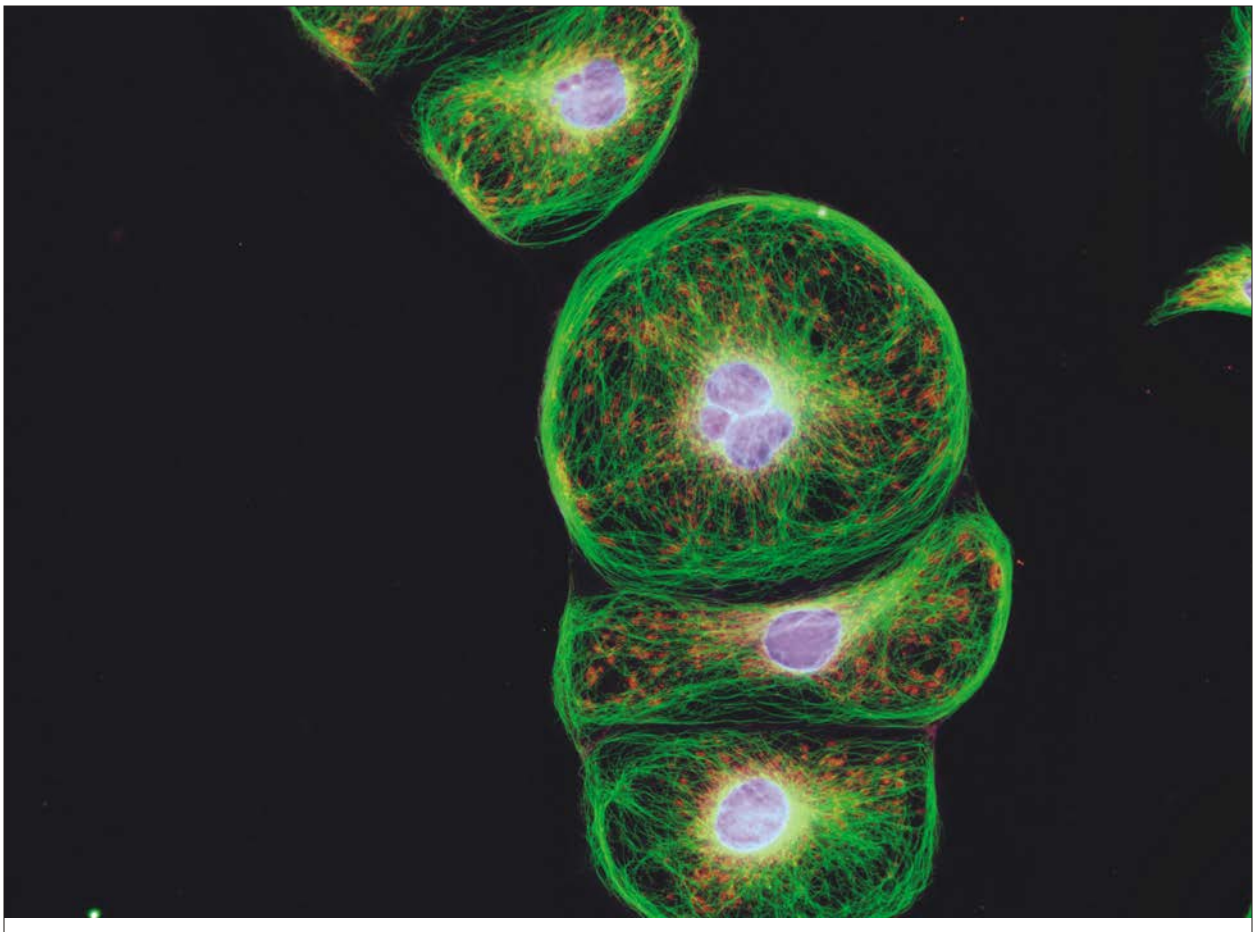

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Towards a C_4 Rice

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ABSTRACT

The success of the green revolution that began in the 1960s led to an increase in rice yield of up to 10 fold that was sufficient to meet the food demand of a continually growing population. This rapid increase in rice yield has not been repeated even after four decades, while the global population continues to grow rapidly. To meet the food demand of the soaring population, rice yields should increase by at least 50% within next four decades. Despite the best efforts of plant breeders, the prospect of a second drastic increase in yield using conventional approaches is unlikely but may be possible if we redesign rice photosynthesis into C_4 type. A C_4 type of photosynthesis is much more efficient than the C_3 type found in wheat and rice and could facilitate an increase in yield potential to levels found in C_4 crops like sorghum and maize. Although, C_4 plants evolved from the C_3, there are distinct differences in their leaf structure (evolution of Kranz anatomy) and biochemistry. The same would be needed for the development of a C_4 rice plant. In 2009, a consortium of scientists was formed to develop C_4 rice. The consortium is using multiple approaches which include but are not limited to; alteration of genomes of C_3 and C_4 plant species, exploration of wild *Oryza* accessions and bioinformatics to discover new genes underlying C_4 photosynthesis. The already known or newly discovered genes of C_4 pathway are being systematically introduced into rice. Here we review the strategies adopted by the C_4 rice project coordinated by the International Rice Research Institute.

Key words: C_4 plant, gene discovery, Kranz anatomy, photosynthesis, rice yield

INTRODUCTION

Rice has been cultivated for more than 9,000 years (Molina *et al*., 2011) and it is the primary source of energy for more than half of the world's population. The well-known green revolution in the 1960s led to an increase in the rice yield from less than 1.5 ton ha^{-1} (Jennings, 1964) to the present rice yield potential of 8-10 ton ha^{-1} (Khush, 1995). This increase in food production sustained the simultaneous increase in global population from 3 billion in 1961 to 7 billion within five decades. The population is expected to reach 9 billion in the next four decades. The major portion of this population increase will be in the rice eating areas of Asia and Africa. To harvest enough rice for the booming population, there is a need for new varieties which can sustainably yield higher, under the perceived situation of less land, water and fertilizer inputs amid the predicted extremes of climatic change. Even with the optimum use of input and management practices, the yield potential of the current inbred *indica* varieties does not exceed 10 ton ha^{-1} (Kropff *et al*., 1993). One way to overcome the current yield ceiling is by genetically introducing the C_4 photosynthetic pathway in rice (Sheehy *et al*., 2000). The concepts and practicalities of

