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Breaking Yield Barriers in Rice: Remodeling Photosynthesis

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Abstract: Yield levels in rice as improved by direct and indirect manipulation of yield and its components by conventional plant breeding have reached a plateau. Since photosynthesis is the least targeted trait for yield enhancement, it can be a potential area for application of biotechnology to harness better yields through genetic manipulation. The key areas of research are plant architecture, duration of photosynthesis, manipulation of PS-I and PS-II, manipulation of Rubisco and driving a C_4 cycle in rice. With the advancement in biotechnology and plant molecular biology, genetic manipulation of these traits especially increasing the efficiency of Rubisco and over expression of C_4 genes in rice have witnessed significant achievements in terms of research and application. The selective advantage of C_4 cycle such as high photosynthetic efficiency, high water and nitrogen use efficiency especially under low CO_2 levels offers a tremendous opportunity for crop modification. Despite obvious limitations with regard to replicating the C_4 cycle in a C_3 plant, rice, which needs gross anatomical changes, the option of engineering C_4 enzymes into rice is really workable. The challenges are enormous, but the opportunities are even more.

Key words: Rice, photosynthesis, C_4 cycle, Rubisco, transgenics

Introduction

Photosynthesis is perhaps the most important process in biological system defining the limits of biomass production and consequently the crop yields. It is pivotal to production of food and fibre as it provides raw materials for all plant products. Thus in any crop breeding strategy for higher yield, we are invariably breeding for higher net photosynthesis. Recent advances in plant molecular biology have led to better understanding of plant metabolic pathways and opened up several areas which are amenable for genetic manipulations aimed at directed changes in plant process culminating in higher yield potential.

The majority of crop plants including rice, assimilate the atmospheric CO_2 via a photosynthetic pathway in which a 3-carbon compound (3 phosphoglyceraldehyde) is the first stable intermediate formed and are thus designated as C_3 plants. This process is called as calvin cycle or photosynthetic carbon reduction (PCR) cycle. In C_3 plants, all the photosynthetic reactions from light capture to assimilation of carbon into carbohydrates take place in chloroplasts of mesophyll cells. The primary enzyme catalyzing CO_2 fixation is Ribulose-1,5-biphospate carboxylase (Rubisco) which is incidentally the most abundant protein on earth (Parry *et al*., 2003). The C_3 plants grow well in temperate climates, but at higher temperatures, the affinity of Rubisco increases for oxygen relative to CO_2, which results in loss of fixed carbon by a process called as photo respiration. Though photo respiration is a mechanism to protect photosynthesis from photo inhibition (Osmond and Grace, 1995), yet it decreases the efficiency of CO_2 assimilation in C_3 cycle.

In contrast to C_3 cycle, some plant species such as maize, sugarcane, sunflower etc have evolved to adapt to the lower CO_2 concentrations in atmosphere (Ehleringer and Manson, 1993) and have higher photosynthetic efficiency than C_3 plants. They are designated as C_4 plants because the first

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stable product of carbon fixation pathways is a 4-C compound such as oxalate or malate. The leaves of C_4 plants have a unique feature called as Kranz-Anatomy in which the photosynthetic enzymes are present in both mesophyll and bundle sheath cells. The enzymes of C_3 cycle are localised in bundle sheath cells whereas the enzymes of C_4 pathway are present in mesophyll. The three dicarbox-ylation enzymes of C_4 cycle are NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenol pyruvate carboxykinase (PEP-CK). The type of carboxylation enzyme present is a criterion to classify C_4 plants into three sub-types. Accordingly maize and sugarcane use NADP-ME as major decarboxylation enzyme and are classified as NADP-ME type. The other enzyme which are an integral part of C_4 cycle are ; NADP-malate dehydrogenase (NADP-MDH) which reduces oxaloacetate to malate and Aspartate aminotransferase (ASP-AT) which transaminates oxaloacetate to Aspartate during the export of C_4 acid from mesophyll to bundle sheath. Regeneration of pyruvate is catalysed by pyruvate orthophosphate dikinase (PPDK) located in chloroplasts of all C_4 type plants. Hatch (1987) outlined three basic steps of C_4 cycle as :

- Fixation of CO_2 in cytosol of mesophyll cells by PEPC to form 4-C acid such as Oxaloacetate.
- Decarboxylation of C_4 acid in bundle sheath cells either by NADP-ME, NAD-ME or PEP-CK with subsequent release of CO_2.
- Regeneration of pyruvate (PEP) by PPDK.

Transfer of C_4 Traits to C_3 Plants-The Concept

Given the difficulties in improving the yield ceilings by manipulation of inputs, it is believed that the second 'Green revolution' will more heavily be driven by the development of cultivars with better yielding ability. Thus increasing the yielding ability is an important if not vital, part of any strategy aiming to achieve the increased yield (Khush and Peng, 1996). The important components of such breeding approach are manipulation of harvest index and net photosynthesis. Since the harvest index in crops like rice is approaching a ceiling value (Horton, 2000), the increase in yield must come from higher net photosynthesis.

