Review Article

Revolutionizing Rice: The Synthesis of Nature's Evolution and Biotechnology in C4 Rice

ABSTRACT

Agriculture plays a fundamental role in meeting the essential needs of humanity, yet numerous factors continuously influence agricultural production and productivity. Over time, evolving agricultural practices have significantly increased yields of staple food crops, thereby enhancing global food security. However, as the world's population continues to grow, there is a pressing need to further augment crop yields. Historically, increases in yield were achieved by expanding cultivated land, but rapid industrialization and urbanization have diminished available agricultural land and depleted natural resources essential for farming. In response to these challenges, enhancing crop photosynthetic efficiency has emerged as a pivotal strategy. Photosynthesis, the process by which plants convert sunlight into energy, has been optimized in various crops through evolutionary and biotechnological approaches. One such innovation is the adaptation of the C4 photosynthetic pathway into C3 rice, a staple food for billions worldwide. The C4 pathway, found in several efficient crops like maize and sugarcane, enhances carbon dioxide uptake and reduces photorespiration, leading to improved water and nitrogen use efficiency. This review explores the integration of nature's evolutionary principles with cutting-edge biotechnological advancements in the development of C4 rice. By introducing the C4 pathway into rice—a traditionally C3 plant—scientists aim to dramatically increase its productivity and resilience to environmental stressors. This transformative approach not only holds promise for meeting future food demands sustainably but also addresses the challenges posed by climate change and resource scarcity. Moreover, the review examines the scientific basis, technological innovations, and potential agricultural implications of implementing C4 rice. It highlights the collaborative efforts of researchers, agronomists, and biotechnologists worldwide in reimagining rice cultivation to secure global food supplies while minimizing environmental impact. Ultimately, the integration of C4 photosynthesis into rice represents a significant step forward in agricultural innovation, poised to redefine food production capabilities and enhance global food security in the face of escalating population growth and environmental change.

Keywords: photosynthetic pathway; environmental stressors; food security ; agricultural production and productivity

1. INTRODUCTION

Agriculture is considered as foundation of human society as food is basic need of the society. One of the major challenges of agriculture is to ensure the food security, reduce hunger and poverty.Addressing food risk requires tackling both food security [availability and access] and food safety [quality and hygiene] (Miron *et al*., 2023).Major crops contributing to food security are cereal crops like wheat, rice and maize.Cereals are a good source of nutrients that are essential for proper functioning of the bodyand are the reliable sources of calories. Hence considered as important staples for the survival of billions of people(Farooq *etal*.,2023). Food security can be achieved by increasing agriculture productivity. Productivity of agriculture is based on water availability, climate and majorly directed by products of agriculture research (Sheehy *et al*., 2008). Climate factors such as temperature, precipitation and uncertain events like increase in carbon dioxide level in the atmosphere are the direct factors affecting cereal production and productivity(Adams *et al*., 1998).While the indirect factors are soil fertility, floods, droughts and land under cereal production (Kumar *et al*., 2021).Rice crop has gained the attention as primary mouth feeder among the three major cereals(rice,maize and wheat) as the other cereals are also used for animal feed or industry purpose (Sheehy *et al*., 2008). The rice dependent global population is estimated to reach 3.5 billion

in the year 2030 (FAO).While climate extremes have various impacts on rice cultivation according to different geographical regions, in India rice yield is expected to a drop of 30 to 60 percent whereas in some parts of Myanmar an increase was expected maybe due to precipitation(Farooq *etal*.,2023).According to The International Food Policy Research Institute (IFPRI) global yield loss of rice would be 10 -15 % due to climate change (Wang *et al*.,2018).

