Genetic engineering of "C$_4$ rice":

Rationale and performance of transgenic rice plants expressing cyanobacterium and maize CO$_2$ concentrating mechanism genes

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Rice is the staple food for half of the world's population, and 90% of the world's rice is grown and consumed in Asia. Thus, rice production is important to the food security of the world. The world's population is projected by FAO to rise from the current 6.5 billion to 9 billion in the next 40-50 years, with most of the population increases occurring in rice-consuming countries. Thus, rice yield must be increased by at least 50% over the next 40 years to prevent malnutrition for the 700 million Asian that currently rely on rice for more than 60% of their daily calorific intake. After the successful application of the hybrid rice technology in recent years, rice yields have been substantially improved and the wide adoption of the technology will further increase the global rice production. However, hybrid rice technology alone can not meet the demands in the long run. Furthermore, traditional breeding approach is time-consuming and ineffective in solving the food shortage problems. In contrast, molecular breeding, a technology combining genetic engineering and traditional breeding, will be a powerful and effective approach to overcome the food production problems. Theoretical analysis has suggested that the yield increases required to match the projected

population growth can only be achieved by increasing the efficiency with which photosynthesis uses solar energy. Our interest is to increase rice growth and productivity by placing the C_4 pathway of photosynthesis into rice.

Rationale for genetic engineering of "C4 rice"

Photosynthesis is the most important metabolic process relative to plant growth and crop productivity because carbohydrates account for more than 85% of the dry weight in plants. The C_3 pathway of photosynthesis evolved first in an environment enriched in CO_2. However, over geologic time atmospheric CO_2 levels decreased and consequently plants evolved several CO_2 concentrating mechanisms (CCMs) in response to decreases in atmospheric CO_2 level: bicarbonate transport system in cyanobacteria, algae and aquatic plants and the C_4 pathway and Crassulacean acid metabolism (CAM) in terrestrial plants (Price *et al.*, 2008). Under low CO_2 or alkaline water conditions, cyanobacteria use high affinity bicarbonate transporters (e.g., inorganic carbon transporter – ictB or sodium bicarbonate transporter – sbtA) to pump in bicarbonate as a major carbon source. This adaptive CCM allows cyanobacteria to survive unfavorable growth conditions. Higher vascular plants have two main photosynthetic mechanisms, namely the C_3 and C_4 pathways. The most productive crops, such as corn, sorghum and sugarcane, use the C_4 pathway while most of the important agronomic crops, such as rice, wheat and potatoes, use the C_3 pathway. The photosynthetic efficiency is much higher in C_4 than in C_3 plants, due to the C_4 CO_2 concentrating mechanism, which suppresses the photorespiratory activity associated with Rubisco. Therefore, C_4 plants are more productive and use CO_2 (**Fig. 1**), minerals (**Fig. 2**) and water with a higher efficiency (250-350 g H_2O used/g dry matter produced as versus 550-850 g H_2O used/g dry matter produced for C_3 plants), especially under high light, warm temperature (**Fig. 3**) and drought environments. As compared to the C_4 crop corn, rice is a C_3 crop and has a lower photosynthetic efficiency and thus a lower yield.
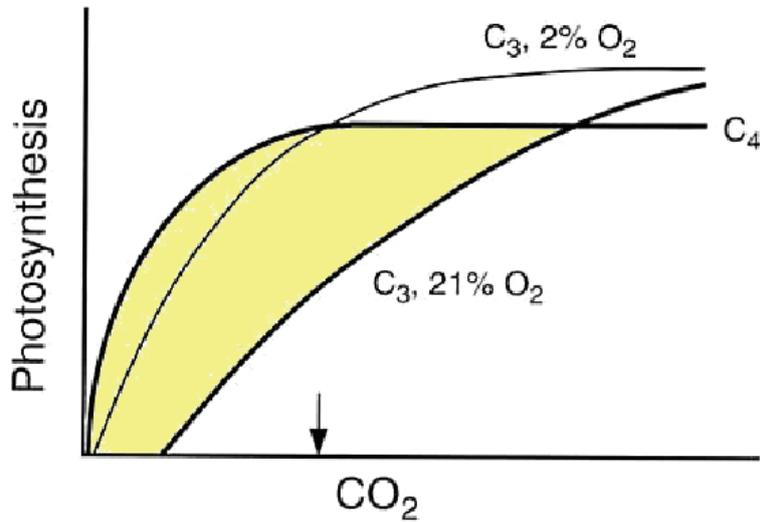
Fig. 1. Photosynthetic CO_2 responses of C_3 and C_4 photosynthesis at 2 and 21% O_2 (adopted from Ku *et al.*, 1996).
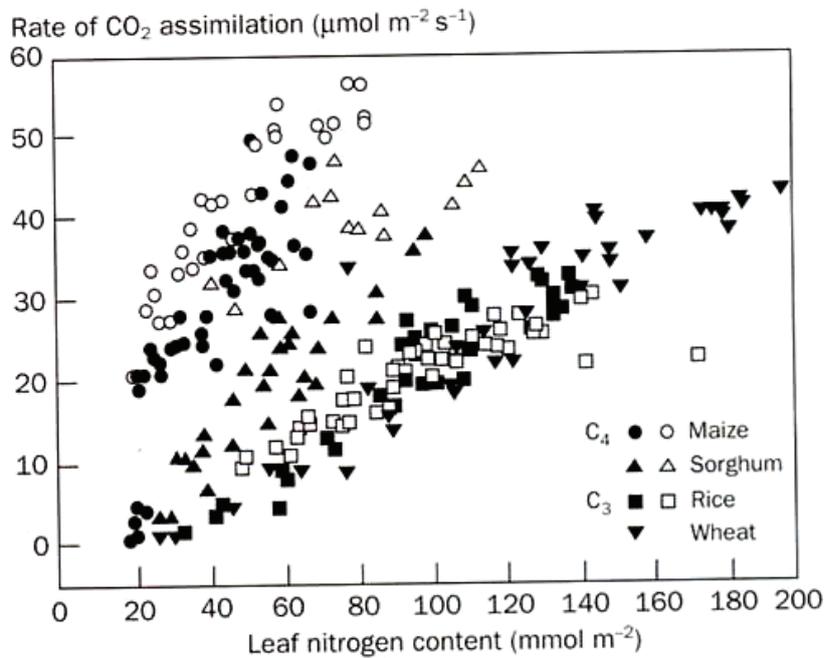