Since the establishment of the fact that C_4 cycle is more efficient than C_3 cycle, it was conceptualized to transfer C_4 traits to C_3 plants to improve the photosynthetic efficiency of C_3 plants (Miyao, 2003). The initial efforts in this direction by conventional crossing between C_4 and C_3 plants were seriously limited by the infertility of C_3-C_4 hybrids (Brown and Bouton, 1993). With the advent of recombinant DNA technology, gene transfer between desperate crop species has become possible. Significant advances have been made in our understanding of the process of photosynthesis and isolation of genes encoding enzymes of C_4 pathways. A number of efficient transformation protocols are now available in rice. Thus the concept of re-designing photosynthesis in rice for higher yields does offer great opportunities.

Redesigning Rice Photosynthesis- The Rationale

Since the rediscovery of mendelian principles, the genetics based crop breeding has led to a significant improvement in crops such as rice, wheat and maize in terms of increased yields. However, there are two surprising aspects of such yield increases (Richards, 2000).

- The greater understanding of photosynthesis has not yet contributed significantly to yield increases.
- The genetic increase in the rate of photosynthesis has not been required to achieve the increased productivity.

The increase in yields has been made possible either by increased/extended photosynthesis per unit land areas or increased partitioning of crop biomass to the harvested product. The first has been achieved by improved cultural practices such as irrigation, fertilization and of course increased CO_2 levels in the atmosphere. The latter has been made possible by breeding for higher harvest index, but as stated earlier, the ceiling limits of harvest index in rice have almost been achieved. Thus a strategy to redesign the photosynthesis in rice, basically a C_3 plant is an area, which can be worked upon to boost up rice productivity to meet booming population increases.

The tools of genetics based plant breeding which resulted in great increases in rice yields have seemingly, lost their edge. Bold efforts to bioengineer crops seems the only hope for a surge in harvests (Mann, 1999a). The evolutionary change or selection have not brought any fundamental changes in photosynthesis as the breeders have primarily focused on pushing harvest index of rice as high as they could thus reengineering photosynthesis is the great white hope of future agriculture because this area of crop physiological tools may provide efficient options for manipulating various structural and physiological components of photosynthesis to realize a new surge in crop productivity.

Recent comparative studies in various crops especially Gramineae have revealed a high degree of conservation of gene order and content in grass family. The biochemical studies had revealed that C_4 enzymes are specific to C_4 plants and follow different kinetics but recent studies have shown that C_3 plants like rice do posses C_4 specific genes but the expression level is too low to be detected. Based on such studies, Ku *et al*. (1996) postulated that C_4 genes have evolved from C_3 genes with modifications in expression in leaves and enzyme kinetics. In fact highly homologous forms of maize PPDK (Pdk-1 and Pdk-2) have been found in rice (Sheen, 1991; Imaizumi *et al*., 1997). Moons *et al*. (1998) isolated a C-DNA clone from rice roots which was highly homologous to maize Pdk-2. The evolution of C_4 specific maize pdk-1 and pdk-2 is shown in Fig. 1. The common evolutionary history suggests that in order to alter the photosynthetic carbon metabolism in C_3 plants, C_4 enzymes have to overproduced in these cells. Thus introduction of intact C_4 specific genes into C_3 plants would lead to high level cell-specific expression of C_4 enzymes. This has been experimentally demonstrated in rice by over expression of C_4 genes from maize (Ranade *et al*., 1998; Ku *et al*., 1999) and *Urochloa panicoides* (Suzuki *et al*., 2000).

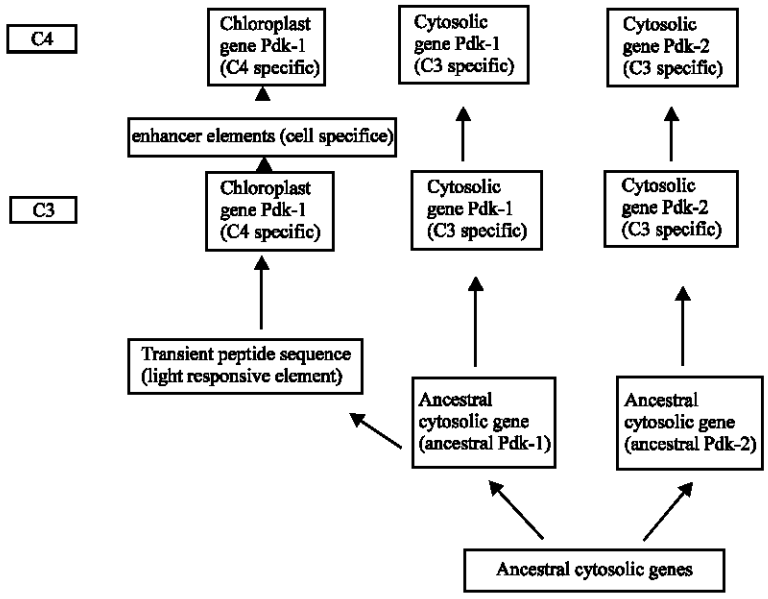

Fig. 1: Schematic pattern of evolution of C4 genes from ancestral C3 genes (modified from: Miyao, 2003)