There is need for increased yield due to increase in population, CO^2concentration, urbanization,temperature and disasters(Kortright, C. M.2012).In mid-sixties increasing the foodgrains yield was proportional to the land area and extension of irrigation later this has been surpassed by the agricultural revolution that used high yielding variety seed of cereal crops, agro chemical fertilizers (inorganic fertilizers) and mechanization which is termed as 'The Green revolution'.Further increase in the yield of these varieties is not observed under the breeder trials in IRRI indicating that these varieties have reached a yield plateau (Sheehy 2001a, Kropff *et al* 1994).Hence there is need for the new method or mechanism to increase rice production.John Sheehy a plant physiologist and Head of the Applied Photosynthesis Group at the International Rice Research Institute (IRRI) from the year 1995 to 2009 conducted a workshop to discuss the idea of increasing the photosynthetic efficiency of the rice crop by incorporating C_4cycle into rice crop thus increasing the yield by 50%. Later in the year 2006, this led to the formation of C_4Rice Consortium by IRRI with a group of 16 laboratories in 11 countries(Furbank *et al*.,2023).

2. EXPLORING PHOTOSYNTHETIC STRATEGIES

The plants are physiologically categorized into three types based on their photosynthetic pathways into CAM, C_3 and C_4 plants. Among these C_4 plants are highly efficient in converting the solar energy into biological energy(Biswal *etal*.,2018).Atmospheric carbon fixation in C_3 pathway (photosynthetic carbon reduction cycle) is catalyzed by the enzyme ribulose bisphosphate (RuBP) carboxylase oxygenase(Rubisco)converting the five-carbon compound ribulose bisphosphate (RuBP) and CO_2 into two molecules of 3-phosphoglycerate (PGA). PGA is reduced into triose phosphate further leads to the formation of stable organic compounds(Raines*et al*.,2011).However,the limiting factor of the C3 cycle is dual activity of Rubisco enzyme (Portis and Parry, 2007). It acts as carboxylase and oxygenase depending upon the temperature and availability of the substrate; carbon dioxide is exchanged by oxygen molecules. This oxygenase activity (photorespiration)of Rubisco catalyzes oxidation of RuBP to form one carboxyl P-glycolate and one molecule of 3-Phospho-glycerate, this process is known as C_2 oxidative photosynthetic carbon cycle. The oxygenation pathway allegedly uses 3.5 ATP and 2 NADPH each RuBP regenerated but provides no additional organic carbon, the carboxylation pathway of photosynthesis uses 3 ATP and 2 NADPH per RuBP regenerated and yields a carbon in hexose. Photorespiration is typically regarded as a wasteful process since it yields 2PG, a substance that is "toxic" to numerous enzymes involved in photosynthetic metabolism, and oxidizes organic carbon without appearing to produce ATP (Shi, X., & Bloom, A. (2021).While the fate of CO_2uptake in C_4 cycle is made more efficient by the enzyme phosphoenolpyruvate (PEP) carboxylase(PEPC).

C4 pathway and its Evolutionary origins:

All the flora and fauna evolve eventually for the survival under natural selection followed by diversification. Similarly,C3 plants took the advantage to thrive in arid environments and reduced atmospheric CO_2 through adaptive changes in some traits, these traits may have predepositedin some plant species whichincrease CO_2 concentration mechanism,traits includeclose vein spacing and larger bundle sheath cell layer to form Kranz-like anatomy. All the C4 plants found are categorized approximately under 18 angiosperm families and in 90 genera.Thirty different evolutionary origins of C4 pathway were found i.e. all these families have evolved independently (Sage, R. F. (2001).C3 ancestral photosynthetic pathway has been modified into C4 pathway through 64independent multiple lineages (parallel evolution or convergentevolution occurs when two independent species happen to evolve in the same direction and hence independently obtain the same traits)(Niklaus, M., & Kelly, S. (2019). The driving forces for C4 evolution are phylogenetic developments and environmental conditions(Sage, R. F. (2001).