Fig. 2. Photosynthetic nitrogen responses of two C_3 plants (rice, wheat) and two C_4 plants (maize, sorghum) (adopted from Sage, 2004).
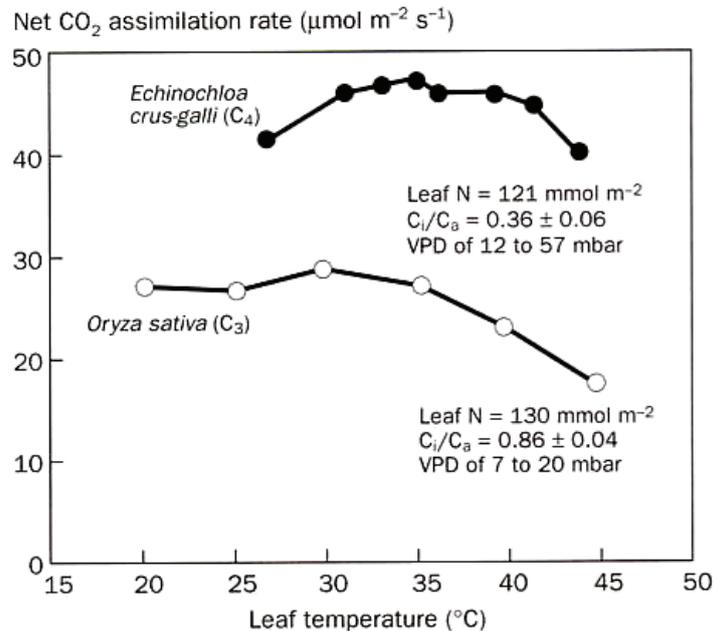
Fig. 3. Photosynthetic temperature responses of rice (C_3) and *Echinocloa crus-galli* (C_4) (adopted from Sage, 2004).