Target Traits/Genes for Genetic Manipulation

Photosynthesis as a process is so complex that identification of individual components that can be modified by genetic manipulation is not an easy task. From the initial process of light capture to CO_2 fixation to carbohydrate accumulation, various components of this process are linked in a complex manner where the components interact with each other. The regulatory mechanisms operating upon these components culminate in a balanced process so as to have control concentrations of intermediates produced during the process (Horton, 1994). Such regulatory mechanisms control the excitation energy of Ps-1 and Ps-II in the beginning and partitioning of storage carbohydrate and sucrose export to sink tissue. However, the regulatory mechanisms operate on the process falling between these input-output terminals (Horton, 2000). Thus any approach aiming at genetic fine tuning of photosynthesis or its sub-systems should analyze the process in detail in order to pin point the areas of limitation under different conditions which can be modulated by genetic manipulation. This should include:

- Identification of photosynthetic losses, in order to identify areas which limit the plant's capacity to achieve maximum photosynthetic efficiency under field conditions.
- Study of canopy structure and leaf orientation to evolve a plant type for better light capture.
- Study of light saturation. It has been found that despite erect leaves, photosynthesis of rice leaves is light saturated for several hours of the day and thus represents an important photosynthetic loss.
- Study of lea temperature, diurnal depression in photosynthesis and photo inhibition.

A number of selectable traits for efficient manipulation of photosynthesis have been identified:

Plant Architecture

The new plant type of rice is characterized by erect leaves, which facilitate greater light penetration to lower leaves thereby optimizing canopy photosynthesis (Duncan, 1971). The erect leaf architecture provides more open canopy as compared to some existing indica rice cultivars such as IR72. This not only lowers the light saturation of upper leaves but also the lower leaves receive sufficient radiation to drive photosynthetic process significant enough to make a difference in total daily photosynthesis (Sinclair *et al*., 2004). Thus genetic manipulation involving plant architecture would seek to identify genes which confer such characteristic to rice plants. Peng (2000) stated that even though there is a correlation between canopy net photosynthesis and biomass production but the present high yielding rice varieties bred through conventional breeding are close to optimum canopy architecture. Thus biotechnology can help modify plant canopy to optimize net canopy photosynthesis for higher biomass production.

Duration of Photosynthesis

Extending the duration of crop photosynthesis is perhaps the simplest genetic way to enhance total photosynthesis, crop biomass and yield. This is due to increase in available solar radiation during crop growth period (Richards, 2000). In case of rice it has been found that crop biomass increases by 0.2 t ha^{-1} for each growth day extended. The extension in crop duration means delaying the leaf senescence. Since the stay green phenotype is conditional by several hormones (Cytokinin and ethylene) and N and C status of plant, genetic manipulation of such process can be anticipated to bring substantial increase in biomass production. In fact genetic manipulation of cytokinin synthesis in tobacco resulted in delayed senescence and increase growth rate (Gan and Amasino, 1995). Similarly in tomato, antisense suppression of ethylene synthesis led to delayed senescence but no change in biomass production (John *et al*., 1995). It has been argued that in order to manipulate leaf longevity for modulation of crop photosynthesis, various factors have to be considered, especially N-economy, water supply, critical growth periods (Horton, 2000; Richards, 2000). The manipulation of crop phenology should be such that periods of high radiation should coincide with critical growth stages.

Similarly, maximizing growth when conditions are cool and vapour pressure deficit is low will increase WUE and biomass production. Another important factor is that photosynthesis per unit N should be enhanced as well as increase leaf nitrogen because maximum photosynthesis has been found to be positively correlated with leaf nitrogen (Peng *et al*., 1995). However, there are contrasting arguments on leaf nitrogen enhancement as a target trait for manipulation of photosynthesis, because a high N application associated with high LAI and high respiratory expenditures thus lower rate of photosynthesis (Ying *et al*., 1998).

Light Harvesting Systems (PS-1 and PS-II)

The light harvesting photsystem-1 (PS-1) and Photosystem II (PS-II) are pigment-protein complexes embedded in thylakoid membrane of chloroplasts and function in series to convert solar energy into biologically utilizable form of chemical energy. They posses certain regulatory systems which optimize the delivery of incoming light energy to electron transport systems.

Higher plants certain different sub-units of PS-1 (PS1-G, PS1-H, PSI-N, PSI-O). The functions of most of these sub-units have been determined using antisense, Co-suppression and knock-out technologies to down regulate these nuclear-encoded PS-I polypeptides (Haldrup and Scheller, 2002). They proposed that the transformation technology is an excellent tool to study the function and regulation of sub-units of PS-I and same can be then used to develop transgenic plants able to grow under more extreme conditions such as cold or high light levels.

The PS-II consists of products of six Lhcb genes (Lhcb1-6) assembled in four complexes namely LHCIIa, LHCIIb, LHCIIc and LHCIId. The LHCIIa, LHCIIc and LHCIId are monomeric complexes known as CP29, CP26 and CP24 respectively. The LHCIIb is a trimeric complex (Jansson, 1994).