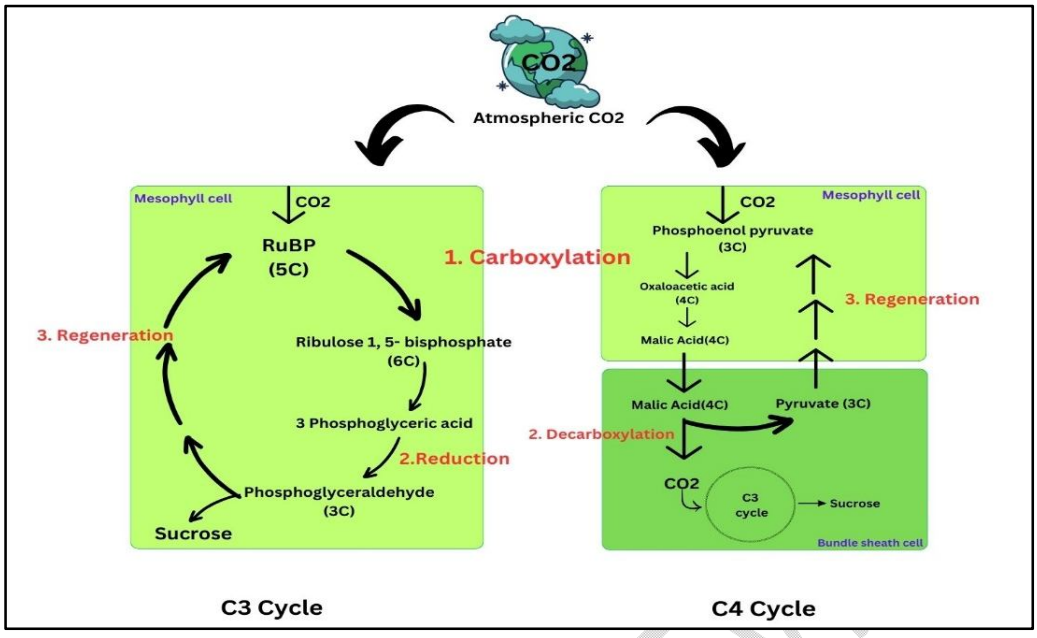

Fig. 1. C3 and C4 pathway

C4 Pathway: The C4 Photosynthesis is a complex trait; have significant characteristics defining it a sefficient mechanism of all the photosynthetic pathways.Study of C4 cycle is important as we further dwell into the study of research held to produce these raits into C3 rice crop. C4 cycle was discovered by Hal Hatch and Roger Slack in the year 1966.C4 plant types possess distinctive anatomical feature known as Kranzleaf anatomy, having two photosynthetic cell types, bundlesheath (BS) cells and mesophyll (M) cells. Mesophyll cells form the outermost layer, encasing bundle sheathcells that surround the vascular bundles(this ring like arrangement in German is referred as Kranz)(Maai *et al*.,2011). These structural frameworks in C4 leaves serve to functionally divide the of processes of carboxylation and decarboxylation. The assimilation of atmospheric CO2 by BS and M cells is very efficient due to the selective expression of essential photosynthetic genes in these cells. This expression results in the specialized accumulation of critical photosynthetic enzymes that catalyze distinct sets of cell-type-specific reactions (Garner *et al*.,2016). The PEPC and C4 pump associated components are located in the mesophyll cells while enzymes required to decarboxylate C4 acids plus Rubisco and photosynthetic carbon reduction cycle (C3 cycle) are present in bundle sheath cells (Rao, X., & Dixon, R. A. 2016).The enzymes associated are carbonic anhydrase(CA), phosphoenolpyruvate (PEP) carboxylase, biochemical subtypes of primary decarboxylating enzymes are NAD-dependent malic enzyme (NAD-ME) , NADP-dependent malic enzyme (NADP-ME) and PEP carboxykinase (PEPCK), pyruvate orthophosphate dikinase (PPdK).The carbonic anhydrases (CAs) in mesophyll cells convert CO2 into bicarbonate, then reacts with PEP by PEPC to form four carbon oxaloacetic acid (OAA). Malate dehydrogenase (MDH) catalyses' the conversion of OAA into malate. Malate is diffused to BS cells. NADP-ME inBS cell catalyzes the oxidative decarboxylation ofmalate producing pyruvate, CO_2 and NADPH (Drincovich*et al*.,2001).Pyruvate is diffused back into mesophyll cell, regeneration of PEPfrom pyruvatebypyruvate orthophosphate dikinase (PPDK)while CO_2enters into C3 cycle (Wang *et al*.,2014).However, a number of transportersbetween the two specialized cells play an important role in order to maintain the coordinated movement of metabolites.