redesigning rice photosynthesis are reviewed in three classic books (Raghavendra and Sage, 2011; Sheehy, 2008; Sheehy *et al*., 2000). A number of reviews have underscored the importance of engineering C_4 pathway in rice (Gowik and Westhoff, 2011; Hibberd and Covshoff, 2010; Kajala *et al*., 2011b; Langdale, 2011; Matsuoka *et al*., 2001; Sage and Zhu, 2011). All of these literature highlight that C_4 photosynthesis is much advantageous than C_3 in rice growing areas under high temperature and bright sunshine. This led to the idea to upgrade C_3 rice to C_4. No doubt this is a complex process as multiple genetic changes are required to alter leaf biochemical and anatomical modifications that occurred during the evolution of C_4 plants from their C_3 ancestors. C_4 photosynthesis has evolved multiple times independently during the evolution of plants (Sage, 2004) and has involved duplication of genomes in whole or part that has created redundancy in genes, evolution of Kranz anatomy, a decrease in number of Mesophyll (M) cells between veins, a spatial shift in the metabolism of M and Bundle Sheath (BS) cells including the relocation of carboxylase enzymes (Gowik and Westhoff, 2011) and evolution of transporters to facilitate metabolic movement between the two cell types (Fig. 1b). The absence of any of these C_4 traits in close relatives of rice prevents its incorporation through classical breeding strategies. The advancements in genetic engineering and plant molecular biology offer great possibilities to incorporate C_4 photosynthesis in rice. While the process of photosynthesis is widely studied in both C_3 and C_4 plants and the introduction of C_4-like metabolism into M cells of C_3 plants has been attempted (Miyao *et al*., 2011), transforming one photosynthesis type into another is a novel attempt. This review reported the strategies being adopted and progress made towards novel C_4 gene identification and transformation of known genes to integrate C_4 photosynthetic system into C_3 rice.

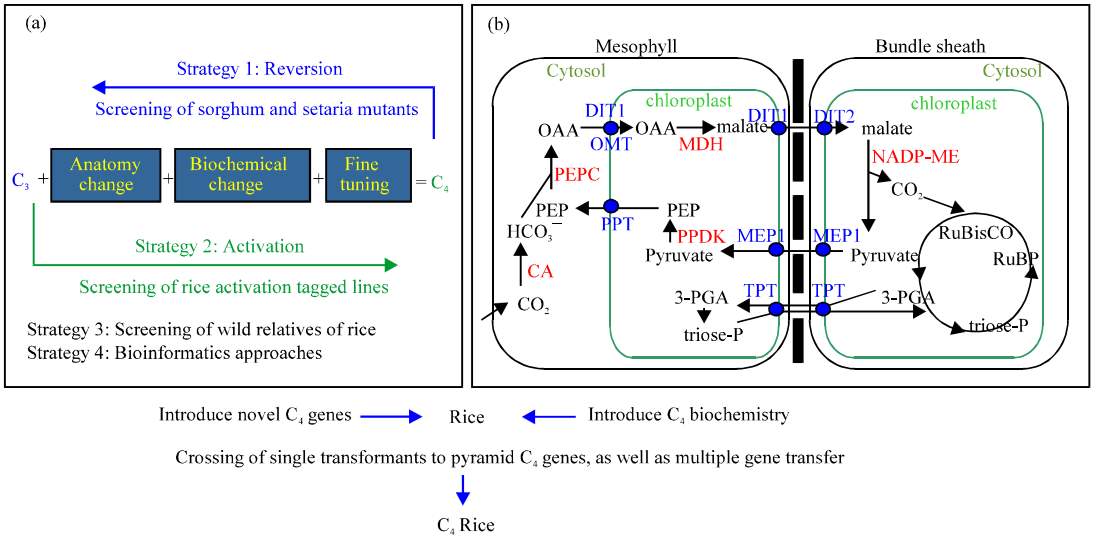

Fig. 1(a-b): Schematic diagram of (a) novel genes related to C_4 pathway, identified by utilizing various resources and (b) the known biochemical components of Nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME) type C_4 pathway, currently being engineered into rice. The core C_4 genes are in red and transporters are in blue color. PGA: Phosphoglycerate, OAA: Oxaloacetic acid, PEP: Phosphoenol pyruvate, Full names of other abbreviations used in the figure are given in Table 1

COMPARISON OF C_3 AND C_4 PHOTOSYNTHETIC PATHWAYS

The C_4 photosynthetic pathway, an evolutionary development from C_3, has undergone modifications in terms of biochemistry as well as leaf anatomy (Gowik and Westhoff, 2011). Most plants are either C_3 or C_4 depending on the type of photosynthetic pathway they use which are classified based on the first compound formed from the assimilation of atmospheric CO_2. In C_3 plants, CO_2 is assimilated by Ribulose-1,5-bisphosphate Carboxylase Oxygenase (RuBisCO) and forms 3-phosphoglycerate (3PGA), a 3 carbon compound. The entire process is completed within M cells which are exposed to atmospheric CO_2 via intercellular air spaces and have an abundance of RuBisCO enzyme. RuBisCO fixes both CO_2 and O_2 depending on their availability thereby facilitating both carboxylation and oxygenation reactions. The amount of RuBisCO in C_3 plants is much more than the plant actually uses was evident from an experiment where tobacco plants survived normally when RuBisCO was reduced by up to 50% (Quick *et al*., 1991). The oxygenase activity of RuBisCO increases photorespiration and causes energy loss (Edwards *et al*., 2004) although this can be beneficial in times of stress as a means to dissipate excess energy (Yokota and Shigeoka, 2008). In contrast, RuBisCO in C_4 plants is not expressed in M cells rather its expression is restricted to BS cells. The atmospheric CO_2 is first fixed in M cells by phosphoenolpyruvate carboxylase (PEPC) forming oxaloacetate, an organic compound with 4 carbon units (Fig. 1b). Unlike RuBisCO, PEPC is an oxygen-insensitive carboxylase which has a much higher affinity for CO_2 and can continue carboxylation even when the leaf internal CO_2 concentration is very low (Ku *et al*., 1996). The CO_2 fixed in the form of 4 carbon acid is transported to BS cells where it is decarboxylated by one of the three decarboxylating enzymes. The CO_2 released after decarboxylation is re-fixed in BS cells by RuBisCO (Fig. 1). This decarboxylation of C_4 acids creates a very high concentration of CO_2 around RuBisCO. This mechanism to concentrate CO_2 in BS cells prevents photo-respiratory oxygenation reactions making C_4 plants generally more productive than C_3 (Peterhansel and Maurino, 2011). Photorespiration is greatly enhanced at higher temperatures due to a lower CO_2/O_2 specificity of the RuBisCO enzyme and the enhanced solubility of oxygen in water compared to CO_2. Therefore, especially in warmer climates, C_4 plants have elevated photosynthesis compared to C_3 plants in the same ecology (Long, 1999).

