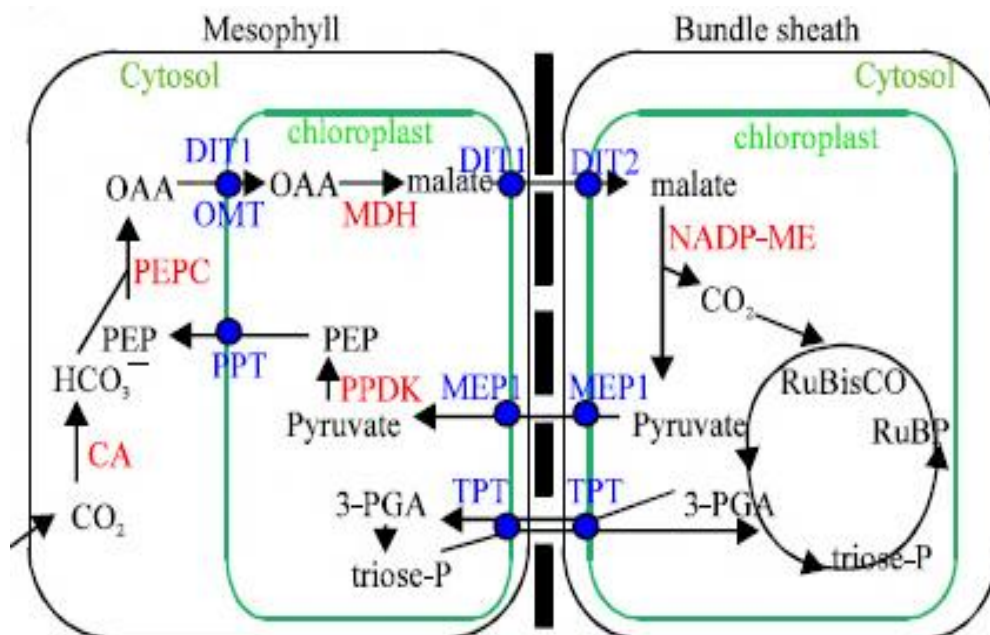


Fig.1 Basic Schematics of NADP-ME C₄ pathway (Source Rizal *et al.*, 2012)

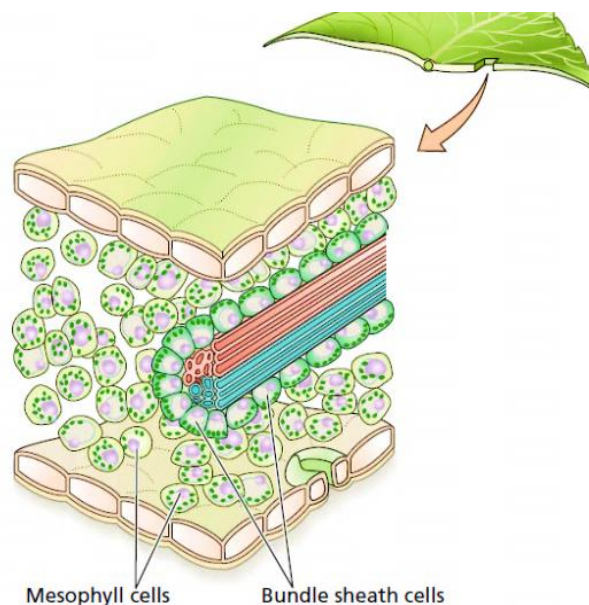


Fig.2 3D model of a C4 leaf (Source Taiz and Zeiger, 3rd edition)