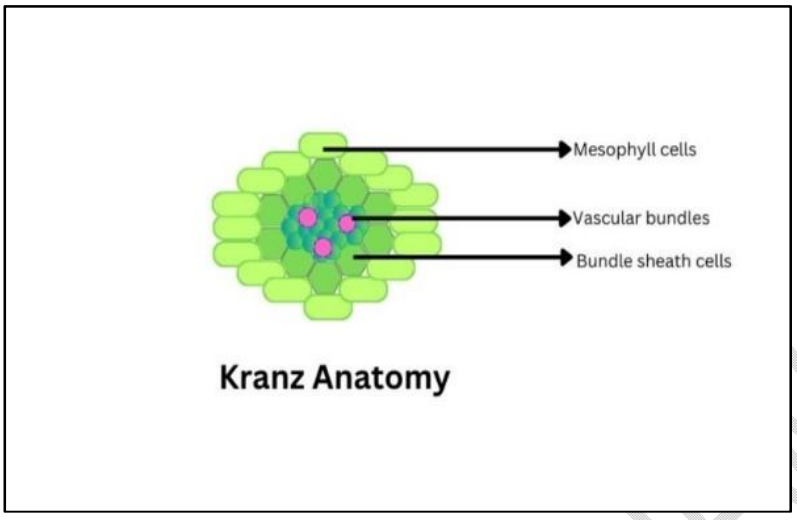

Fig. 2: Kranz anatomy present in crop

Why the C_4 Pathway Outperforms C_3 Pathway"

In C3 cycle rubisco acts both for carbon dioxide and oxygen which leads to the loss of 25 percent of fixed CO_2 along with ATP molecules during the respiration process while in C4 cycle this is fixed by PEP making all the CO_2molecules available for the further reduction thus increased photosynthetic efficiency. Apart from biochemical differences the anatomical difference between the two dark reactions is quite significant. That is C_3 is a single cell process while C_4 is dual cell cycle requiring two different cells types for completing one cycle of carbon fixation.This special anatomical feature “Kranz anatomy” acts as CO2 pump. Other adaptive advantages of C_4 plants over C_3 plants is high water use efficiency(ability to produce more dry matter per unit water used or transpired) which has been experimentally proven by Shantz and Premise and high nitrogen use efficiency (carbon fixed per unit nitrogen in the plant) observed by Colman and Lazenby (Brown, R. H. ,1978).The other factors are altering vein patterns that is close vein spacing and high vein density, low MS:BS ratio (this reduces the path length and ensure rapid intercellular diffusion of C4 metabolites) McKown, A. D., & Dengler, N. G. (2010).

Overview of C4 rice breeding initiatives:

Now that we have known the importance of producing C4 rice we shall explore the efforts put forward to make it possible till date. Now the question lies is it possible to produce such a miracle rice, what could be the possible ways of producing it. A vast range of knowledge has been gathered by a number of scientists working world wide making a possible change every day thus creating path of C4 rice and walking towards the better future. Approaches for C4 rice research are described below.

Feasibility of producing C4 Rice: The evolutionary aspects of C_4 plants show that they have been originated through multiple lineages from C_3 plants under different environment conditions that is high levels of atmospheric oxygen making it more challenging for the plants to take CO^2 thus making an evolutionin carbon fixation method i.e. C_4 Carbon fixation cycle (Osborne, C. P., & Beerling, D. J. (2006). Some genera such as Flaveria and Cleome, contain both C_3 and C_4 species making it feasible for producing interspecies hybrids.However this method have not shown any significant improvement for C_4 engineering due to its major drawbacks such as producing sterile hybrids making it impossible to maintain the inheritance of the genes, some showing higher yields but mostly resembling the C_3 parents,segregation of the genes generation after generation as C_4 traits are quantitative traits controlled by number of genes and interspecies hybrids can only be produced if the C_3 species are closely related to C4 species making it as the major drawback of this method as not all important crops have close C_4 relatives. One of the methods to overcome the sterility is protoplast fusion however protoplast fusion, however, does not solve the problem of sterility and instability of the C4 trait, which has hindered its application in C3-to-C4 engineering(Cui, H. (2021).