The ability of C_3 (rice) and C_4 (sorghum) plants to assimilate photosynthates was studied at different light intensities measured as Photosynthetic Photon Flux Density (PPFD) using infrared gas analyzer. The PPFD was gradually decreased from 2500 $\mu mol_{photon}\ m^{-2}\ sec^{-1}$ to zero, CO_2 was kept constant at 400 ppm, a flow rate of 400 $\mu mol\ sec^{-1}$ was maintained and the leaf temperature was 30°C. It showed that the C_4 plants can also utilize maximum level of sunlight because their rate of photosynthesis sharply increases with increasing light intensity and does not appear to saturate (Fig. 2). Radiation Use Efficiency (RUE) is up to 50% higher than that of C_3 due to reduced number of photons required to fix each molecule of CO_2 (Sheehy, 2008). A typical C_4 plant produces 1 g of biomass for every 250-350 g of water transpired, whereas to produce the same quantity of biomass C_3 plants transpire 650-800 g of water (Ehleringer and Monson, 1993). Plants with C_4 photosynthesis are more efficient in using photosynthetic nitrogen than C_3 plants largely due to reduced amounts of RuBisCO protein required to achieve the same rate of photosynthesis (Sage *et al*., 1987). The C_4 CO_2 concentrating mechanism therefore confers more efficient photosynthesis, combined with greater nitrogen, water and radiation use efficiency and this frequently translates into higher yields in crop plants.

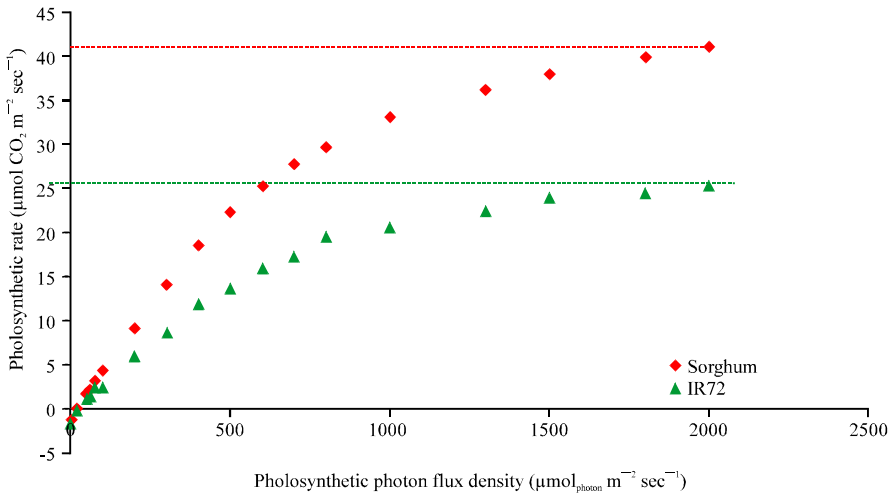

Fig. 2: A comparison between light response curves of a C_4 plant (sorghum) and C_3 plant (rice, IR72) generated using readings obtained from infrared gas analyser

SEARCH FOR GENETIC FACTORS CONTROLLING C_4 LEAF ANATOMY

While the metabolic process of photosynthesis has been extensively researched and photosynthetic enzymes well studied, most of the genetic factors regulating C_4 anatomy are still unknown (Langdale, 2011). We have adopted a dual approach to identify these genetic factors. The first approach is to mutate C_4 plants (sorghum and *Setaria viridis*) to randomly hit some of the C_4 characteristics and to identify the responsible genetic factors (Fig. 1a). The second approach is to use rice DNA activation tagging to over-express random rice genes and look for C_4-like characteristics (Fig. 1b). Both of these approaches require screening of large populations to find desirable phenotypes. For mass screening of these populations, we searched for induced alterations in leaf Vein Density (VD). The veins of C_4 plants are generally much more closely spaced than C_3 plants. This is required to facilitate the precise spacing of M and BS cells (vein-BS-M-M-BS-vein) and the general requirement for a 1:1 stoichiometry of BS and M cells. Alterations to vein spacing can arise through a number of anatomical changes including alterations to M cell number between the veins, altered BS size and number or increased vein size. All of these are important changes that have occurred in C_4 plants and so identification of genes that disrupt or induce these changes in C_4 (sorghum) or C_3 (rice) respectively could help us to understand how leaf anatomy is regulated at the genetic level. Fortunately, analysis of vein density is a relatively simple procedure that can be undertaken very quickly with a hand-held microscope in the field or laboratory and is suited to screening large populations.