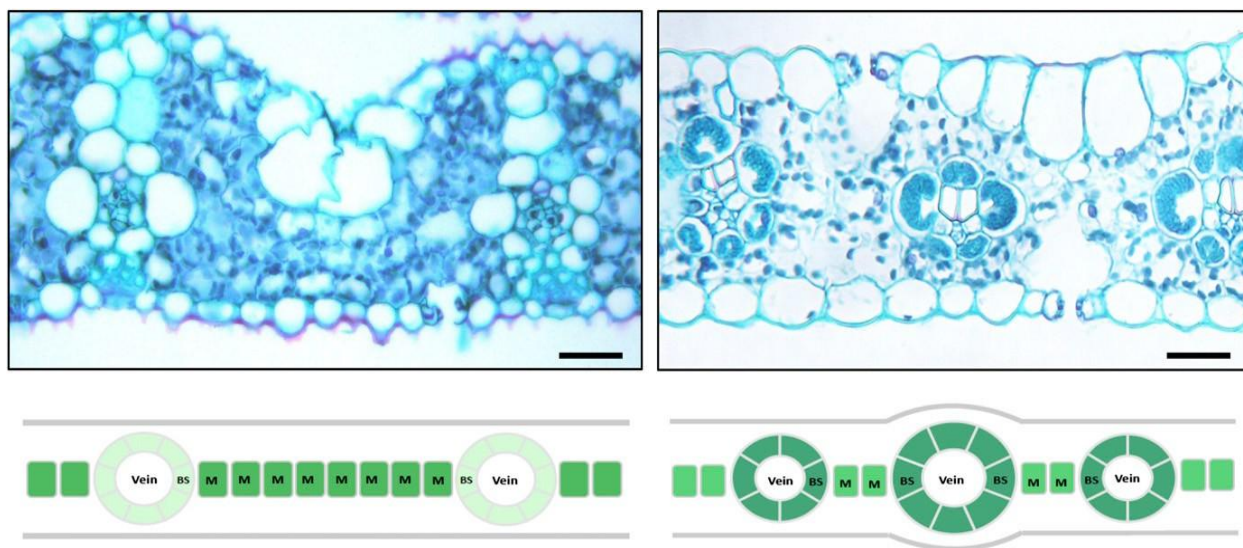


Fig.3 Transverse Leaf Sections and Corresponding Schematics of C3 Rice and C4 Maize. Rice (left) and maize (right). Bars = 30 mm (Source- Langdale 2011)

Table.1 The mean number of spikelets, spikelets at maturity, filled spikelets and 1000- grain weight in crops of IR72 in the dry seasons of 1997 and 1999⁽⁵⁷⁾ (source - Sheehy *et al.*, 2001)

Item	Mean Number
Number of juvenile spikelets (m ⁻²)	113,848
Spikelets at maturity (m ⁻²)	51,372
Filled pikelets at maturity (m ⁻²)	38,793
1000-Grain weight (g, 14% moisture content)	24.0

Table.2 List of genes currently being transferred into rice to build NADP-ME type of C₄ Photosynthesis in rice⁽⁴⁹⁾ (Source- Rizal *et al.*, 2012)

Gene Name	Function	References
Carbonic anhydrase (CA)	Conversion of CO ₂ to bicarbonate (HCO ₃ ⁻) in the cytosol of M cells	Ku <i>et al.</i> , (1996)
Phosphoenolpyruvate carboxylase (PEPC)	Catalyzes the formation of oxaloacetate (OAA) using HCO ₃ ⁻ and Phosphoenolpyruvate (PEP) in M cells	Ku <i>et al.</i> , (1996)
Dicarboxylate translocator 1 (DiT1)	Exchanges OAA with malate in M cells	Taniguchi <i>et al.</i> , (2000)
2-oxoglutarate/malate transporter (OMT)	OAA transporter across the chloroplast membranes of M cells	Taniguchi <i>et al.</i> , (2000)
Malate dehydrogenase (MDH)	Catalyzes the reduction of OAA to malate in chloroplasts of M cells	Agostino <i>et al.</i> , (1992)
Dicarboxylate translocator 2 (DiT2)	Exchanges Glutamate with malate in BS cells	Brautigam <i>et al.</i> , (2011)
NADP-malic enzyme (NADP-ME)	Decarboxylation of malate in chloroplasts of BS cells	Ku <i>et al.</i> , (1996)
Mesophyll envelope protein 1 (MEP1)	Transport of pyruvate in chloroplasts of M and BS cells	Brautigam <i>et al.</i> , (2011)
Pyruvate, orthophosphate dikinase (PPDK)	Conversion of pyruvate to PEP in chloroplasts of M cells	Ku <i>et al.</i> , (1996)
Phosphoenolpyruvate/phosphate translocator (PPT)	Imports PEP from cytosol	Brautigam <i>et al.</i> , (2011)
Triosephosphate/phosphate transporter (TPT)	Transports triose phosphate in a 1:1 counter exchange with phosphate	Brautigam <i>et al.</i> , (2011)
Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBis CO)	Refixation of CO ₂ in chloroplasts of BS cells	Hatch (1987) and Kanai and Edwards (1999)

