Engineering A C4 Rice

William Paul Quick

C4 Rice Center, International Rice Research Institute, Los Baños, Philippines. Email: W.P.Quick@Irri.Org

Abstract

The demand for food of the growing population continues to increase over the years. Rice being the staple food of most Asian countries has not reached its full potential being a C3 crop. C4 plants on the other hand have a higher photosynthetic capacity and are more efficient in utilizing water and nutrients. Our research is a part of a large multinational consortium tasked with introducing C4 characteristics into rice. This seminar provides an overview of the many approaches employed by the C4 Rice Consortium: namely, metabolic C4 engineering and identification of determinants of leaf anatomy by mutant screens. The aim of the metabolic C4 engineering approach is to generate a two-celled C4 shuttle in rice by expressing the classical enzymes of the NADP-ME C4 cycle in a cell-appropriate manner. The aim is also to restrict RuBisCO and glycine decarboxylase expression to the bundle sheath (BS) cells of rice in a C4-like fashion by specifically down-regulating their expression in rice mesophyll (M) cells. In addition to the changes in biochemistry, two-celled C4 species show a convergence in leaf anatomy that include increased vein density and reduced numbers of M cells between veins. By screening rice activation-tagged lines and loss-of-function sorghum mutants we endeavour to identify genes controlling these key traits. In this paper, we outline the strategy being adopted by the C4 rice consortium to engineer a more efficient photosynthetic pathway into rice. We also summarize related research in this area.

Keywords: C4 photosynthesis, rice, genetic engineering

Introduction

Rice is an important staple for half of the world's population and the major increases in human population will occur mainly in the rice-consuming countries. Failure to meet the rising rice demands of Asia will result in misery for hundreds of millions, the slowing down of economic development, and quite probably political instability. Furthermore, during this century, it is widely predicted that climate change could adversely affect agriculture in many developing countries (Easterling 2007). An increase in the frequency of weather-related disasters, driven by climate change, could seriously damage future rice production. Given that water and nitrogen use efficiencies have to increase and that future crops are likely to grow in a hotter world, how can the projected food demands of the developing world, and in particular Asia, be met?

Experimental and theoretical analyses of the rice crop indicate that the required increase in yield potential cannot be achieved with rice using the C3 photosynthetic pathway. We have a unique and historical opportunity to use the new molecular tools to change a single mechanism in the rice plant and thereby simultaneously improve yield, water use efficiency, heat tolerance and nitrogen use efficiency. By redesigning rice photosynthesis and eliminating photorespiration, we could achieve a number of important goals that impact on the poor. Yields in all rice ecosystems would rise. Water use efficiency would increase substantially, bringing benefits to the poorest farmers in rainfed and upland rice ecosystems. The water requirements of irrigated rice would fall and so protect yields in the irrigated systems that feed the urban poor, crucial at a time of increased competition for water. Nitrogen use efficiency would rise significantly, which would greatly assist poor farmers and contribute to the reduction of a range of nitrogen pollutants including emissions of nitrous oxide, a powerful greenhouse gas. It is useful to demonstrate that such improvements would not only help solve current humanitarian problems but prevent future ones emerging. This paper gives a brief description of the C4 Rice Project and discusses recent developments in the C4 engineering efforts.

Review of recent advances in research

In the majority of plants, including rice, CO_2 is first fixed into a compound with three carbons (C3) by the photosynthetic enzyme ribulose bisphosphate carboxylase oxygenase (Rubisco) - this is known as C3 photosynthesis. Rubisco is inherently inefficient because it can also catalyze a reaction of its substrate with oxygen in the air, giving a wasteful process known

as photorespiration (rather than photosynthesis). At temperatures in excess of 20°C, there is increasing competition by O_2, with a dramatic reduction in CO_2 fixation and photosynthetic efficiency. Thus, in the hot tropics where most rice is grown, photosynthesis becomes very inefficient.