INDUCED MUTATION ALTERS C_4 LEAF ANATOMY

Induced mutations have greatly facilitated gene discovery, the understanding of complex traits and enhanced the speed of novel gene identification. We treated sorghum (*Sorghum bicolor* (L.) Moench) seeds with Ethyl Methanesulfonate (EMS) and gamma rays separately to generate two mutant populations. Based on the alteration in vein spacing from wild type VD (more than 8 veins mm^{-1} at the widest part of the youngest fully expanded leaves) to low VD (LVD) (less than

7 veins mm^{-1}), we screened more than 70,000 mutant lines in the M_2 generation. It was found that mutagenesis had caused a reduction in VD, largely due to increased M cell number between the veins, in 24 independent lines that we are currently analyzing in more detail. To reduce the number of non-specific mutations we are currently generating backcrossed (BC_2F_2) populations. To identify mutations a number of techniques are available, such as genotyping single nucleotide polymorphisms (Kwok, 2001), denaturing HPLC (DHPLC) or TILLING (McCallum *et al*., 2000), endonuclease cleavage method (Oleykowski *et al*., 1998), cel 1 based TILLING method (Till *et al*., 2004) and high-resolution melt analysis (Ririe *et al*., 1997). With the improved speed and reduced cost of large-scale DNA sequencing we have opted for next generation genome sequencing to identify the responsible mutated genes as the reference genome sequence for sorghum-BTx623 is publicly available (Paterson *et al*., 2009). We are currently analyzing the sequence data and hope to identify the relevant gene or genes that will then be transformed into rice and the mutant sorghum lines to analyze the effects of their expression. This direct approach of mutation discovery quickens the process of gene identification.

EMERGENCE OF C_4 CHARACTERISTICS IN MUTANT RICE LINES

The most direct method in functional gene discovery is to look for a correlation between phenotype and genotype within a specific mutant. Elimination or activation of gene function through insertional mutagenesis using transfer DNA (T-DNA) or transposable elements has proven to be an extremely valuable research tool. The foreign DNA acts as a mutagen and a tag for the site of insertion allowing for a function to be assigned to a specific DNA sequence and for genes associated with a specific phenotype to be isolated. The insertion of these elements is a random event and the mutations are stable through multiple generations (Azpiroz-Leehan and Feldmann, 1997). Such approaches have their limitations, particularly when they are compensated by gene redundancy, lethality due to loss of function or where specific unknown conditions are required for the gene activity such as biotic/abiotic stresses. To overcome these limitations modified insertional elements have been developed like the gene trap systems that fuse the tagged gene with a reporter gene, such as β-glucuronidase (GUS) or Green Fluorescent Protein (GFP) (Springer, 2000). Activation tagging technique uses T-DNA or a transposable element containing multimerized Cauliflower Mosaic Virus (CaMV) 35S enhancers. They enhance gene functions in both orientations at considerable distance from their site of insertion. This results in the transcriptional activation of genes and dominant gain-of-function mutations. The resulting mutant phenotypes can reveal the normal function of the gene while the tag facilitates the identification of the location in the genome.

A considerable number of rice mutant resources have been created using chemical mutagenesis (Wu *et al*., 2005; Till *et al*., 2007), T-DNA insertion (An *et al*., 2003; Chen *et al*., 2003; Chern *et al*., 2007; Hsing *et al*., 2007; Jeong *et al*., 2002, 2006; Mori *et al*., 2007; Wan *et al*., 2009; Wu *et al*., 2003), the retrotransposons Tos17 (Hirochika, 2001), maize transposon Activator/Dissociation (Ac/Ds) elements (Kolesnik *et al*., 2004; Park *et al*., 2007; Qu *et al*., 2008; Upadhyaya *et al*., 2006), maize enhancer/suppressor mutator elements (En/Spm) (Kumar *et al*., 2008) and the Full-length cDNA Over-expressor gene (FOX) hunting system (Hakata *et al*., 2010; Kondou *et al*., 2009; Nakamura *et al*., 2007). The first rice genes identified using insertional mutagenesis were discovered simultaneously using forward (Agrawal *et al*., 2001) and reverse (Takano *et al*., 2001) genetic screens of separate Tos17 mutant populations. Genes have also subsequently been identified in forward (Jung *et al*., 2003), reverse (Lee *et al*., 2003), expression based (Kang *et al*.,

2005) screens of T-DNA populations and populations utilizing the Ac-Ds transposon (Zhu *et al.*, 2003, 2004) and the FOX-hunting systems (Nakamura *et al.*, 2007) highlighting the value of these resources for functional gene discovery. Databases are available for each of these individual populations as well as databases that combined data from multiple insertional mutagenesis projects such as RiceGE/SIGnAL (http://signal.salk.edu/cgi-bin/RiceGE), OryGenes DB (http://orygenesdb.cirad.fr) and Gramene (http://www.gramene.org). Together these resources represent an extensive and diverse resource in various genetic backgrounds (Droc *et al.*, 2006; Ware *et al.*, 2002). The availability of mutant resources, advanced analytical techniques and the large number of databases aid to the identification of the genes of interest.

We are screening activation insertion mutants from Korea (http://postech.ac.kr/life/pfg/risd/) and Taiwan (http://trim.sinica.edu.tw). While the Korean collection consists of 47,932 T-DNA activation lines in the japonica varieties Dongjin and Hwayoung (Hsing *et al.*, 2007), the Taiwan collection contains approximately 70,000 T-DNA insertions in the japonica variety, Tainung-67 (Jeong *et al.*, 2006). Approximately 80,000 Flanking Sequence Tags (FSTs) are available for the Korean population and 25,000 for the Taiwan collection. The aim is to identify mutants with alteration in leaf anatomy that resemble C_4 traits in rice by activation of specific genes. This could lead to the identification of novel genes controlling C_4 leaf anatomy, cell biology and ultra structure. To date approximately 17,000 lines have been screened for mutants with a stable increase in VD. From the two populations more than 60 mutants have been identified with increased VD. In some of these lines, the increase is due to a reduction in the number of M cells between veins, a feature characteristic of C_4 leaf anatomy. Such mutants can be used to identify the genes controlling the phenotype either through sequencing of mutagenised lines and/or transgenic testing of candidate genes. In future we will also screen Ac/Ds mutant lines from Australia (Upadhyaya *et al.*, 2006) and FOX lines from Japan (Nakamura *et al.*, 2007).

