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Review

Constructing C_4 rice - the challenge of new green revolution

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The new green revolution is looking for a breakthrough in the world. Constructing C_4 rice based on good plant type is a reliable and effective approach to enhance photosynthetic efficiency in leaf. The paper discusses the significance, techniques, physiological characteristics and future research directions of transgenic rice with C_4 gene and makes some suggestions on breeding new cultivars with super-high yield and photosynthetic efficiency in high light intensity and high temperature area of China, especially in Africa.

Key words: Rice, C_4 photosynthesis gene, photosynthesis, physiological breeding.

INTRODUCTION

Because of the application of semi-dwarf gene in the 1960s and heterosis in the 1970s in China, Chinese rice yield process leaped twice, rising 20%, respectively, from the previous level. Nowadays, average efficiency of light use in high-yield varieties is about 1.5%, while theoretically it should reach 3 - 5% (Qiu, 1992). Thus, photosynthetic productions have prodigious potential to be increased. It is obvious that, in the perspective of photosynthesis, yield consists of two components, “source” and “sink.” At present, Chinese super-hybrid rice archives high yield mainly because of the increase in sink, for example, by adjusting plant architecture to obtain a maximum number of grains. However, in the major hybrid rice combinations used so far, the panicles are big, but the empty-seed rate is high as well. To further increase yield, the emphasis should logically be shifted to an increase in “source”. In previous years, Ku et al. (1999) introduced key enzymes of the maize C_4 pathway to rice and achieved a significant increase in photosynthetic capacity. We developed a new approach to introduce genes for the C_4 enzymes, phosphoenolpyruvate carboxylase (PEPC) and pyruvate Pi dikinase (PPDK), into sterile and restorer lines, respectively, and enhanced photosynthetic efficiency up to 50% in the F_1 by crossing the two lines (Wang et al., 2004). Therefore, we believe that, to increase the source, we can integrate C_4 photosynthetic pathway into conventional C_3 rice on the current basis of more efficient plant architecture.

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GENETIC ENGINEERING OF C_4 ENZYMES IN C_3 PLANT

Since 1960s, it has been a noticeable research topic to attempt to enhance the photosynthetic efficiency of C_3 plants by incorporating C_4 photosynthetic traits into them, but there has been no striking progress for long terms. After that, the hybridization between C_4 and C_3 - C_4 intermediate plants was made in the genus of *Atriplex* and *Flaveria* and it was discovered that photosynthetic characteristic in the hybrids F_1 was similar to that in C_4 plants with C_4 plant as the male parent (Brown, 1986, 1993). However, in Gramineae, the hybrids between C_3 and C_4 plants usually exhibited infertility due to reasons such as irregular chromosome pairing or genetic barriers. Thus, employing conventional breeding methods to incorporate C_4 traits into C_3 crop cannot still bring into effect.

In the nineties of the 20th century, with the rapid development of molecular biological technology, transfer of foreign genes into crops has become increasingly routine, making it possible to introduce the genes encoding C_4 photosynthesis enzyme into C_3 plants. Gehlen et al. (1996) observed that the PEPC gene from

Corynebacterium glutamicum was expressed in potato by transgenic technique, but the activity of PEPC in transgenic potato was only 5 times that in the untransformed potato, not significantly increasing. Using an Agrobacterium-mediated transformation system, Ku et al. (1999) firstly successfully introduced the intact gene of maize PEPC, which is the key enzyme in C_4 photosynthetic pathway in maize, into the C_3 crop rice. The transgenic rice plants showed high-level expression of PEPC gene and exhibited reduced O_2 inhibition of photosynthesis, which opened up broad prospects for improving the photosynthetic productivity of crop by genetic engineering. Until now, PEPC from maize (Ku et al., 1999; Ding et al., 2007; Yuan et al., 2007), from sorgum (Zhang et al., 2003) and from *Echinochloa crusgalli* (Zhang et al., 2005), have been successfully introduced into C_3 rice. There are four reports on transgenic plants, which overproduce PPDK derived from higher plants; transgenic *Arabidopsis* (Ishimaru et al., 1997), potato (Ishimaru et al., 1998), rice (Fukayama et al., 2001) overproducing the maize C4-specific PPDK and transgenic tobacco overproducing PPDK from a CAM plant *Mesembryanthemum crystallinum* (Sheriff et al., 1998). Three sets of transgenic rice plants overproducing the maize C_4-specific isoform (Takeuchi et al., 2000; Tsuchida et al., 2001) and overexpressing sorghum C_4 specific NAD-malic enzyme (NADP-ME) (Chi et al., 2004) and the rice C_3-specific isoform of NADP-ME (Tsuchida et al., 2001) have been reported. Based on this studies, the technology to express the C_4 enzymes at high levels and in the desired locations in the leaves of C_3 species is becoming well established and it is now possible to produce transgenic C_3 plants that express at least a set of key enzymes of the C_4 pathway.