The C4 pathway involves the initial fixation of atmospheric CO_2 into C4 acids using an enzyme that is insensitive to O_2. In the next stage of the pathway, CO_2 is released from the C4 acids for fixation by Rubisco. The two stages are spatially separated, allowing a high concentration of CO_2 in the vicinity of Rubisco. The buildup of CO_2 by this "CO_2 pump" requires extra energy from sunlight and therefore it is only in warm climates that the C4 pathway is beneficial. The two stages of C4 photosynthesis are partitioned in morphologically distinct photosynthetic cells. In C4 grasses such as maize and some C4 dicots, enlarged bundle sheath (BS) cells surround the veins (V) and the BS cells are then surrounded by mesophyll (M) cells. Each pair of veins is thus separated by two bundle sheath and two mesophyll cells in a V-BS-M-M-BS-V pattern referred to as Kranz anatomy. Furthermore, the specialized organelles carrying out photosynthesis (chloroplasts) are very different in these two cell types. Mesophyll cell chloroplasts support the capture of CO_2 by the C4 cycle, whereas BS chloroplasts support fixation of CO_2 by Rubisco. The anatomy of C3 plants is typically a V-BS-M-M-M-M-M-M-M-M-BS-V pattern; the BS cells are smaller than in C4 plants and they contain few chloroplasts. However, there is variation in vein spacing in C3 species and differences in C4 plants have been observed between photosynthetic organs (e.g., between the husk and foliar leaves in the C4 plant maize). A fully functional C4 pathway requires a coordinated change in tissue structure and metabolic biochemistry. In an evolutionary context, the transition from C3 to C4 photosynthesis has occurred independently in more than 60 different plant taxa (Sage 2011). This provides hope that humanity can replicate the process using genetic engineering.

There have been attempts to transfer C4 traits into C3 plants by conventional plant hybridization between two species. This approach was useful for a limited number of plant genera such as *Atriplex*, *Brassica*, *Panicum*, *Moricandia*, and *Flaveria* (reviewed in Brown and Bouton 1993). Unfortunately, most of the C3-C4 hybrids showed infertility due to abnormal chromosome pairing and other genetic barriers. Because many of the major traits associated with C4 photosynthesis are absent from all rice species assessed to date and wide hybridization between sorghum and rice, oat and maize failed to transfer the C4 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 C4 traits into C3 plants.

Genomic and transcriptional sequence comparisons of cell-specific and leaf-developmental gradient transcription profiles are being used to identify C4 specific regulatory genes (Langdale 2011). In a recent study, four key genes of the C4 pathway were introduced into rice mesophyll cells to generate a C4 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 a C4 type photosynthesis in C3 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 C4 biochemistry and anatomy. This requires discovery of 1) additional genes working in coordination with the known C4 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.

Although isoforms of genes encoding C4 enzymes are also present in C3 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 C4 enzymes into C3 plants using transgenic techniques (Matsuoka et al 2001; Miyao et al 2011). These efforts have deepened the understanding of the mechanism of C4 photosynthesis and provided valuable information about the functions and evolution of these C4 genes. This has enabled scientists to express enzymes involved in the C4 pathway at high levels comparable to C4 species and in desired locations even in the leaves of C3 plants.

C4 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 C4 pathway. Based on primary C4 acid decarboxylating enzymes used, the C4 pathway is divided into 3 subtypes: NAD-malic enzyme, NADP-malic enzyme and PEP carboxykinase types (Huber and Edwards 1975). In a typical NADP-malic enzyme C4 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 C4 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 C4 specific genes localized in BS cells can retain their property of cell specificity even in a C3 plant suggesting that C3 plants still possess a regulatory mechanism for gene expression of BS cell specific C4 genes at their correct sites.

Comparison of gene expression in leaves of C3 and C4 species could lead to the identification of genes that have turned on or off during the evolution of multiple C4 species independently, or genes whose overall expression has significantly changed. There are two previous such studies in closely related dicot species, namely C3 and C4 species of *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 C4 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 down regulation 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 C4 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. A few plastidic and mitochondrial transport proteins were largely up-regulated in C4 leaf tissue (Brautigam et al 2011). Among the proteins with regulatory functions, 43 were significantly upregulated in either C3 or C4 *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 & 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 C4-associated genes.

Transfer of C4 photosynthetic metabolic pathway to non-C4 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 C4 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 C4 genes into rice that would allow expression of 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 C4 rice engineering programme.