HARNESSING THE DIVERSITY OF RICE

Rice is one of the cultivated species of genus *Oryza* of Poaceae family. Genus *Oryza* is composed of 10 different genomes with 23 wild and 2 cultivated species. Although C_4 evolved independently more than 60 times (Monson, 2003) and there are symptoms of C_4 characteristics in the stem and petiole of C_3 plants like tobacco (Hibberd and Quick, 2002), there are no occurrences of C_4 or C_4-like species within this genus *Oryza*. However, *Oryza* possesses a wide range of variation for different traits even among the accessions of a single species. Ram *et al.* (2007) have reported 56% genetic diversity among its diverse genomes. Among these species, *Oryza rufipogon* and *O. nivara* are currently being screened for C_4-traits as these two species have higher intra-species genetic diversity; 46% for *O. rufipogon* (Gao *et al.*, 2002) and 78% for *O. nivara* (Juneja *et al.*, 2006) and differences in the branching pattern (Yamaki *et al.*, 2010) and M cell conductance (Scafaro *et al.*, 2011) were found to be correlated with yield and availability of CO_2 for carboxylation, respectively. The advantage of working with wild rice species is the readily available information about genome sequences and genetic resources in Gramene database (http://www.gramene.org), Oryzabase (http://www.shigen.nig.ac.jp/rice/oryzabase/top/top.jsp), Rice-BRCdb (http://rice-brcdb.mpl.ird.fr/brcdb/) and the Oryza Map Alignment Project (OMAP, http://www.omap.org/). A high resolution Universal Core Genetic Map (UCGM) was developed by Orjuela *et al.* (2010) using 165 anchors and 16 accessions from *O. sativa, O. barthii, O. rufipogon, O. glumaepatula* and *O. meridionalis*. This group also developed the Paddy Map for easily generating sets of polymorphic SSR markers in any interspecific or intraspecific cross of *Oryza* species. Several Chromosomal

Segment Substitution Lines (CSSLs) have been developed for rice by small wild rice chromosomal introgressions which were found to be widely useful for identifying QTL and genes for different traits. These resources are still underutilized for physiology and photosynthesis screening. Therefore, there exists huge potential to modulate rice physiology using these resources to improve C_3 photosynthesis of rice or to make it more suitable for C_4 photosynthesis. Many agronomically important traits have already been successfully introgressed into rice by conventional breeding programs (Amante-Bordeos *et al*., 1992; Brar and Khush, 1997, 2002; Jena, 2010). A wide variation in leaf morphology in wild *Oryza* species provides good reason to explore these materials in more detail that would help in rice leaf anatomy and physiology manipulation. Several studies are ongoing world-wide to look for C_4-like properties in wild rice. The pioneering work of Yeo *et al*. (1994) showed increasing PEPcase activity in *O. australiensis* and much lower photorespiration rate in *O. rufipogon,* both of which are of direct interest in the study of C_3 to C_4 interconversion. They also observed large differences among *Oryza* species in transpiration at the single leaf level. Recent studies have shown that there is a wide variation in the photosynthetic characteristics among the wild rice (Zhao *et al*., 2010). They assessed photosynthesis related traits like net photosynthetic rate, leaf chlorophyll content, specific leaf area, flag leaf area and leaf N_2 concentration and reported higher photosynthetic rates in *O. rufipogon* and *O. australiensis*. Both studies suggest *O. rufipogon* as one of the suitable candidates for improving photosynthesis in rice. At International Rice Research Institute (IRRI), the first attempt of mass screening of wild rice accessions for VD change and lower CO_2 compensation point was carried out in 2008. After mass screening of CO_2 compensation point, we found a reduction in CO_2 compensation point for a few *O. nivara* accessions. These results indicate that genes responsible for leaf anatomical changes cause changes in observed CO_2 compensation point and could be identified from wild rice relatives and can be used to enhance our C_4 pathway engineering efforts into cultivated rice.

GENOMIC APPROACHES FOR UNDERSTANDING REGULATION OF C_4 PHOTOSYNTHESIS

In a recent study, four key genes of the C_4 pathway were introduced into rice M cells to generate a C_4 cycle between the chloroplast and cytoplasm. Although this did not result in enhanced photosynthesis or reduced compensation point it did demonstrate the possibility to achieve C_4 type photosynthesis in C_3 plants (Taniguchi *et al*., 2008). With a two cell system, we need to observe the desired cell specific expression patterns, suitable level of expression and activity and the metabolic connectivity of the two cell types. So, it becomes important to also have knowledge of the regulatory network controlling C_4 biochemistry and anatomy. This requires discovery of (1) additional genes working in coordination with the known C_4 genes, (2) transcription factors regulating the activities of genes of network and their respective binding sites and (3) involvement of gene silencing mechanisms. Several functional genomics and bioinformatics approaches have been or are being applied to meet these objectives.

For the discovery of genes associated with any phenotypic trait, gene expression profiling has been one of the most commonly used approaches. Although it was known for a long time that the mature leaves relay signals of environmental cues to new leaves of the same plant (Lake *et al*., 2001), the differential expression within the same leaf are being studied recently (Li *et al*., 2010). Our experimental designs involve comparison of gene expression between (1) C_3 and C_4 leaves, (2)

M and BS cells (3) developmentally distinct regions of a leaf and (4) different growth stages within a leaf. These experimental designs are meant to capture different and/or overlapping biological processes underlying C_4 photosynthesis. A few gene expression data has been generated by microarray analysis (Sawers *et al.*, 2007), however, majority has been generated by next generation sequencing of the transcriptome (RNA-seq), as discussed in detail below.