Leaf Vein density: One of the key properties of C4 is higher vein density and low M:BS cell ratio.In order to search these properties in rice leaf, one of the possible sources is screening mutagenized population of rice crop.Two rice mutant populations were screened, a deletion mutant library with a cv. IR64 background (12,470 lines) and a T-DNA insertion mutant library with a cv. Tainung 67 background (10,830 lines). The mutants leaf width and vein density were 4-6mm and 6.5 veins per mm respectively, while the wild type leaf width was 8-9 mm and vein density was less than 6.5mm. High vein density trait is found to be linked (linkage was incomplete) with narrow lead width thus can further be used as a phenotypic marker for finding high vein density variants of C4 rice. These mutant populations can be used for exploration of C4 relevant traits and as breeding lines (Feldman *et al*.,2014).

Leaf Anatomical diversity of rice crop contributing to C4 engineering:Rice crop possess wider genetical and ecological diversity of both wild relatives and cultivated species and show large variation in leaf architecture. The study of the leaf architecture or anatomy was conducted in 24 *Oryza* species and possible evolutionary trends were examined. It was found that the features of newly evolved rice species include: a greater number of bundle sheath cells, longer and laterally elongated mesophyll cells, and fewer veins overall. *Oryza coarctat* - Additional unique veins,*Oryza grandiglumis*- Increased vein density,*Oryza minuta*-thicker leaves, more veins per mm, fewer mesophyll cells,*Oryza brachyantha*- Larger and laterally elongated mesophyll cells,*Oryza australiensis*- Increase in total mesophyll area, *Oryza rhizomatis*-larger bundle sheath cell number.These variations in key anatomical traits among *Oryza* species contribute to the potential development of C4 rice by providing insights into leaf structural diversity. Traits such as increased vein density, mesophyll size, and bundle sheath cell number can be manipulated to mimic C4-like leaf structures in rice. By understanding and utilizing these anatomical variations, researchers can work towards engineering rice plants with enhanced photosynthetic efficiency similar to C4 plants, potentially leading to improved yields and agricultural sustainability (Chatterjee *et al*., 2016).

Single cell C4 photosynthetic pathway: Hydrilla verticillateis an aquatic facultative C4 plant i.e. it shifts the photosynthetic mechanisms from C3 to C4 depending upon the availability of $C0_2$. Under low CO_2 conditions it performs C4 photosynthesis without any structural changes by upregulating the genes encoding C4 specific isozymes of PEPC, PPDK, NADP-ME (Rao *et al*., 2002, 2006; Estavillo *et al*., 2007). Efforts has been made to introduce this mechanism into rice by overexpressing the C4 enzymes.The four C4 enzymes (i.e. PEPC, PPDK, NADP-MDH, and NADP-ME) have been successfully overproduced in the rice transformants however of all the combination of these enzymes only the quadruple transformants showed increased photosynthetic rates (Taniguchi *et al.,* 2008). However, the increased rates were not due the introduced CO_2 concentration mechanisms.Overproduction of C4 specific NADP-ME led to inhibition of photosynthesis serous stunting, bleaching of the leaf color(Miyao *et al*.,2011).

A synthetic biological tool: A system for rice has been developed to regulate transgene expression. The dTALE1-STAP1 and dTALE2-STAP2 systems are molecular tools used in synthetic biology. They are designed to enable specific and tunable gene expression in plants, such as rice, by utilizing synthetic transcription activator-like effectors (dTALEs) to regulate gene expression. These systems are part of the toolkit of synthetic biology, allowing for precise control and manipulation of gene expression in plants for various applications, including crop improvement and engineering novel biological pathways. The system, consisting of synthetic designer transcription activator-like effectors (dTALEs) and cognate synthetic TALE-activated promoters (STAPs), can activate STAP-driven reporter gene expression in stable transgenic rice lines. The strength of individual STAP sequences varies between cell-types, requiring empirical evaluation. The dTALE-STAP system offers a powerful approach to fine-tune transgene expression and introduce different synthetic circuits into distinct developmental contexts. The implementation of the dTALE-STAP systems allows for tissue-specific and tunable expression of multiple genes in rice, which is crucial for modifying gene expression in various tissues to achieve C4 photosynthesis. The advantages include the potential to accelerate the project goals by enabling the modified expression of multiple genes in several tissues, which is essential for the successful implementation of C4 photosynthesis in rice(Danila *et al*.,2018).