Innovative studies underway

The C4 rice project

The C4 rice project was set-up to introduce a C4 photosynthetic pathway into a C3 crop (rice). In October 2008, IRRI was delighted to learn that the Bill & Melinda Gates Foundation (BMGF) had decided to support the C4 Rice Project. The project is a large collaborative program involving sixteen separate organizations world-wide. The members of the C4 Consortium (Table 1) were confident that we had the scientific ability to deliver our objectives. It will take about 15-20 years of coordinated research carried out at IRRI and in the laboratories of the C4 Rice Consortium to deliver C4 rice to plant breeders in the developing world. The C4 Rice project has accomplished its objectives in the 3-year plan for Phase I and has recently received funding approval for the proposed Phase II research program.

Progress in Phase I

While the metabolic process of photosynthesis has been extensively researched and photosynthetic enzymes well studied, most of the genetic factors regulating C4 anatomy are still unknown (Langdale 2011). We have adopted a dual approach to identify these genetic factors.

Table 1. The members of C4 rice consortium

	Affiliation
1. Prof. Gynheung An	Kyung Hee University
2. Dr. Thomas Brutnell	Donald Danforth Plant Science Center
3. Prof. James Burnell	James Cook University
4. Dr. Asaph B. Cousins	Washington State University
5. Prof. Gerry Edwards	Washington State University
6. Dr. Robert Furbank	CSIRO, Council of Scientific and Industrial Research Organization
7. Dr. Julian Hibberd	University of Cambridge
8. Dr. Yue-Ie Hsing	Academia Sinica Taiwan
9. Dr. Steven Kelly	University of Oxford
10. Prof. Jane Langdale	University of Oxford
11. Prof. Richard Leegood	University of Sheffield
12. Dr. Erik Murchie	University of Nottingham
13. Dr. Chris Myers	Cornell University
14. Dr. William Paul Quick	IRRI, International Rice Research Institute
15. Prof. Rowan Sage	University of Toronto
16. Prof. Tammy Sage	University of Toronto
17. Dr. John Sheehy	IRRI, International Rice Research Institute
18. Prof. Susanne von Caemmerer	Australian National University
19. Prof. Daniel Voytas	University of Minnesota
20. Prof. Peter Westhoff	Heinrich Heine University Düsseldorf
21. Dr. Su-May Yu	Academia Sinica Taiwan
22. Dr. Xinguang Zhu	Shanghai Institutes for Biological Sciences

The first approach is to mutate C4 plants (sorghum) to randomly hit some of the C4 characteristics and to identify the responsible genetic factors. The second approach is to use rice DNA activation tagging to over-express random rice genes and look for C4–like characteristics. Both of these approaches require screening of large populations to find desirable phenotypes.

Work in the first year of the project was focused on generating mutant plant populations and one major component was the creation of two very large populations of mutagenized sorghum. The second year has focused on screening these mutagenized populations and our focus has been on leaf anatomical screens. For mass screening of these populations, we searched for induced alterations in leaf vein density (VD). The veins of C4 plants are generally much more closely spaced than C3 plants. This is required to facilitate the precise spacing of mesophyll and bundle sheath 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 mesophyll cell number between the veins, altered BS size and number or increased vein size. All of these are important changes that have occurred in C4 plants and so identification of genes that disrupt or induce these changes in C4 (sorghum) or C3 (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. The groups working in Australia, USA, and the UK focused on the detailed phenotyping of the different rice and sorghum backgrounds. Detailed photosynthetic and anatomical characteristics have been established and protocols developed for screening mutant lines. Antibody generation has been started and is in use for validation of transgenic lines. These antibodies have been transferred to other members of the consortium and are currently being used to test cell specificity of bundle sheath and mesophyll-specific promoters using immunolocalization of embedded sections.

Generation of the transgenic lines with altered C4 biochemistry is spearheaded by groups in the UK, Germany, and IRRI. The first batch of 18 transgenic constructs have been transformed into rice, 6 more are in progress. Lines are now being grown to identify homozygous lines, to increase seed stocks for distribution to the consortium and to cross lines for gene stacking. The main C4 biosynthetic pathway genes have now been expressed in rice and the next molecular part is to insert the metabolite transporters.