TRANSCRIPTOMES OF C_3 AND C_4 LEAVES

Comparison of gene expression in leaves of C_3 and C_4 species could identify genes that have turned on or off during the evolution of multiple C_4 species independently, or genes whose overall expression has significantly changed. There are two such studies between C_3 and C_4 species of genera *Cleome* and *Flaveria* (Brautigam *et al.*, 2011; Gowik *et al.*, 2011). Choice of such species minimizes the differences in expression due to species-specific features, thus allowing more precise discovery of genes associated with differences in photosynthetic pathway. The findings from both studies unveiled that in addition to the core C_4 pathway, there are several other functional gene classes which are affected (Brautigam *et al.*, 2011). Among the classes which showed lower steady state mRNA level included Calvin-Benson cycle, photorespiration, protein synthesis, primary metabolism, while those which showed higher level included photosynthetic classes of photosystem1 (PSI) and cyclic electron flow, starch metabolism, nitrogen metabolism, cofactor synthesis, glucan metabolism and lipid transfer proteins. There were some deviations too: *Flaveria* additionally showed downregulation of PSII, attributed to different ATP and NADPH demand related to the different mode of photosynthesis in the two species. Apart from the affected gene classes, some genes were also reported as candidate C_4 genes. The discovery of transporters was most important among them, as they ensure the availability of metabolites to the enzymes present in different cellular compartments. Few plastidic and mitochondrial transport proteins were largely up-regulated in C_4 leaf tissue (Brautigam *et al.*, 2011). Among the proteins with regulatory functions, 43 were significantly up-regulated in either C_3 or C_4 *Cleome*, whereas in *Flaveria*, several hundreds of such proteins were found to be differentially expressed and some of the important ones include auxin response factor2 (ARF2), golden2 like (GLK2), plastidic Sigma-70 like factors (SIG1 and SIG5) (Gowik *et al.*, 2011). Other genes related to chloroplast positioning, such as giant chloroplast1 (GC1) and chloroplast unusual positioning1 (CHUP1) were also reported to be candidates for C_4-associated genes.

TRANSCRIPTOMES OF MESOPHYLL AND BUNDLE SHEATH CELLS

The comparison between C_3 and C_4 whole leaf tissue is likely to miss genes expressed in different locations (or specific cell types), unlike their expression levels. Consequently to identify such genes, a more appropriate experimental design would be needed. From the perspective of the C_4 pathway, isolation of RNA from M and BS cells separately are obvious choices. The findings in the case of maize by using either microarray or RNA-seq showed that about one-fifth of genes, expressed in leaves, are differentially expressed between M and BS cells (Sawers *et al.*, 2007; Li *et al.*, 2010). Several functional classes were partitioned between the two cell types including light harvesting complexes, respiration, secondary metabolism and transport. Comparison of the transcriptome data with proteome datasets of maize plastids (Friso *et al.*, 2010) showed near-perfect correspondence between cell-specific enrichment and high correlation between differential expression ($0.68<r<0.98$) (Li *et al.*, 2010). This indicates that genes involved in C_4 photosynthesis are largely regulated at the transcriptional level.

TRANSCRIPTOMES ACROSS LEAF DEVELOPMENTAL GRADIENT

In addition to identifying genes involved in functioning of the photosynthetic pathway, it is also important to understand the factors involved in leaf development. This will help in identifying factors involved early in leaf development for building the cellular infrastructure where photosynthesis could operate. One such study was conducted by Li *et al.* (2010) in maize leaves examining the expression profile of genes in four developmentally distinct segments, namely basal, transitional, maturing and matured zones. While the fraction of functional genes was slightly higher in segment undergoing development (leaf base) compared to the fully mature segment, alternate splicing events were observed in about half of the expressed genes with introns. About eighteen distinct expression profiles were identified; six of them were considered main clusters accounting for 82% of the total genes expressed. Two clusters represented genes that are expressed at the highest levels towards the basal zone and include genes that encode enzymes for cell wall biosynthesis, DNA synthesis, cell cycle regulation and chromatin structure, protein metabolism, potential signaling proteins, auxin and brassinosteroid biosynthesis and signaling, respiratory pathways and vesicle transport. A marked change in the nature of enriched functional categories was observed for cluster showing peak expression of genes in the transition zone. Similarly, in the maturing or matured segments, the enriched gene classes included isoprenoid biosynthesis, the Calvin cycle, redox regulation and the light reactions of photosynthesis (Li *et al.*, 2010).

REGULATORY ELEMENTS ASSOCIATED WITH C_4 GENES

Known regulatory elements are often of poor resolution: The cell-specific expression in M and BS cells is generated by both transcriptional and post-transcriptional mechanisms and only for some of the C_4 enzymes is the mode of regulation known, at least partially (Hibberd and Covshoff, 2010). For the regulation at the transcriptional level, cis-regulatory elements form a key component and these often reside in the range of 0.5-2 kb upstream of the transcriptional start site. Here we summarize the available information about transcriptional regulation of C_4 related genes that involve *cis*-elements. In the case of PEPC, in C_4 *Flaveria*, a 41 bp long region named M-enhancing module (MEM1) has been identified which drives expression in M cells (Gowik *et al.*, 2004; Akyildiz *et al.*, 2007). This module is located in the distal region of the core promoter and is constituted of a tetranucleotide (CACT) insertion and a nucleotide substitution (G→A). In maize, however, the M cell specificity is attributed both to a 0.6 kb upstream region (Taniguchi *et al.*, 2000; Kausch *et al.*, 2001) and some epigenetic modifications (Langdale *et al.*, 1991). For PPDK, while in maize a very specific region (-301 to -296 from translational start site) is reported to be important (Matsuoka and Numazawa, 1991), in C_4 *Flaveria* a 1 kb long region (-1212 to +279 from transcriptional start site) accounts for higher M cell expression relative to BS cells (Rosche *et al.*, 1998). For NADP-ME, the behavior of two C_4 *Flaveria* species were different and the one showing transcriptional regulation, namely *F. trinervia*, required a 2 kb region (-1758 to +305 from translational start site) for strong BS-specific expression (Lai *et al.*, 2002). Like NADP-ME, the regulation of small subunit of RuBisCO was found to be complex and different regions for M cell repression and BS expression were reported (Viret *et al.*, 1994). Other C_4 genes are currently being studied within the C_4 Rice program to derive more cell specific promoters with appropriate expression levels.

PREEXISTING ELEMENTS RECRUITED FOR CELL SPECIFIC EXPRESSION OF C_4 GENES

Recent studies on the origin of *cis*-elements recruited by C_4 genes have unveiled some interesting findings. Based on the experiments on NAD-ME and NADP-ME, it was reported that

genes from C_3 species contain *cis*-elements sufficient for BS specificity in C_4 leaves (Brown *et al.*, 2011). The *cis*-elements were found to be located in the coding region and required to be transcribed to be functional which strongly suggest a post-transcriptional nature of regulation (Brown *et al.*, 2011). When the hypothesis was tested in two more C_4 genes, namely PPDK and CA, one or both Untranslated Regions (UTRs) were found to be sufficient for enhanced expression in M cells (Kajala *et al.*, 2011a).