Both light harvesting systems drive photosynthetic processes that follow light capture. In limiting irradiance, the photosynthesis is allowed to function with maximum efficiency but in high light, the excess excitation energy is converted to heat by a process called Non-photochemical quenching (designated as qE) involving various carotenoid compounds such as violaxanthin and zeaxanthin (Horton *et al*., 1996). A great variability occurs among different species in such rate limiting process. Thus it would be desirable in a crop improvement programme to develop varieties with higher qE in marginal habitats and to reduce or eliminate it in favorable habitats to reduce regulatory costs involved.

The genetic manipulation of the PS-I and PS-II in rice would involve structural or functional changes in components of these light harvesting systems. Zhang *et al*. (1997) reported that 90% reduction in Lhcb 1 protein of PS-1 by the expression of an Lhcb4 gene. Similarly, Horton *et al*. (1999) recorded reduced content of CP29 and CP26 complexes of PS-II by use of antisense expression. All these complexes have possible role in non-photochemical quenching (qE) and thus their manipulation can be effective in alternation of regulatory characteristics of light harvesting. However, a major problem with such a manipulation is that non-expression of any of these light harvesting proteins of PS-I and PS-II might cause structural disruptions in macrostructure of PS-I and PS-II. A possible approach is to effect expression of other Lhcb proteins in these complexes so that only regulatory mechanisms of light harvesting complexes are altered. An alternative strategy is to express designer Lhcb genes in plants to regulate qE (Horton, 2000).

Manipulation of RuBisCo

RuBisCo is the principal enzyme for carboxylation in photosynthesis. It is arguably the world's most important enzyme since nearly all life on earth depends ultimately on its action (Voet and Voet, 1995). It is estimated to be synthesized at the rate of about 4×10^9 tons/year and fixes CO_2 to the tune of 10^{11} tons/year. It consists of 8 large (55 kd each) and 8 small (12 kd each) sub-units in higher plants. The carboxylation activity is associated with larger sub-unit where as the smaller sub-unit has a regulatory function (Sane, 1993). The larger sub-units are plastid derived while as smaller sub-units are nuclear derived. It is the most prevalent enzyme in this planet and constitutes about 40% of total

protein. The X-ray crystallography studies of Branden *et al*. (1991) revealed that the L_8S_8 enzyme has a symmetry of a square prism. The large sub-unit contains catalytic site as is shown by its enzyme activity in absence of small sub-unit. It consists of 477 residues. The residues 1-150 form a mixed five stranded β sheet and residues 151-475 fold into α β barrel. The active site is present at the mouth of this barrel near C-terminus of its β strands. Since photosynthesis as a process is inherently not so efficient, it offers various targets for manipulation to improve it, right from light capture to processing of final product. The bioengineering of RuBisCo offers most appealing target due to several considerations (Mann, 1999 b).

- It is the most abundant protein that initiates biochemical reactions that creates carbohydrates, proteins and fats that sustain plants and other living beings including humans. Thus any attempt at manipulation RuBisCo will surely have significant implications for efficiency of photosynthesis.
- RuBisCo is so slow in its activity that according to T.J. Andrews, it is nearly the worlds worst, most incompetent enzyme. Its slow rate, low affinity for atmospheric CO_2 and use of O_2 as an alternative substrate for competing process of photo respiration makes it very inefficient. But since being a rate limiting factor, it is viewed as one of the potential targets for genetic manipulation of photosynthesis (Natu and Ghildiyal, 2005). Thus manipulation of Rubisco by genetic engineering is a key area of investigation. In fact attempts are being made to produce Rubisco which is 5-times more active in CO_2 fixation (Super RuBisCo). If genetic engineers will be able to develop such enzyme with faster and efficient CO_2 fixation capacity of Rubisco, it will be a major leap towards increasing crop yields by producing more biomass at a faster rate. Besides, since it makes about half of leaf nitrogen, the bulk of N_2 requirements of plants come from the need to produce this vital enzyme. If N_2 demands will be lowered down. Thus plants will have not to invest heavily in Rubisco to fix sufficient carbon (Mann, 1999b). A number of research groups began exploring ways to put in place an efficient Rubisco. An important finding was that Rubisco from various red algae such as *Chromatium vinosum*, *Galdieria sulphuraria* and *Phaeodactylum tricornutum* was 2.5-3 times more efficient even though the reason is not yet known. Transferring such RuBisCo into chloroplasts of higher plants like rice, even though a workable approach is not destined to be successful. It involves engineering of 16 genes and assembling them properly in an enkaryotic genetic background. Besides such a product has to be connected to the regulatory system of chloroplasts. A number of reports about transformation of tobacco with genes encoding large (rbc L) and small (rbc S) sub-units of Rubisco have been published (Table 1).

An efficient CO_2 fixing super-Rubisco was generated by *in vitro* site directed mutagenesis of rbc L from *Chromatium vinosum* (Which is by far the fastest ever Rubisco reported). The mutagenised gene was introduced into the *cyanobacteria* and subjected to selection for transformants under low CO_2 conditions. They could isolate a Rubisco which was five times more actively fixing CO_2 than that of higher plants. However, a major problem concerning engineering efficient Rubisco into higher plants like rice is that the assembly of the polypeptides from nucleus and chloroplast derived subunits into holoenzyme does not take place for effective expression probably due to the differences in chaperone system involved in the assembly of holoenzyme.