The Bioinformatics and Metabolic modeling teams in China (Shanghai Institutes for Biological Sciences), IRRI and USA (Cornell) have been collaborating closely to provide excellent bioinformatic support to the C4 project and to develop new insights into unforeseen areas of C4 engineering requirements through development of mechanistic models. We have developed a highly mechanistic model of C4 photosynthetic metabolism and a reaction diffusion model of single-cell C4 photosynthesis. Using these models, we identified a number of critical design features required for high efficiency of C4 photosynthesis: a) PGA and T3P transport between bundle sheath and mesophyll cells are critical to maintaining a high C4 photosynthetic efficiency due to its role in balancing ATP and NADPH generation in these two cell types; b) the leakage rate between bundle

sheath cells and mesophyll cells needs to be considered together with the metabolite transport rate between them to gain a high efficiency of CO_2 uptake; and c) both the amount and location of carbonic anhydrase inside cells are critical for an efficient CO_2 concentrating mechanism.

The recent advances in high throughput sequencing (not foreseen during the conception of this project) have meant that the consortium has invested considerable effort in applying this technology to their research within and without of the C4-Rice project. To facilitate this, we have developed a set of critical informatics tools to support high throughput RNA-seq data analysis: Plant miRNAs have many features different from animal miRNAs; we have developed models with the statistical features that allow plant miRNA prediction and developed a user friendly and publicly accessible platform for plant miRNA prediction. We have developed pipelines to identify enriched motifs in promoter regions of chosen gene sets (several have been produced within the consortium). We have developed an RNA-seq assembly pipeline for non-model species and started using the pipeline to study the RNA-seq data from C3 and C4 related species produced by the 1000 transcriptome project. Currently, these pipelines are not only used within the C4 rice consortium, but are also under discussion to be deployed by the iPlant Collaborative (http://www.iplantcollaborative.org) to enable their application to the larger plant research community.

Phase II Plans

Our proposal for Phase II builds on our current successes; develops ongoing work and incorporates several new research avenues brought on by scientific developments. The range of key activities covered within the C4 program for Phase II are highlighted in Fig. 1.