Recently, the International Rice Research Institute invited scientists from various countries to discuss the possibility of constructing C_4 rice in 10 to 15 years to lead a new “Green Revolution” (Dennis, 2006).

PHYSIOLOGICAL CHARACTERISTICS OF TRANSGENIC RICE EXPRESSING C_4 GENES

In the last decade, more attention has been paid to the introduction of C_4 photosynthetic gene into C_3 plants to raise their photosynthetic capacity (Matsuoka et al., 2001). Due to the development of recombinant DNA technology, PEPC (Ku et al., 1999), PPDK (Fukayama et al., 1999), NADP-ME (Tsuchida et al., 2001) and PEPC+PPDK (Ku et al., 2000) transgenic rice plants have been obtained. As shown by the first study of Ku et al. (1999), they obtained transgenic C_3 plants with high level expression of the maize C_4-specific Ppc gene (encode phosphoenolpyruvate carboxylase, PEPCase) and rice plants obtained exogenous PEPC gene from C_4 maize plant enhance photosynthetic capacity by increase of stomatal conductance (Ku et al., 2001). But recent experimental result (Jiao et al., 2003) showed that there are no relationships between the increase of photosynthetic rates and the enhancement of stomatal conductance of leaves in PEPC transgenic rice. Physiological studies (Zhang and Jiao, 2002) showed that, the transgenic plants transformed with Ppc gene displayed a light saturation rate higher by 55% and a CO_2 compensation point lower by 27%. Also Zhang et al. (2003) introduced sorghum intact C4-pepc gene, including its promoter, into two Chinese cultivars of rice. Preliminary data from transformed lines showed that the sorghum C_4-pepc gene had been transcribed and translated in rice and that the transgenic rice gained low CO_2 compensation point and high photosynthesis efficiency. These findings were supported by the results of Bandyopadhyay et al. (2007).

Fukayama et al. (2001) introduced the maize intact C_4-ppdk gene, which contained its own promoter and terminator sequences and exon/intron structure, into rice in 2001. The PPDK activity in the leaves of some transgenic lines was greatly increased, in one line reaching 40-folds over that of wild-type plants. In a homozygous line, the PPDK protein accounted for 35% of total leaf-soluble protein or 16% of total leaf nitrogen. In maize and transgenic rice plants carrying the intact maize gene, the maize C_4-ppdk gene was expressed in a similar organ-specific manner. Ku et al. (2001) reported that transgenic rice plants expressing the maize PEPC and pyruvate, orthophosphate dikinase (PPDK) exhibit a higher photosynthetic capacity (up to 35%) than untransformed plants in 2001. However, the reaction of PPDK is freely reversible, depending on concentrations of substrates, activators and inactivators (Burnell and Hatch, 1985). This could be the reason why the overexpression of PPDK does not result in significant effects on carbon metabolism in the leaves.

As pointed out by Ku et al. (1991), the activity of NAPD-ME shows negative correlation with the activity of photorespiration. It thus appears that transfer of NADP-ME gene into C_3 plants might be an effective way of lowering photorespiration and improving photosynthetic efficiency of C_3 plants. There are many reports on the successful transfer of photosynthetic enzymes of C_4 plants into C_3 plants and their high level expression in the latter by means of gene engineering techniques (Ku et al., 1999; Takeuchi et al., 2000; Furayama et al., 2001; Zhang et al., 2003). Yet inconsistent results have been obtained in studies aimed at elucidating the underlying physiological mechanisms. Takeuchi et al. (2000) tried to account for some of the physiological characteristics of transgenic rice expressing high level maize NADP-ME in terms of chloroplast development (Takeuchi et al., 2000). A report suggests that NADP-malic enzyme could be detrimental in the development of normal chloroplasts when expressed at high levels (20 - 70 folds increases) in a C_3 plant (Takeuchi et al., 2000). Chi et al. (2004) confirmed the effective expression of sorghum C_4 type

NADP-ME in rice, with the enzyme activity being elevated 1 - 7 folds. However, no appreciable change was demonstrated in carbon assimilation of the transgenic rice though increased photoinhibition was noted under high light intensity. These studies indicate that the efficiency of photosynthesis of C_3 plants can be increased by transformation with C_4-type genes of C_4 plants.

Interestingly, it has been observed that the photosynthetic rate of PPDK transgenic rice with high expression of PPDK enzymes did not significantly increase (Jiao et al., 2002), while the photosynthetic rate decreased with the introduction of the NADP-ME gene (Chi et al., 2004). In a previous study we showed that the photosynthetic rate in transgenic rice expressing both the PEPC and PPDK genes could be greatly increased with the use of adenosine triphosphate (ATP) or ATP promotive substance (Ji et al., 2005; Zhang et al., 2009). In the present study, exogenous ATP ensured the adequate supply of ATP for the C_4 cycle and consequently greater CO_2 fixation occurred and the Pn in transgenic rice expressing both the PEPC and PPDK genes was increased. Further experiment is required to test this hypothesis. Nevertheless, our findings demonstrated that ATP was a key limiting factor for further promotion of the photosynthetic capacity of transgenic rice expressing C_4 genes and construction of C_4-like rice.