Table.3 Various C₄ enzymes with their roles in C₃ plants⁽³⁷⁾ (Source Langdale, 2011)

S.N.	Name of the C ₄ pathway enzymes	Function in C ₃ plants
1.	Chloroplast localized CA	<ul style="list-style-type: none"> Ensures a supply of carbon dioxide into Calvin-Benson cycle
2.	PEPC	<ul style="list-style-type: none"> Generates malate as a photosynthetic product which is used to provide carbon skeletons to the TCA cycle and for ammonium assimilation PEPC activity contributes to extension of fibers in cotton It is also involved in salt and drought responses in wheat and Arabidopsis
3.	PEP-CK	<ul style="list-style-type: none"> Mobilization of sugars from lipids in seeds during germination Provision of PEP to shikimate pathway
4.	PPDK	<ul style="list-style-type: none"> PPDK generated PEP has been shown to contribute to seed metabolism

Rationale of C₄ rice development

In addition to the biochemical complement C₃ plants also exhibit certain anatomical hints towards C₄. For example roots of C₃ plants show significant thickening and suberization of cell walls in endodermis, mimicking the thickening of cell walls in BS cells⁽⁸⁰⁾. Thus C₃ plants in principle already have the basic elements to build C₄ machinery. At this level two different research strategies can be agreed upon to engineer C₄ rice. These two strategies are in turn based upon two evolutionary hypothesis suggested for C₄ evolution. One of them called incremental gain hypothesis, states that plants gained C₄ features gradually i.e. one by one before they developed the full C₄ complement⁽⁵¹⁾. This is supported by the existence of C₃, C₄ intermediates, C₄ like species (e.g. *Parthenium* which is a C₃ plant having Kranz anatomy⁽²⁴⁾). Going by this hypothesis, building C₄ rice requires stepwise integration of C₄ biochemistry into a common rice cultivar and then manipulating other components of the transport process of C₄ photosynthesis. Other hypothesis is the 'master switch' hypothesis which states that a single large mutational event led to C₄ development. This is supported by the relative ease of convergent evolution of C₄ photosynthesis. (C₄ systems evolved more than 50 times in 19 families)⁽⁴⁵⁾⁽⁵¹⁾. If this hypothesis holds true then engineering C₄ rice requires identification of that genetic switch, which would enable the cascade of actions leading to C₄ differentiation.

The checklist

Following are the essential features that need to be genetically engineered in C₃ plants to make them C₄.

Compartmentalization- a compartment to concentrate carbon dioxide around Rubisco is needed. The envelop must have the

biochemical component to release carbon dioxide from the carrier which will bring transiently fixed carbon dioxide towards the envelope. Also the compartment must have carbon dioxide proofing system to restrict carbon dioxide release from BS cell.

Transportation- the system must possess a pool of metabolites for capture, transport and release of carbon dioxide, and these metabolites must have high transfer affinity between BS and M cells.

Energy- Active light energy driven carbon dioxide fixation system is required. Energy supply must be enough to last the metabolite transportation and carbon dioxide acceptor regeneration process

Stoichiometry- ratio of M cell to BS cell must be 1:1 to maximize the efficiency of transport process.