The evolution of C_4 plants from C_3 plants occurred at least 45 times in angiosperms, but the changes are complex. Most C_4 plants compartmentalize photosynthetic reactions between two morphologically distinct cell types that are arranged in concentric circles around the vascular veins, so called Kranz leaf anatomy (**Fig. 4**). This anatomical change coupled with the C_4 pathway of photosynthesis endow C_4 plants a superior photosynthetic capability. There is no doubt that it is extremely ambitious to generate C_4 rice. However, the polyphyletic evolution of C_4 pathway suggests that the transition from C_3 to C_4 is relatively simple and provides cause for optimism. Genes encoding all the enzymes involved in C_4 pathway are present in C_3 plants, although their expression levels are much lower than in C_4 plants. Also, some plants, such as *Eleocharis*, exhibit plasticity in switching from C_3 to C_4 under certain environmental conditions. With a better understanding of the formation of Kranz leaf anatomy and gene regulation in C_4 plants, it may be possible to generate C_4 rice with enhanced photosynthesis, growth and yield.
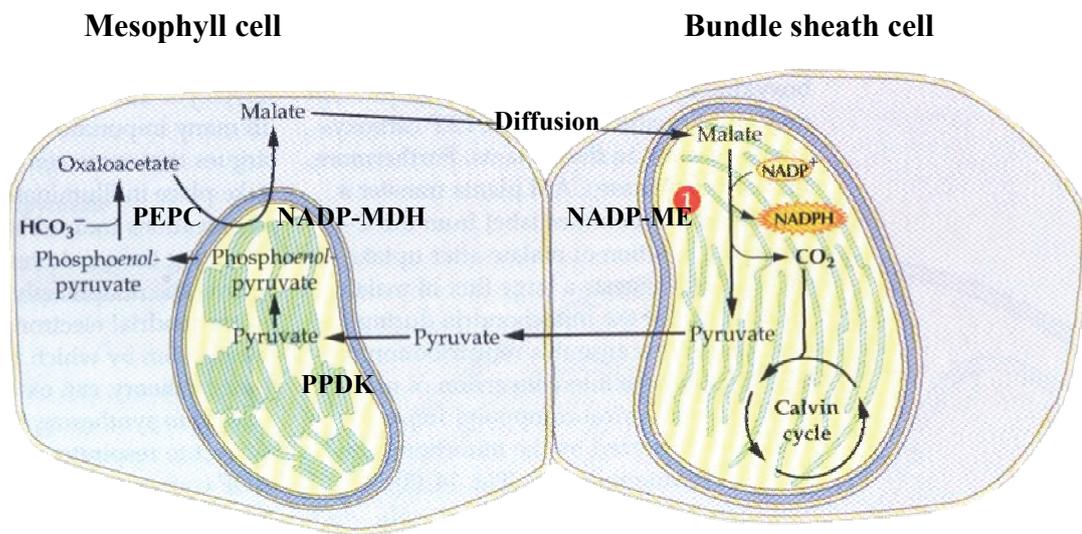
Fig. 4. The C_4 pathway in NADP-malic enzyme (ME) type C_4 plants, such as maize, sugarcane and sorghum. The key enzymes of the pathway are phosphoenolyruvate carboxylase (PEPC), NADP-malate dehydrogenase (NADP-MDH), NADP-malic enzyme (NADP-ME), and pyruvate orthophosphate dikinase (PPDK) (adopted from Bucanon *et al.*, 2001).

Strategies for creating C4 rice

Since rice is a C_3 plant, which lacks Kranz leaf anatomy, it will be rather difficult to introduce the C_4 pathway of maize (**Fig. 4**) in the single mesophyll cells of rice. However, the C_3 aquatic plant *Hydrilla verticillata* is capable of inducing a primitive C_4 pathway in the mesophyll cells when grown under low CO_2 conditions (**Fig. 5**). This primitive C_4 pathway in Kranz-less leaf is apparently effective in reducing photorespiration. It is also clear that this primitive C_4 pathway is very similar to that of maize, but have all the reactions taking place in a single mesophyll cell. The earlier strategy on engineering C_4 rice has been concentrating on mimicking this system in rice. Transgenic rice lines have been produced from Kitaake (japonica) to overexpress several maize C_4 photosynthesis genes independently or simultaneously: phosphoenolpyruvate carboxylase (PEPC, Ku *et al*., 1999)), pyruvate Pi dikinase (PPDK, Fukayama *et al*., 2001),