An alternative approach aiming at increasing efficiency of Rubisco is manipulation of Rubisco activase (Salvucci *et al*., 1985) which conditions carbamylation of Rubisco, essential for activity of this enzyme. Recently antisense constructs have been used to reduce expression of Rubisco with the objective of saving leaf nitrogen and to determine as to what extent of this enzyme limits the rate of photosynthesis. It has been found that decreasing the Rubisco increases the N-use efficiency both at

Table 1: Manipulation of RuBico in tobacco in terms of expression and activity of rbc genes from various sources (Parry *et al*., 2003)

Introduced genes	Transformed organelle	Protein expression	Protein activity
Tobacco rbc L	Nucleus	+	+
Tobacco rbc S	Chloroplast	+	+
Chromatium vinosum rbc L	Nucleus	-	-
Cyanobacteria rbc L	Nucleus	-	-
Cyanobacteria ebc L	Chloroplast	-	-
Galdieria sulphuraria rbc L and rbc S	-do-	+	-
Phaeodactylum tricornutum rbc L and rbc S	-do-	+	-
Helianthus annus rbc L	-do-	+	+

current and elevated levels of CO_2 (Mitchell and Sheehy, 2000). In rice, the antisense rbc S construct driven by endogenous rbc S promoter resulted in increased photosynthetic rate at high CO_2 concentration for a given leaf N-content (Makino *et al*., 1997). Recently, the rice variety Notohikari was transformed with rice rbc S antisense construct, by particle bombardment, driven by rice rbc S promoter. In some transformants the Rubisco to leaf N ratio was decreased by 30% compared to wild type, but showed 5-15% higher rates of photosynthesis (Makino *et al*., 2000). They also found that the incremental change in photosynthetic rate corresponded exactly with the ratio of decreased fraction of leaf N present as Rubisco. It was thus concluded that rice plants with optimal Rubisco content at elevated CO_2 show higher N-use efficiency of photosynthesis under CO_2 enrichment. However, it was found that such an improvement does not necessarily lead to increased biomass.

- During the evolution of photosynthesis, the atmosphere was virtually devoid of oxygen but oxygen being pumped into it by photosynthesis, Rubisco's weakness was revealed (Mann, 1999b). The enzyme started combining with O_2 rather than CO_2, a process called as 'photo respiration'. Even though, in plants like rice, Rubisco is 100 times more likely to pick up CO_2 than O_2 but higher concentration of O relative to CO takes out this advantage. Thus, if Rubisco's combination with CO_2 incorporates carbon into plant, its opposite reaction virtually drains out carbon from it because photo-respiration produces Glyoxylate which is partly converted to CO_2. It has been found that carbon dioxide loss through this process is 20-25% of the apparent rate of photosynthesis. Thus it offers an area of limitation with Rubisco which can be manipulated by genetic engineering to produce a Rubisco which reacts more specifically with CO_2 than O_2 to prevent loss of CO_2. It is believed that if genetic manipulation could double this specificity, 20% increase in V_{max} can be expected theoretically (Parry *et al*., 2003).

The attempts at manipulation of CO_2 specificity of Rubisco face many problems. First the assembly of larger and smaller sub-units into a hexadecamer holoenzyme following manipulation is problematic. Secondly the X-ray crystallography studies have shown such numerous differences that we can not easily pick up the target difference for manipulation. Thirdly, neither O_2 nor CO_2 binds directly to Rubisco. Even the formation of enediol of RuBP, with which O_2 and CO_2 react directly is same for both oxygenation and carboxylation. Nevertheless many factors influencing specificity have been identified and are target traits for manipulation. One such specificity factor is C-terminal loop 6 region of larger sub-unit. A number of residues of this sub-unit such as valine 331. Lysine 334, Leucine 326, methionine 349, Isolucine 326, leucine 335, alanine 340 etc have been found to affect the specificity but the *in vitro* mutagenesis studies have found that only 3-13% increase in specificity factor occur due to such manipulations. Other studies have reported that position of residues and length and charge of C-terminus also affects specificity. It was found that an extended C-terminus creates additional interaction sites on the protein surface.

The structural manipulations by mutations and genetic engineering have failed to produce a Rubisco with better specificity. Thus it was realized that considerable improvement in Rubisco specificity can be achieved by exploiting natural variation for this enzyme (Parry *et al*., 2003). The

highest specificity factor Rubisco was reported from red alga *Galdieria partita* which is three times than that of crop plants. Other sources from which high specificity Rubisco has been isolated include *Chromatium vinosum, Galdieria sulphuraria, Phaeodactylum tricorntum* and *Helianthus annus.* Significant advances have been made in choroloplast transformation of crops like tobacco which encourage scientists to use such manipulation in rice also. The challenges however are complexities of sufficient expression, post-transnational modifications, interactions and assembly of introduced Rubisco sub-units form foreign sources.