GRAS transcription factor SCARECROW (SCR) protein:A study aimed to evaluate the role of SCARECROW(SCR) in rice development by designing four CRISPR guide RNAs (gRNA) targeting OsSCR1 (OS: *Oryza sativa)*and OsSCR2. These guides were cloned into constructs to generate and assess knockout mutants. The role of SCR in maize and Arabidopsis were different it regulates the

development of the endodermis in Arabidopsis and maize roots, but also controls the formation of other cell types,mesophyll in maize and bundle-sheath in Arabidopsisduring leaf development.The study highlights the diverse roles of the SCARECROW (SCR) gene in plant development across species. In rice, SCR is involved in stomatal patterning, and two duplicated SCR genes function redundantly in this process.The study indicates that SCR genes play a crucial role in stomatal development in rice, which is essential for optimizing photosynthesis efficiency. Understanding the specific functions,the role of SCR in stomatal patterning, researchers can potentially manipulate this pathway to improve photosynthetic efficiency and overall crop productivity in C4 rice (Hughes, T. E., & Langdale, J. A. (2022).

To create C4 rice, number of techniques and technologies have been used to achieve different objectives that converges into one and act as stepping stones for the success of the miracle rice some of these are listed in the table below.

Table 1: Techniques and purposes of conversion of C3 to C4 rice

S.no	Techniques	Purpose	Reference
1	**Flip-Flap imaging method**	The Flip-Flap method involves mounting plant samples between two coverslips with spacers to prevent squashing, then imaging them from both sides using a laser scanning confocal microscope (LSCM) to produce two z-series (Stack A/Flip and Stack B/Flap). Stack B is rotated and inverted before sticking Stack A to produce a full 3D image. This method enables dual-view imaging of cleared and stained thick plant samples, allowing for full 3D reconstruction up to 300 µm in thickness.	Serra *et al.,*2022
2	**Bright field microscope (Olympus BX51 compound microscope)**	Leaf anatomical study of 24 *Oryza* species	Chatterjee *et al*.,2016
4	**Ortho Finder**	Clustering genes into Ortho groups.	Emms *et al*.,2016
5	**MAFFT (Multiple Alignment using Fast Fourier Transform)**	Bioinformatic tool used for construction of multiple sequence alignments.	Emms *et al*.,2016
6	**Phyldog Bio software**	inference of gene trees and reconciliation of gene trees with species tree.	Emms *et al*.,2016
7	**Nomarski optics/ Nomarski interference contrast (NIC) / differential interference contrast (DIC) microscopy,**	Study the bundle sheath cells.	Wang *et al*.,2017
8	**Zeiss Axioplan light microscope with Olympus cell Sens**	Quantification of number of chloroplasts in the bundle sheath cells.	Wang *et al*.,2017

	software		
9	**Leica DMRB microscope**	number of veins across the medio-lateral leaf axis was counted	Wang *et al*.,2017
10	**Chlorophyll fluores -cence imaging**	used to study the density of stomata.	Rizal *et al*.,2017
11	**RC DC (reducing agent and detergent compatible) protein assay**	Protein quantification	Levey *et al*.,2018
12	**LI-6400 system(LI-COR)**	Used to measure the rates of photosynthesis and transpiration i.e leaf gas exchange measurements.	Levey *et al*.,2018
13	**GC-MS(gas chromatography-mass spectrometry (GC-MS) system) analysis using the 7200 GC-qTOF system (Agilent)**	Metabolites quantification	Levey *et al*.,2018

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