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NADP-malic enzyme (ME, Takeuchi *et al.*, 2001), CK (PEPC x PPDK) and CKM (PEPC x PPDK x ME). In general, the photosynthetic efficiency and grain yield of these transgenic plants are higher than that of untransformed rice plants (Ku *et al.*, 2000; Ku *et al.*, 2001, Jiao *et al.*, 2001), but high level expression of ME causes photooxidation in transgenic plants due to metabolic imbalance (Takeuchi *et al.*, 2000; Tsuchida *et al.*, 2001). This is due to the competition between PSII and NADP-ME for NADP in the mesophyll chloroplast of ME transgenic rice plants. Depletion of NADP pool in the chloroplast by high activities of NADP-ME renders electron being accepted by oxygen molecule for production of harmful oxyradicals. NADP-ME type C_4 plants avoid this problem by modifying its bundle sheath chloroplast without having PSII activity in the non-stacked thylakoid membrane (**Fig. 1**) whereas the aquatic plant *H. verticillata* may avoid this problem by living in a relatively low light condition. Thus, a functional C_4 CO_2 concentrating is still lacking in these transgenic rice plants.
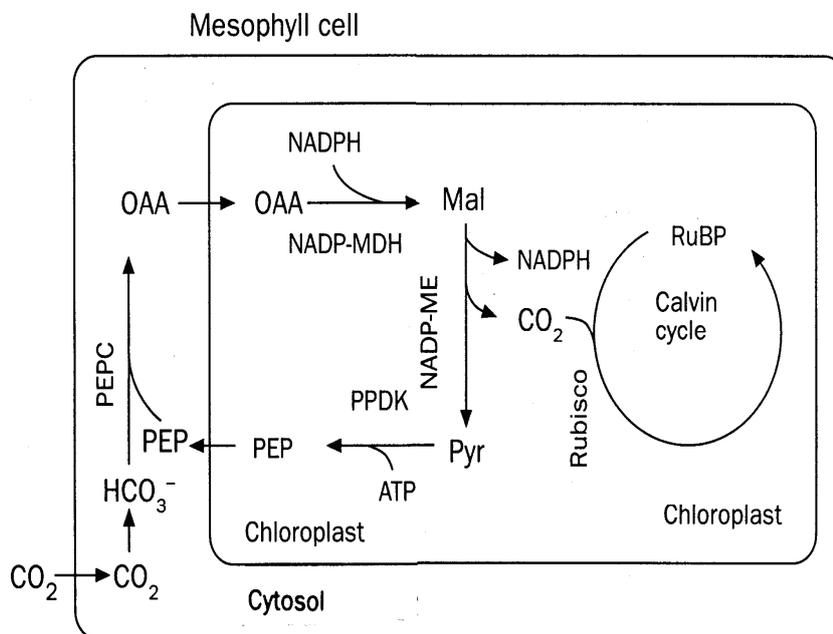

Fig. 5. The primitive C_4 pathway in *Hydrilla verticillata,* an aquatic plant (Magnin *et al.*, 1997)

To avoid metabolic imbalance in engineering C_4 rice by mimicking the C_4 pathway of *Hydrilla* or maize, our recent efforts have been concentrated on creating a synthetic CO_2 concentrating mechanism in rice by combining the CCMs from both cyanobacterium and maize. In this simplified scheme (**Fig. 6**), inorganic carbon transporter from cyanobacteria is expressed on plasmamembrane of mesophyll cells to pump in bicarbonate, followed by carboxylation of bicarbonate in the cytosol by maize PEPC to form oxaloacetate (OAA) and subsequent decaroxylation of OAA by maize PEP carboxykinase in the chloroplast (PCK). With this synthetic pathway, bicarbonate, the substrate for PEPC, is directly transported into the cell and OAA formed is decarboxylated by PCK upon entering the chloroplast without the need to be reduced to malate with
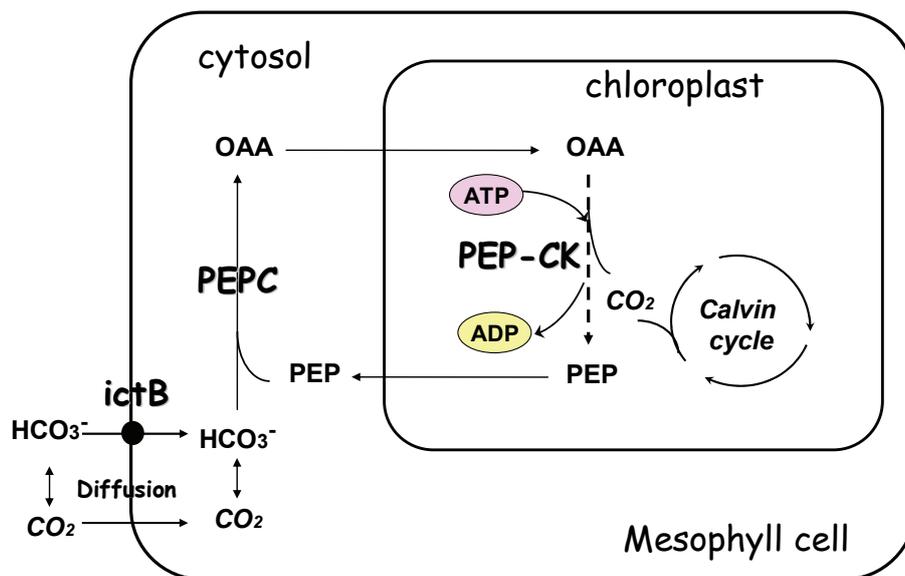