Driving C_4 Photosynthesis in Rice

One of the reasonable and realistic approaches proposed for genetic manipulation of photosynthesis is expression of C_4 photosynthesis genes. On account of its obvious advantages such as greater biomass per unit of intercepted radiations, greater quantum yields, higher rates of CO_2 assimilation and similar protein costs of C_4 enzymes (Evans and Von Caemmerer, 2000), Engineering C_4 cycle in rice offers a compelling opportunity, although there are increased requirements of ATP/NADPH for C_4 cycle (Horton and Murchie, 2000). The Radiation Conversion Factor (RCF) for rice is 2.2 in comparison to a C_4 plant like maize (3.3). It has been proposed that if we can engineer rice to achieve the RCF of that of maize, 50% increase in yield would be conceivable (Mitchell and Sheehy, 2000). Thus engineering rice for C_4 photosynthesis is one of the radical approaches mainly because C_3 cycle suffers from O_2 inhibition due to oxygenase reaction of Rubisco and subsequent loss of CO_2 by photo respiration. (Poolman *et al*., 2000).

A number of C_4 enzymes already described have been identified as target genes for manipulation, especially PEPC, PPDK and PEPCK. Matsuoka and Minani (1989) first cloned the PEPC gene from maize genomic libraries and analysed its primary structure. The sequence of the gene spans 6781 bp and consists of 10 exons and 9 introns. The analysis revealed that it has two sites of initiation of transcription at position-81 and position-84 upstream form first nucleotide. The 5′ flanking region of the gene lacks typical TATA and CCAAT elements but there is a TATA-similar sequence TATTT as well as a homologous sequence of Sp_1 binding site (CCGCCC). The Southern Blot analysis revealed that PEPC exists as a small multigene family because it also contains light response elements but the enzyme is actually encoded by a single-copy in maize genome. The over expression of PEPC is the first step for establishing a C_4 cycle in a C_3 plant like rice (Hausler *et al*., 2002). Although, the first report of transformation with PEPC came in tobacco by introduction of maize PEPC driven by endogenous mesophyll specific chlorophyll a/b binding protein gene (cab) but no apparent increase in CO_2 assimilation were observed even at 2 fold increase in PEPC expression. However, The encouraging finding was that there was no decline in yield at higher temperatures indicating that respired CO_2 had been refixed by PEPC in transformed cells (Hudspeth *et al*., 1992). In rice, the over expression of maize PEPC gene was achieved by Ranade *et al*. (1998), by incorporating intact maize PEPC gene by *Agrobacterium* method and recorded improved photosynthesis at higher temperatures. Ku *et al*. (1999) developed transgenic rice lines containing complete maize PEPC gene (8.8 Kb) driven by its own promoter and recorded 110 fold increase in PEPC activity or 3 fold as compared to maize activity. The idea behind using an intact maize gene was that maize gene promoters drive high level expression of a reporter gene in transgenic rice plants. The maize PEPC gene was inherited in a mendelian fashion and remained active in subsequent generation. The increased activity of maize PEPC gene is accompanied with corresponding increase in protein which accounts for about 12% of total leaf soluble protein in transgenic rice plants. Furthermore, the high level of expression of maize PEPC gene in transgenic rice plants did not interfere with the growth and fertility of most of these plants. The reason for use of PEPC promoter as against CaMV35 S is the phenomenon of gene silencing associated with latter due to its invasive nature which triggers protective measures for maintaining genome integrity. Furthermore the presence of introns in intact maize gene containing introns is also regarded as a reason for such high level expression in transgenic rice plants. Similarly, Suzuki *et al*. (2000) introduced

PEPCK form *Urochloa panicoides* into rice. This enzyme, in addition to decarboxylating oxaloacetate to PEP which acts a substrate for non C_4 PEPC occurring naturally in cytosol of rice mesophyll cells, also provides Rubisco with higher CO_2 environment. It was found that 20% of CO_2 (labeled C_{14}) was incorporated into 4C compound in the transformed lines as compared to 1% in control. However, in this case also, no significant increase in net photosynthetic rate was observed (Table 2). They proposed that co-expression of PEPC and PEPCK in C_3 mesophyll and chloroplasts respectively could bring about such desired results.

Ku *et al.* (2000) using an alternative approach introduced PEPC and PPDK in two transgenic lines of rice and hybridized them to obtain, in segregating generations, a line over expressing both maize enzymes and observed upto 35% higher rates of photosynthesis as compared to 10-30% with PEPC only and 30-35% with PPDK only. The preliminary data revealed as much increase in grain yield over control suggesting that taken together, C_4 genes in rice can substantially increase photosynthesis and yield upon overexpression.

Successful overexpression of maize PPDK in rice resulted in elevated activity when intact C_4 maize PPDK including introns, exons, promoter and terminator sequences was introduced. The enzyme constituted about 35% of leaf protein and had no effect on growth or fertility (Fukayama *et al*., 2001). Similarly another chloroplast enzyme, C_4 maize NADP-ME was expressed at high levels in transgenic rice, driven by CaMV35 S or cab promoter (Tsuchida *et al*., 2001). However, in this case, there was severe decline in growth and chlorophyll content presumably due to excessive NADPH production. Table 3 shows some of the C_4 enzymes, over expressed in transgenic rice plants.