DISCOVERY OF *CIS*-ELEMENTS

The locations of *cis*-elements of several C_4 genes are available but at a poor resolution. For a successful transplantation of C_4 pathway in rice we would require high resolution information about these *cis*-elements. The experimental approach of promoter-deletion assay provides reliable information, however, is of poor resolution in terms of location and requires significant research efforts. *In silico* approaches greatly help in accelerating their discovery using the prior information with improved accuracy. The recent findings on the presence of *cis*-elements in the transcript region (Brown *et al.*, 2011; Kajala *et al.*, 2011a) will further prevent the typical searches from being restricted only to the upstream regions. Availability of relevant gene expression data between photosynthetic cell types, C_3/C_4 leaf tissues and across the developmental gradients has helped in identification of co-expression clusters which in turn could be used for *cis*-element prediction. Moreover, the availability of draft genome sequences of various grass species like maize, sorghum and *Setaria*, also allows for sequence conservation studies of the upstream sequences housing the regulatory elements.

GENE SILENCING: EPIGENETICS AND NON-CODING RNAS

C_4 differentiation occurs along a developmental gradient with undeveloped proplastids found in cells at the leaf base and fully differentiated C_4 M and BS chloroplasts at the leaf tip (Li *et al.*, 2010). C_4-specific genes were recruited from existing C_3 genes to encode the components necessary to the C_4 mechanism. A true C_4 cycle requires the co-ordinated activity of multiple enzymes in different cell types and in response to diverse environmental and metabolic stimuli. The selective expression of specific C_4 pathway genes in specific cell types requires multiple levels of regulation. Although cis-acting DNA elements are important for gene regulation, chromatin configuration also plays a vital role. The expression of PEPC has been linked to epigenetic control, histone modification and chromatin remodeling in maize (Danker *et al.*, 2008). These histone modifications significantly contribute to gene regulation by acetylation of specific histone lysine residues. Interestingly, this pattern is not dependent on gene activity, but is already established in etiolated plants. Cell-type specific chromatin modifications potentiate subsequent light activation of transcription during differentiation of photosynthetic tissues in C_4 plants.

There is another gene regulatory mechanism which is likely to be involved in functioning of C_4 specific features: the down-regulation of gene expression through non-coding RNAs (ncRNAs). Among various ncRNA types, the micro RNAs (miRNAs) are relatively well characterized and often show conservation across plant and animal species (Jones-Rhoades *et al.*, 2006). Since majority of miRNA families have been reported to be involved in plant development including the vascular development (Jones-Rhoades *et al.*, 2006; Rubio-Somoza and Weigel, 2011), their role in development of Kranz anatomy and down-regulation of photosynthesis related genes cannot be ruled out. To investigate this aspect, small RNA sequencing of appropriate leaf samples is being

pursued within the C_4 consortium. The sequencing data will further be analyzed to discover novel and existing miRNAs, for which a protocol is in place for high accuracy prediction of plant miRNAs from deep-sequencing data (Thakur *et al.*, 2011).

MOLECULAR ENGINEERING APPROACHES TOWARDS DEVELOPMENT OF C_4 RICE

Introducing C_4 photosynthesis genes into rice: In the past there have been attempts to transfer C_4 traits into C_3 plants by conventional plant hybridization between the two species. This approach was useful for a limited number of plant genera such as *Atriplex*, *Brassica*, *Panicum*, *Moricandia* and *Flaveria* (Brown and Bouton, 1993). Unfortunately, most of those C_3-C_4 hybrids showed infertility due to abnormal chromosome pairing and other genetic barriers. Because many of the major traits associated with C_4 photosynthesis are absent from all rice species assessed to date and wide hybridization between sorghum and rice, oat and maize failed to transfer the C_4 cycle as a whole, the use of conventional breeding to achieve this goal seems unlikely. Therefore, a genetic engineering approach seems to be the most appropriate technology to transfer C_4 traits into C_3 plants. Although isoforms of genes encoding C_4 enzymes are also present in C_3 plants, they are usually expressed at very low levels and in the wrong cell types. Recent developments in plant molecular biology and genetic engineering have made it possible to introduce the desired genes encoding C_4 enzymes into C_3 plants using transgenic techniques (Matsuoka *et al.*, 2001; Miyao *et al.*, 2011). These efforts have deepened our understanding of the mechanism of C_4 photosynthesis and provided valuable information about the functions and evolution of these C_4 genes. This has enabled scientists to express enzymes involved in the C_4 pathway at high levels comparable to C_4 species and in desired locations even in the leaves of C_3 plants. C_4 photosynthesis depends on synchronized division of labor between M and BS cells which is achieved by differential expression of the genes encoding the enzymes and transporters of the C_4 pathway. Based on primary C_4 acid decarboxylating enzymes used, the C_4 pathway is divided into 3 subtypes: NAD-malic enzyme, NADP-malic enzyme and PEP carboxykinase types (Huber and Edwards, 1975). In a typical NADP-ME C_4 type plant, e.g maize, 21% of genes are differentially expressed between BS and M cells (Li *et al.*, 2010). Promoters with BS or M specific activity from the C_4 grasses can be used to drive tissue specific transgene expression in rice leaves. For example, the promoter of PEPCK gene from *Zoysia japonica* fused with β-glucuronidase expressed selectively in vascular tissues and BS cells of transgenic rice (Nomura *et al.*, 2005). This result demonstrates that some of the C_4 specific genes localized in BS cells can retain their property of cell specificity even in a C_3 plant suggesting that C_3 plants still possess a regulatory mechanism for gene expression of BS cell specific C_4 genes at their correct sites.