Approaches for engineering C₄ rice

Mutagenesis and Rice DNA activation tagging

Scanning for a correlation between phenotype and genotype within a specific mutant is one of the most direct methods of functional gene discovery. Together, mutagenesis and rice DNA activation tagging account for both loss of function and gain of function mutants. In mutagenesis loss of function C₄ mutants are made 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. Since the process involves screening of very large populations measurements of vein density (VD) (that can be undertaken with just a handheld microscope) is used for mass screening. Selection is based on alteration in VD from wild type (VD > 9 veins mm⁻¹) to low VD

(VD < 7 veins mm⁻¹). Backcrossing for two generations (upto BC₂F₂) is sufficient to reduce the number of non-specific mutations⁽⁴⁹⁾. Identification of mutants can be done via genotyping of SNPs⁽³⁶⁾ (single nucleotide polymorphisms), Denaturing HPLC (DHPLC), and target induced local lesions in genome (TILLING)⁽⁷⁰⁾. Mutants can be cross referenced with publicly available databases like Sorghum BTx623⁽⁴⁷⁾.

Gene redundancy limits the efficacy of insertional mutagenesis using T-DNA or transposable elements in activating gene functions. Gain of function mutants for C₄ rice is being created through DNA activation tagging. This technique uses T-DNA or transposable element containing multiple copies of cauliflower mosaic virus (CaMV) 35S enhancer. Since they can enhance gene function in both orientations from the site of their insertion, this leads to dominant gain of function mutations i.e. C₄ like characters in C₃ rice. The tag facilitates identification of gene location and the phenotype of the mutant reveals function of gene. Retrotransposons Tos 17⁽²⁸⁾, maize Ac/Ds elements⁽³⁴⁾⁽⁴⁸⁾ and Full length cDNA over expressor gene (FOX) hunting systems have been used to generate a number of rice mutant resources. The activation insertion mutants are then cross referenced with databases from related projects like RiceGE/SIGnAL (<http://signal.salk.edu/cgi-bin/RiceGE>), Ory GenesDB (<http://orygenesdb.cirad.fr>) and Gramene (<http://www.gramene.org>).

OMICs approaches

To discover the next level of correlation between the gene and associated phenotypic trait gene expression profiling is required. Comparative transcriptome analysis of C₃ and C₄ leaves, M and BS cells and other C₃ and C₄ tissues can yield the exact number of genes differentially expressing between C₃ and C₄

plants. For this purpose maize is the chosen C₄ plant because maize and rice have similar developmental trajectories (both mature from tip to base). 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⁽⁷⁶⁾. Different modeling techniques have been developed using the transcriptome data available. Among these morphogenesis models are more popular as they can integrate genetic control, leaf anatomy and processes such as diffusion simultaneously⁽²⁰⁾.

Epigenetic approaches

In addition to cis acting DNA elements, chromatin configuration also has a bearing on C₄ differentiation and associated gene regulation. Expression of PEPC has been linked to histone modification, epigenetic control and chromatin remodeling in maize. Light specific activation of transcription during differentiation of photosynthetic tissues may be potentiated by cell type specific chromatin modification like acetylation of specific histone lysine residues. The role of miRNAs (micro RNAs) in vascular differentiation is also well characterized⁽³⁰⁾⁽⁵⁰⁾. On this basis potential role of miRNAs in Kranz anatomy development is being explored⁽⁶⁹⁾.

What we know upto now?

One of the initial and most important discoveries was the identification of putative transporter proteins that were upregulated in C₄ species in order to facilitate translocation of C₄ pathway intermediates between BS and M cells⁽⁵⁾⁽¹⁴⁾⁽¹⁷⁾. The identified transporters include OMT1 (2-oxoglutarate/malate transporter), DiT2 (dicarboxylate transporter2), PPT1 (PEP/phosphate transporter), MEP (mesophyll envelop