The experimental approaches for transfer of C_4 enzymes from maize and other organisms have revealed following observations, which are of considerable significance :

Table 2: Photosynthetic and Agronomic traits of transgenic rice (Source : Suzuki *et al*., 2000)

Trait	Transformants	Control
Net photosynthetic rate (μ mol CO_2 $m^{-2}scc^{-1}$)	29.50	28.20
CO_2 compensation point (μL L^{-1})	53.90	52.30
Chlorophyll content (mg g^{-1} fresh wt.)	3.92	4.12
Culm length (cm)	60.00	64.15
Panicle length (cm)	14.90	16.50

Table 3: Overexpression of C_4 enzymes in transgenic rice leaves (Source: Miyao, 2003)

		Highest enzyme activity (in folds)		
C_4enzyme with location	Introduced construct	Over rice	Over maize	References
PEPC (Mesophyll)	Intact maize gene	110	3-4	Ku *et al.* (1999)
PPDK (Mesophyll)	Rice cab promoter + maize full length C_4 c-DNA	5	<0.1	Fukayama *et al.* (2001)
PPDK (Mesophyll)	Maize Pdk-1 C_4 promoter + maize full length C_4 c-DNA	5	<0.1	-do-
PPDK (Mesophyll)	Intact maize gene	40	0.5	-do-
NADP-ME (Bundle sheath)	Rice cab promoter + rice full length C_3 c-DNA	<5	<0.1	Tsuchida *et al.* (2001)
NADP-ME (Bundle sheath)	Rice cab promoter + maize full length C_4 c-DNA	30 70	0.6 ---	-do- Takeuchi *et al.* (2000)
PEPCK Bundle sheath)	Rice cab promoter + *Zoysia* full length C_4 c-DNA	---	0.1	Miyao *et al.* (Unpublished results)
PEPCK (Bundle sheath)	Maize C_4 PEPC promoter + Urochloa C_4 c-DNA	---	0.5	Suzuki *et al.* (2000)
PEPCK (Bundle sheath)	Maize pdk-1 C_4 promoter + *Urochloa* C_4 c-DNA	---	0.5	-do-

- Overproduction of C_4 enzyme in C_3 plants is greatly affected by phylogenetic distance. Maize C_4 enzymes are over expressed more in rice than tobacco due to problems of incorrect transcription initiation and splicing. Since maize and rice have evolved from a common ancestor and share genome synteny, intact maize C_4 enzymes are readily over expressed in rice.
- Introduction of chimeric gene containing c-DNA for a C_4 enzyme with a strong promoter alone or with enhancer sequences also increase activity of C_4 enzymes in leaves of C_3 plants (Matsuoka *et al*., 2001).
- Double transformants generated by the introduction of maize PEPC and NADP-ME from *Flaveria pringlei* showed 5 fold increase in the activity of both enzymes. Similarly studies with multiple transformants in tobacco and potato have revealed that all the enzymes were over expressed. The detailed analysis of such experimental findings will give us a clue as to what extent we can be able to put in place a fully operational C_4 cycle in C_3 plants.
- The expression levels of C_4 enzymes are drastically reduced by transgene rearrangement and in some case level of expression was found to be even less than non-transformants (Ku *et al*., 1999), especially with long gene constructs with complex intron-exon construct.

Improving CO_2 Concentration Mechanism

The studies so far with manipulation of Rubisco or C_4 enzymes have established, beyond doubt, the fact that such manipulations are not enough to make C_4 cycle operational in C_3 plants like rice. A mechanism must be put in place which can concentrate CO_2 near Rubisco (supercharging photosynthesis). In fact the C_4 plants have a mechanism wherein the specialized mesophyll cells temporarily fix CO_2 and jam it into the bundle sheath cells at such high concentrations that the oxygenation reaction is effectively blocked (Mann, 1999b). The genetic manipulations for creating an in cell CO_2 concentrating mechanism would require introduction of at least some of structural features of C_4 plants into C_3 plants like rice because it seems unlikely that engineering of CO_2 concentrating mechanisms would succeed without such manipulations (Leegood, 2002). Such an attempt would require manipulation of genes governing complex cell differentiations and development of different cell types in C_4 plants. Even though such factors may be governed by only a few genes but the fact is the none of them has been yet identified. Thus even if the bundle sheath and mesophyll cells are appropriated to express specific enzymes, it will still be structurally inappropriate for an operational C_4 cycle due to poor cell to cell communication. Several research groups working on this aspect of photosynthesis redesigning have proposed different C_4 system as in case of *Borszezowia* but this would require reworking of rice leaf. Another approach is to introduce pyrenoids or carboxysomes, from algae and cyanobacteria, into the chloroplasts of rice. A gene *col A* was cloned from cyanobacterium *Synechocystis*, which encodes a protein extrusion engaged in CO_2 concentration. CO_2 responding genes have also been cloned from the same bacterium. With these novel genes, it should be possible to improve CO_2 concentrating mechanism in rice.