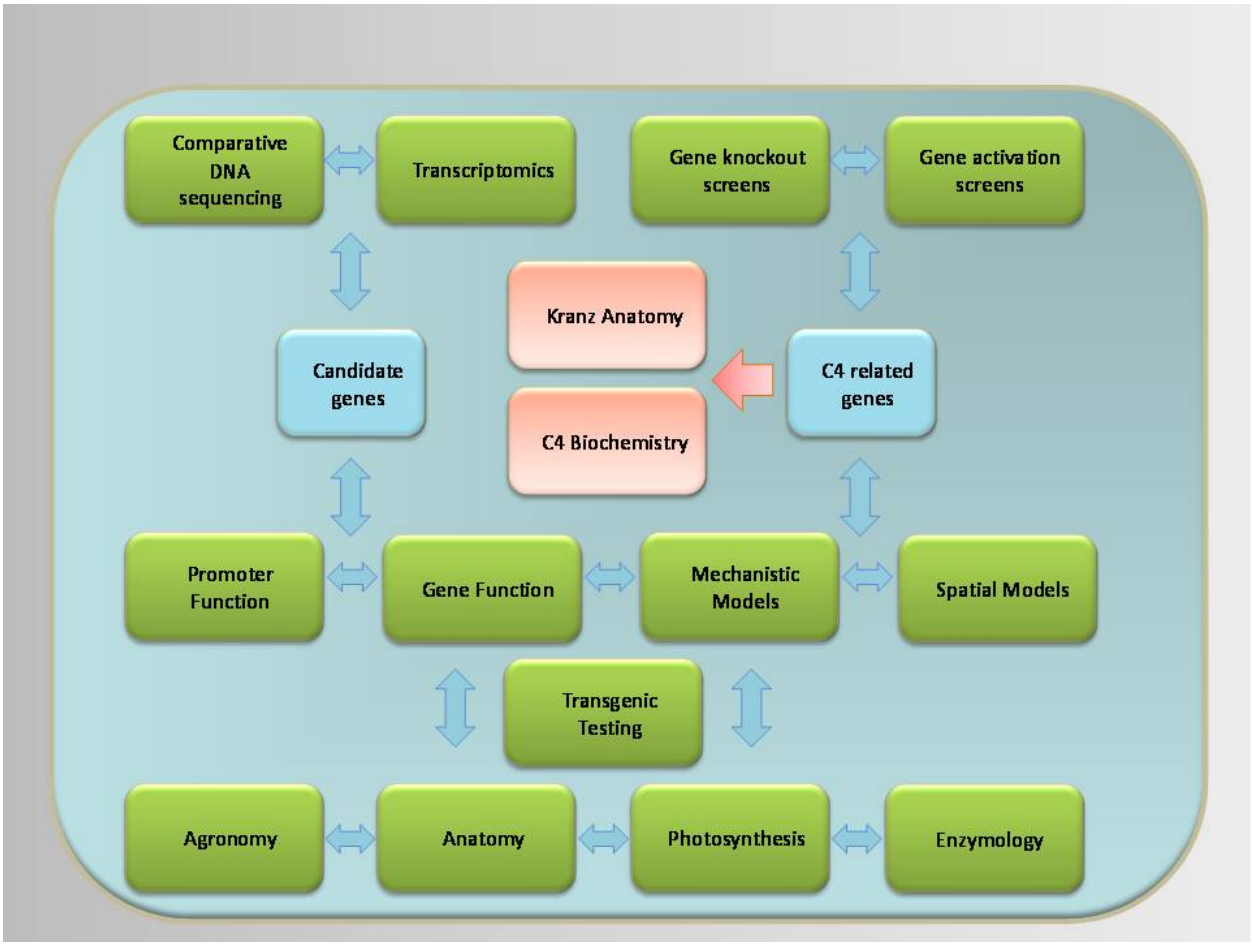

Figure 1. The range of key activities in the C4 Phase II program incorporating a systems biology approach to the C4 rice research.

This clearly demonstrates our ambition to incorporate a more systems biology approach to our research where all activities and groups are linked together to serve the common goal of producing a C4 rice plant and to utilize multi-disciplinary approaches to inform this complex engineering program.

A circular bidirectional flow of information allows critical testing and exploitation of research results. Whilst we endeavor to exploit our current knowledge towards our end goal, it is also realized that if we are to be successful in building fully functional C4 rice, then a significant aspect of the C4 project also needs to be generating new knowledge and gene discovery. It is important therefore that the two processes of engineering and generation of new information are tightly coupled. The bioinformatics of transcriptomes and the screening of mutagenised or transgenically modified populations of rice and *Setaria/Sorghum* are central to generation of new data in both Phase I and II. Transgenic testing and systems modeling provide the means to evaluate and test these results and allow a holistic systems biology approach to contribute to this bio-engineering task.

Ensuing, genetic engineering provides the means to test our models and achieve our objectives. At present, the main focus of the C4 rice project follows two major themes: the introduction of the cell specific function of the two-cell biochemical pathway and alteration of leaf anatomy to allow the correct mesophyll and bundle sheath cell alignment.

The subsequent three years will pursue gene discovery using a variety of approaches that include high-throughput physiology, DNA and RNA sequencing, bioinformatics, mathematical modeling and system to optimize the C4 pathway in rice. We will also assemble the basic C4 pathway in rice to build the first prototypes for testing. Our vision of success is the generation of C4 prototype(s) rice that has the basic biochemical pathway and the acquisition of knowledge required to optimize this pathway in the subsequent phase.

Prospects of achieving the breeding goal

Given the access to advanced technologies and sustainable funding, two decades should be enough time to produce C4 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 C4 rice project under the aegis of C4 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 C4 genes and pyramid all C4 genes into a prototype either by multigene transfer or by multiple crossing or both.

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Citation: Quick WP. 2013. Engineering a C4 rice. In: Muralidharan K and Siddiq EA, eds. 2013. *International Dialogue on Perception and Prospects of Designer Rice*. Society for Advancement of Rice Research, Directorate of Rice Research, Hyderabad 500030, India, pp 329-335.