Fig. 6. Creation of a functional C_4 pathway in the C_3 mesopyll cells. The three key steps are: (1) transport of biocarbonate across membrane by inorganic carbon transporter (ictB or sbtA), (2) carboxylation by phosphoenolpyruvate carboxylase (PEPC) in the cytosol, and (3) decarboxylation of oxaloacetate (OAA) in the chloroplast by PEP carboxykinase (PEP-CK).

the input of NADPH energy. Furthermore, the decaoxylation of OAA to release CO_2 and PEP by PCK requires only one ATP and PEP is directly used for PEP carboxylation whereas in the NADP-ME type C_4 pathway it requires two ATP to regenerate PEP from pyruvate after decarboxylation of malate by ME. Thus, energetically this synthetic CO_2 concentrating mechanism will be more favorable. So far, we have produced transgenic rice from Tainoung 67 (japonica subtype) to constitutively express (1) ictB or sbtA gene from cyanobacteria, (2) intact maize PEPC gene and (3) maize PCK gene.

(1) ictB transgenic rice: All transgenic rice lines expressing the cyanobacterium ictB transporter exhibited enhanced activities of Rubisco (25-30%) and PEPC (10-20%), higher carboxylation efficiency (15-20%), higher photosynthesis rates (10-30%), and lower photosynthetic CO_2 compensation points (5-10%). Consistently, the transgenic rice plants produced more panicles per plant (10-120%) and more grains (10-70%), relative to untransformed plants. The enhancements in growth and grain yield are closely related with the increased photosynthetic capacity among the transgenic lines. This study demonstrates that the simple CCM from cyanobacterium can largely improve the photosynthetic efficiency, growth and productivity of C_3 crops, such as rice.

(2) PEPC transgenic rice: Transgenic rice lines that expressed high levels of the maize PEPC with PEPC activities up to three fold higher than that of maize have been obtained. Compared with untransformed wild type rice plants, PEPC transgenic plants showed higher photosynthetic rates (10-20%), especially under high light conditions, higher light and CO_2 use efficiencies, and higher grain yields in the field (up to 50-60%).

(3) PCK transgenic rice: PCK is a cytosolic enzyme. In order to target the enzyme to the chloroplast for effective decarboxylation of OAA in transgenic rice plants, the maize gene is linked to the chloroplast targeting

sequence from tomatao RbcL in the transformation vector. Compared to untransformed rice plants, PCK transgenic plants showed higher total biomass (5-20%), heavier grains (11-16%) and increased grain yield (3-12%). Most importantly, feeding OAA to leaf enhanced the photosynthetic rates of transgenic plants, indicating it is functional in the chloroplast by supplying more CO_2 in the chloroplasts. An earlier study by Suzuki *et al.* (2001) also produced transgenic rice overexpressing PCK gene isolated from PCK subtype C_4 plant *Urochloa panicoides*.

A functional C_4 pathway requires the coordination of several biochemical steps located in different cellular and intracellular compartments. Traditional cross hybridization was used to generate transgenic plants simultaneously expressing these enzymes in the same plants. Homozygous transgenic plants expressing both PEP and ictB or both PEPC and PCK have been obtained for further characterization. Preliminary evaluation showed that these hybrid transgenic rice plants have significantly higher biomass and grain yields. It is anticipated that simultaneous expression of ictB, PEPC and PCK in rice is likely to confer a functional C_4 photosynthesis, leading to increased photosynthesis and productivity. In addition, we have also evaluated the potential of transferring these CCM genes to hybrid rice by crossing the various transgenic lines with elite rice cultivar, such as 93-11, to combine heterosis and C_4 photosynthetic traits. Preliminary observation suggests this is a feasible approach.

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