Single cell CO_2 concentrating mechanism found in aquatic macrophyte *Hydrilla verticillata* has also been proposed for possible genetic manipulation (Leegood, 2000). However, similar anatomical constraints and poor cell to cell communication are a major hurdle. In this context not only the structural specialization of C_4 plants is important but also the various factors such as role of plasmodesmata, intercellular metabolite transport, specialization of metabolite transporters, electron transport pathway and regulation of enzyme activity in C_3 and C_4 plants are very complex and cannot be bypassed. A prototype of such a C_4 cycle in rice will require coordinated expression of C_4 enzymes with all the necessary regulatory mechanisms and metabolite transporters such as Pyruvate, malate/OAA or PPT in place.

Will C_4 Rice be More Productive

Improvement of yield potential is the primary objective of all crop improvement strategies, both conventional and modern breeding methods. Genetic manipulations to produce efficient 'Super - Rubisco' or overexpression of C_4 enzymes have provided enough evidence of increased photosynthetic rates, lower O_2 inhibition and low photorespitation in transgenic rice plants. However, even though the photosynthesis is pivotal for production of plant biomass, it is surprising that yield and the photosynthetic rate are often, not so well correlated (Sharkey *et al*., 2000). In fact photosynthesis is just one factor which determines plant yield which is also influenced by rate of respiration and rate of detachment of plant mass. Besides, photosynthesis as a process is complex in terms of interactions of various factors at input level, the mechanisms of processing of these inputs and the final production of output. Thus in our endeavour to redesign photosynthesis for realizing increased crop yields, the maneuvering has to be done at all feasible levels. In some studies, it has been found that the enzyme 'Sucrose phosphate synthase' which is the immediate product of photosynthesis has been found to be correlated with higher yields. The expression of this enzyme has been found to be especially important in photosynthetic than non-photosynthetic tissues, in consideration to yield increase. The redesigning strategy should thus focus on effecting optimum expression of 'SPS in photosynthetic tissues as two little and too much level of this enzyme has been found to result in lower yields.

There have been enough experimental evidences that C_4 plants produce more biomass per unit intercepted radiation, greater quantum yields than C_3 plants at 30°C besides greater CO_2 assimilation rate (Evans and Caemmerer, 2000). In rice as well significant increases in photosynthesis rates have been recorded in various C_4 enzymes introduced singly or in combination (PEPC, PEPCK, PPDK) and consequently 10-35% increase in grain yield has been recorded in transformed plants due to increase in tiller number (Ku *et al*., 2001).

However, there are no immediate expectations that transgenic rice plants with C_4 photosynthesis will be a field reality in future because even though the component process of photosynthesis have been well understood, their integration into the whole plant process is not (Horton, 2000). In fact in U.S.A., various preliminary data regarding crops with enhanced photosynthesis and yield have been published and more information on agronomic performance should be available soon.

Another important aspect of engineering photosynthesis in rice is the advantageous physiological adaptability of C_4 cycle to various abiotic stresses such as heat, salinity and drought. C_4 photosynthesis confers substantial benefits to crops in tropical environments especially in high light, frequent drought, heat and salinity but not as much in flooded conditions (Sage, 2000), thus C_4 rice would be beneficial for upland conditions only. If the less adaptability of C_4 plants to flooded conditions is not related to photosynthesis, then C_4 rice conditions. It has also been proposed that the engineering of rice vis-à-vis rising CO_2 concentration of atmosphere will also result in increased productive potential and thus enhance yield. In the words of R.F. Sage, the C_4 strategy might be the best approach for increasing rice production in next half century.

Future Perspective

There is a persistent hope among the plant breeders that photosynthetic efficiency of crops can be improved for enhancing yielding ability, by improved energy capture and its subsequent translation into greater harvestable yields (Poolman *et al*., 2000). This is indeed a formidable challenge to plant biotechnologists to modify photosynthesis but the opportunities are enormous. The selective advantages of C_4 cycle such as high photosynthetic capacity and high water and nutrient use efficiency especially under low CO_2 conditions offers an appealing strategy for crop modification. There are numerous reports of successful overproduction of C_4-type enzymes in rice resulting in about 110 fold increase in activity than is actually recorded in non-transformants, but the field applications of such experimental results is far from realizable due to obvious practical limitations. The most important

limitation is the provision of mechanism for CO_2- concentration near the Rubisco which needs to engineer leaf anatomy of rice plant. Thus it is not enough to replicate a C_4-type photosynthesis in rice. Rice possesses mesophyll cells which are photosynthetically inactive, which need to be activated. Some of the changes have been found to be actually deleterious especially in case of NADP-malic enzyme, in which case transgenic rice plants tend to die quickly. But such is not the case with PEPC which actually cuts down the O_2 inhibition by about one-third.

Since most of the proposed strategies for enhancing yield potential in future rice varieties like harvest index, light harvesting efficiency and leaf area index have already reached possible upper limits, altering photosynthesis by putting in place a better Rubisco or engineering C_4 enzymes into rice is an appealing target. In the opinion of Austin, All the relatively obvious steps have been taken. Photosynthesis is what is left". The process has not also been drastically changed by evolution in its fundamental aspects, now it is indeed a challenge before biotechnologists, whether they can redesign it by directed changes to break the yield ceilings.

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