Japanese scientists (Fukayama et al., 2000; Taniguchi et al., 2008) did not observe improved photosynthetic characteristics when PEPC transgenic rice was cultivated in greenhouses under the conditions of 26°C and plant PFD of 500 – 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. In the present study, the plants were cultivated outdoors during the summer in Nanjing, China (temperature 26 – 35°C and PFD 500 – 1400 $\mu mol \cdot m^{-2} \cdot s^{-1}$), thus under different environmental conditions (Zhang et al., 2009). Zhang and Jiao (2002) also showed that PEPC transgenic rice exhibited the characteristics of high photosynthetic capacity, tolerance to photo-oxidation and increased yield. These findings were supported by the results of Bandyopadhyay et al. (2007). Therefore, we contend that transgenic rice expressing C_4 genes should be cultivated and directionally screened under high light intensity and high temperature conditions, which are important technical conditions. Thus this work were carried out valuably in Africa. But the study of nitrogen and water use efficiency on transgenic rice expressing C_4 genes were still blank.

In recent years, what bred cultivars with the high photosynthetic efficiency and grain yield by introducing the C_4 gene into rice have drawn more attention for breeders? A proposal was put forward to promoting super rice yield by introducing C_4 gene from C_4 plant or algae into them (Yuan and Zhao, 2004). In fact, there have been many works on physiological breeding of transgenic rice with C_4 gene in china. It was reported in a series of work that sterile lines (Wang et al., 2004), restore lines (He et al., 2006) and hybrid rice combinations with PEPC gene have been bred in different ecological regions. More importantly, the marker-free transgenic homozygous rice restorer lines with PEPC and PPDK genes by Agrobacterium-mediated transformation using super binary vector were obtained (Yuan et al., 2007). Doubtlessly, it would be an effective approach for super rice with high photosynthetic efficiency and high grain yield.

Astonishingly, some plants can operate either C_4 or C_3 photosynthetic mechanisms. The submerged culms of *Eleocharis vivipara* are C_3, but the emergent culms are C_4 with the Kranz anatomy characteristic of sedges (Ueno, 1998). This represents an inducible system of the C_4 syndrome, surely useful for identifying the gene(s) responsible for the coordinated expression of both the C_4 biochemistry and Kranz leaf anatomy. Aquatic plants in vernal pools such as Orcuttia has been shown to have two types of anatomy: the terrestrial leaves are C_4 with Kranz anatomy but submerged leaves have C_4 biochemistry without Kranz anatomy (Laura et al., 2008). The implication is that C_4 photosynthesis is possible without Kranz anatomy but is beneficial only under water. Study of these species will increase knowledge of the natural range of methods of concentrating carbon dioxide and could aid the design of novel C_4 systems.

FUTURE WORK

Integration of high efficiencies of photosynthetic productivity and plant architecture

Since the scientists have successfully approached the goal of increase “source” by crossing genetically engineered PEPC enzyme contained sterile and restore lines, we can apply such a strategy to the “super-hybrid” rice with high efficient and good architecture. That is, to introduce C_4 enzymes into parental lines of “super-hybrid” rice and integrate the two improved traits together.

Further modification of photosynthetic productivity

In our previous work, we found that photosynthetic rate of PPDK transgenic rice is limited on the increment of light intensity. But such limitation can be released by applying extra ATP (Jiao et al., 2007). Therefore, we guess that if we can increase production of ATP through genetic engineering, the photosynthetic productivity should be further increased. In addition, to further increase the photosynthetic productivity, we can also try to re-fix the CO_2 released by respiration by introducing PEPC of CAM plants, dark activated enzyme, into available C_4-enzyme transgenic rice. In this way, the transgenic plants can carry out C_4 photosynthesis day and night.

Genetic modification of leaf anatomy

So far, all C_4 plants found in nature have specific Kranz structure adapted for their metabolic characteristics. So it

is reasonable to hypothesize that genetic modification of leaf anatomy may also be a useful approach to increase photosynthetic productivity. It was found that in tobacco stems, a C_3 plant, as well as the veins of celery stalks, existed photosynthetic cells with C_4 characteristic, which are just like the bundle sheath cells in maize leaves. They could be engaged in this genetic research aspect of constructing C_4 rice. It is worthy of attention that true C_4 structure of leaves is induced at five leaves stage in the leaves of maize. Recent technique advances such as Laser Capture Microdissection enable us to study the regulatory mechanism of cellular differentiation related not only to the leaf anatomy, but also to the metabolic pathways. We believe that such study will lead us finally to build up an anatomic base for the high efficient photosynthetic productivity of transgenic rice with C_4 pathways.

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