Agrobacterium mediated transformation of immature embryos of *indica* rice varieties has proven to be highly efficient (Hiei and Komari, 2006). This has made it possible to introduce C_4 genes into the *indica* rice varieties unlike in the past where most of the transgenic plants with C_4 genes were developed using *japonica* varieties such as Kitaake (Ku *et al.*, 1999; Fukayama *et al.*, 2001; Taniguchi *et al.*, 2008). One of the major objectives of developing C_4 rice is to enable it to perform efficient photosynthesis under higher temperature and reduced water conditions. As *indica* rice varieties are more widely cultivated under such conditions, introduction of the C_4 pathway is more beneficial. We have chosen IR64, a high yielding *indica* variety to insert the well characterized C_4 genes such as PEPC, PPDK, NADP-ME and MDH from maize following the *Agrobacterium* mediated genetic transformation protocol of Hiei and Komari (2006). The C_4 cycle cannot be established only with these core C_4 enzymes (Miyao *et al.*, 2011). Therefore, we aim to

Table 1: Summary of genes being transformed into rice to build NADP-ME type of C_4 photosynthesis

Gene name	Function	References
Carbonic anhydrase (CA)	Conversion of CO_2 to bicarbonate (HCO_3^-) in the cytosol of M cells	Ku *et al*. (1996)
Phosphoenolpyruvate carboxylase (PEPC)	Catalyses the formation of oxaloacetate (OAA) using HCO_3^- and Phosphoenolpyruvate (PEP) in M cells	Ku *et al*. (1996)
Dicarboxylate translocator 1 (DiT1)	Exchanges OAA with malate in M cells	Taniguchi *et al*. (2000)
2-oxoglutarate/malate transporter (OMT)	OAA transporter across the chloroplast membranes of M cells	Taniguchi *et al*. (2000)
Malate dehydrogenase (MDH)	Catalyses the reduction of OAA to malate in chloroplasts of M cells	Agostino *et al*. (1992)
Dicarboxylate translocator 2 (DiT2)	Exchanges Glutamate with malate in BS cells	Brautigam *et al*. (2011)
NADP-malic enzyme (NADP-ME)	Decarboxylation of malate in chloroplasts of BS cells	Ku *et al*. (1996)
Mesophyll envelope protein 1 (MEP1)	Transport of pyruvate in chloroplasts of M and BS cells	Brautigam *et al*. (2011)
Pyruvate, orthophosphate dikinase (PPDK)	Conversion of pyruvate to PEP in chloroplasts of M cells	Ku *et al*. (1996)
Phosphoenolpyruvate/phosphate translocator (PPT)	Imports PEP from cytosol	Brautigam *et al*. (2011)
Triosephosphate/phosphate transporter (TPT)	Transports triosephosphate in a 1:1 counter exchange with phosphate	Brautigam *et al*. (2011)
Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)	Refixation of CO_2 in chloroplasts of BS cells	Hatch (1987) and Kanai and Edwards (1999)

ensure a proper facilitation of exchange of metabolite fluxes into and out of the M and BS cells by inserting the appropriate transporters of the C_4 pathway (Table 1). Efficient functioning of the induced C_4 cycle in rice will call for a cell type specific suppression of some of the endogenous rice genes. The obvious one is RuBisCO which will be down-regulated in the M cells and over-expressed in BS cells. Other such genes include the subunits of glycine decarboxylase that are involved in the photorespiration.

FOREIGN GENE STABILITY IN THE RICE GENOME

Evidence of stability of alien C_4 genes and their expression have been reported by a number of earlier attempts to place C_4 genes into C_3 plants such as rice (Taniguchi *et al*., 2008), *Arabidopsis* (Ishimaru *et al*., 1997) and tobacco (Gallardo *et al*., 1995). In rice, several C_4 genes have been successfully transformed from maize and other closely related C_4 species that have stably integrated into its genome and expressed over several generations (Taniguchi *et al*., 2008). Furthermore, we have T_3 transgenic rice plants harbouring PPDK and PEPC genes from maize that have been constantly expressing the *ZmPPDK* and *ZmPEPC* protein over the three consecutive generations. Introducing the C_4 genes from phylogenetically close species aids stable integration and expression of the transgenes in the host genome.

PYRAMIDING OF C_4 GENES

Transfer of C_4 photosynthetic metabolic pathway to non-C_4 species would not be complete unless all associated genetic factors are fully inserted in one plant. Multiple transgenes can be stacked in a single plant by crossing of individual transgenic lines and/or by sequential transformation. This strategy of transforming one gene at a time, generating homozygous lines for each and then successive rounds of crossing or sequential transformations to pyramid the necessary C_4 genes in rice might prove to be very time and labour consuming process. Moreover, multiple integration sites would further complicate production of homozygous lines. Once each gene has been transformed

and tested in isolation, a multigene engineering approach could be very useful to simultaneously transfer many of the C_4 genes into rice that would allow us to express multimeric proteins and study the complex genetic regulations. Emerging techniques such as artificial plant chromosome engineering (Naqvi *et al*., 2009), recombination-assisted multifunctional DNA assembly platform (RMDAP) (Ma *et al*., 2011), transcription activator like (TAL) effectors (Scholze and Boch, 2011) and zinc finger nuclease (Zeevi *et al*., 2012) could be applicable. Although none of the above technologies have yet been extensively tested in rice, availability of these novel tools offers new avenues for the C_4 rice engineering program.

Transgenic plants with stable and appropriate level of expression of introduced C_4 genes (Table 1) will be intercrossed. A prototype C_4 rice plant will contain all the known C_4 genes. To ensure proper functioning of the engineered C_4 cycle, it will require detailed molecular, biochemical and physiological characterizations. The biochemistry will need to be assayed for correct level of enzyme function and carbon fixation in order to confirm a successful C_4 cycle activity. The major physiological processes, such as gas exchange and efficiency of photochemistry will need to be analyzed. The proteome, transcriptome and metabolome should also be checked to ensure that the C_4 metabolic system is well in place.

CONCLUSIONS

Given the access to advanced technologies and sustainable funding, two decades should be enough time to produce C_4 rice. However, the immediate need for the next green revolution, volatile rice markets and intensive media coverage is compelling researchers to try even harder for a faster output. The C_4 rice project under the aegis of C_4 consortium has successfully completed the first phase during which molecular tools development, infrastructure development, recruitment of scientists and researchers were completed. More importantly, mass screening of sorghum mutants and establishment of efficient rice transformation system were accomplished. The target in coming years is to transform rice with novel C_4 genes and pyramid all C_4 genes into a prototype either by multigene transfer or by multiple crossing or both.

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