protein), TPT (triose-phosphate phosphate translocator, bile acid: sodium symporters, proton: sodium antiporter, two mitochondrial dicarboxylate carriers, and one plasma membrane intrinsic protein⁽⁶⁾). It was also noted that there is differential upregulation of regulatory proteins like ARF2 (Auxin response factor 2), GLK 1 and 2 (Golden 2 like 1 and Golden 2 like 2), certain Sigma 70 like factors (SIG 1 and SIG5) as well as proteins that influence chloroplast positioning like GC1 (Giant Chloroplast 1) and CHUP1 (Chloroplast unusual positioning 1)⁽¹⁵⁾. There has been discovery of sequences termed as 'candidate sequences'. These are called as such because these can affect BS or M cell specific expression without the need of any promoter sequence. Some of these sequences include Mesophyll expression module 1 (MEM1) that gets expressed cell specifically in M cell⁽¹⁾⁽¹⁸⁾, 5' and 3' UTRs (untranslated regions) of genes encoding PPDK and CA which cause BS cell specific expression of the two enzymes⁽³²⁾. Besides the upregulation of transcript levels of transporters and regulatory proteins, genes related to components of ATP production system are also upregulated. These include PSI components and Cytb₆/Cyt_f complex proteins⁽⁵⁾⁽¹⁷⁾. In addition to above three, there is notable upregulation of maize SCARE-CROW (SCR) and SHORTROOT (SHR) homologs during vascular development of C₄ plants. This highlights the probable role of SHR/SCR pathway in patterning Kranz anatomy⁽¹²⁾⁽⁶⁴⁾⁽⁶⁵⁾.

Comparative transcriptome studies have facilitated plotting of expression trajectories between C₃ and C₄ species and based upon these trajectories it can be concluded that C₄ systems resulted as a consequence of integration of a series of small scale changes⁽³⁵⁾. This inference argues against the potential master regulator and thus a probable flip switch for C₄ rice doesn't hold true anymore. Studies of transcriptome data

combine with flux-balance analysis suggest that a misbalance in nitrogen metabolism between BS and M cells created through compartmentalization of the photorespiratory pathway is a pre-requisite for evolution of C₄ systems⁽⁴⁰⁾⁽⁵²⁾⁽⁵³⁾⁽⁷⁶⁾. Further, transcript profiles of amphibious species *Elocharis baldwinii* reveals that ABA, auxin signaling and DNA methylation play crucial roles in induction of C₄ photosynthesis⁽⁸⁾. On the genetic engineering front, C₄ genes from different plants have been stably integrated into rice and expressed for over at least 3 generations⁽⁶⁸⁾. Enough scientific evidence has been generated regarding T₃ transgenic rice plants harboring PPDK and PEPC genes from maize, constantly expressing ZmPPDK and ZmPEPC over three consecutive generations⁽⁴⁹⁾. During such studies it also has been found that some C₄ specific genes localized in BS cells retain their property of cell specificity even in a C₃ plant. This can be demonstrated in the expression profile of PEPCK gene from *Zoysia japonica*. When the promoter of the gene is fused with β -glucuronidase and inserted into rice, it expresses selectively in vascular tissues and BS cells of transgenic rice⁽⁴⁶⁾. Thus it can be inferred that C₃ plants also possess the regulatory mechanism required for cell specific expression of C₄ genes.

Summary and future perspectives

The idea of transferring C₄ traits into C₃ crops is not new and dates back to early years of this century when Japan Tobacco was granted a US patent for generation of PEP-CK type of C₄ cycle in rice⁽²⁾. The increasing levels of difficulty in meeting the food and fuel demand however, is again stemming up interest regarding C₄ rice. With access to advanced technologies and monetarily self-sufficient international collaborations (like C₄ consortium), much of the ground work like molecular tool development, unraveling the

genetic infrastructure, collection and study of available genetic variability has been done and with the current pace of work C₄ rice seems achievable within two decades from now. Some of the near future steps could be

- More number of cross species comparisons and associated datasets are required
- Transcript profiles need to be correlated with epigenetic signatures i.e. gene discovery need to be more closely correlated with gene function
- A relatively deeper insight into gene regulatory networks is required.

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How to cite this article:

Sudershan Mishra, Mithilesh Kumar Singh, Sikha Snehal and Himanshu Pathak. 2017. C₄ Rice - Tweaking Rice Physiology for Second Green Revolution. *Int.J.Curr.Microbiol.App.Sci*. 6(12): 1161-1176. doi: <https://doi.org/10.20546/ijcmas.2017.612.131>