BRIEF COMMUNICATION

Photosynthetic characteristics of hybrid rice with phospho*enol*pyruvate carboxylase gene

L. LING[*], B.J. ZHANG[**], and D.M. JIAO[***,+]

College of Life Science, Sichuan University, Chengdu, 610064, China[*]
Department of Life Science, Xiaozhuang College, Nanjing, 210017, China[**]
84-401, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, China[***]

Abstract

High level of phosphoenolpyruvate carboxylase (PEPC) gene was stably inherited and transferred from the male parent, PEPC transgenic rice, into a female parent, *japonica* rice cv. 9516. Relative to the female parent, the produced JAAS45 pollen lines exhibited high PEPC activity (17-fold increase) and also higher photosynthetic rates (about 36 %-fold increase). The JAAS45 pollen lines were more tolerant to photoinhibition and to photo-oxidative stress. Furthermore, JAAS45 pollen lines, as well as their male parent, were tested to exhibit a limiting C_4 cycle by feeding with exogenous C_4 primary products such as oxaloacetate (OAA). Thus the PEPC gene and photosynthetic characteristics of PEPC transgenic rice could be stably transferred to the hybrid progenies, which might open a new breeding approach to the integration of conventional hybridization and biological technology.

Additional key words: anther culture; C_4 cycle; chlorophyll fluorescence induction; net photosynthetic rate; *Oryza*; oxaloacetate; photosystem 2; transgenic plants; *Zea*.

In the last decades, with the rapid development of molecular biology and transgenic technology, key genes of the C_4 photosynthetic pathway in maize have been successfully introduced into rice, which is a C_3 crop (Fukayama *et al*. 1999, Ku *et al*. 1999, Takeuchi *et al.* 2000). Till now, only a few systematic studies on photosynthetic characteristics of transgenic rice with maize phospho*enol*pyruvate carboxylase (PEPC) have been carried out. They show that such transgenic rice expresses high PEPC activity similar to that in maize (Chi *et al*. 2001) and has better photosynthetic capacity (Huang *et al*. 2002, Jiao *et al*. 2002) with enhanced tolerance to photo-oxidation (Jiao *et al*. 2002, 2005), as compared with untransformed rice cv. Kitaake. Use of radioactive methods proved that a greater amount of CO_2 was fixed in their primary C_4 photosynthates, indicating the existence of a primitive CO_2 concentrating mechanism (Jiao *et al*. 2003). Moreover, the stable PEPC transgenic rice germplasm has been obtained by systematic selection and identification under natural conditions (Jiao *et al.* 2002). Therefore, rice breeders have tried to transfer the good photosynthetic characteristics of PEPC transgenic rice to conventional rice. Wang *et al*. (2002, 2004) crossed PEPC transgenic rice as male parent with sterile lines and obtained hybrid progenies with optimal photosynthetic characteristics. In addition, PEPC transgenic rice germplasm was crossed as male parent with *japonica* rice cultivar 9516. Then we screened out JAAS45 pollen lines and H137 pollen lines from the anther of F_1 hybrids by anther culture. The aim of the present investigation was to study whether PEPC gene and photosynthetic characteristics of parents could be transferred stably into hybrid progenies.

Seeds of PEPC transgenic rice germplasm, cv. 9516, and JAAS45 pollen lines were grown in 4 000 cm^3 pots in a net-door room in Nantong. There were five hills per pot and one seedling per hill. Plants were watered and fertilized conventionally. According to Wang and Fang (2003),

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[+]Corresponding author; e-mail: jiaodm_123@yahoo.com.cn
Abbreviations: F_v/F_m – the maximum photochemical efficiency of photosystem 2; HI – sprayed with distilled water and exposed to high irradiance [1 400 μmol m^{-2} s^{-1}]; HI + MV – sprayed with 1.5 mM methyl viologen (MV) and exposed to high irradiance [1 400 μmol m^{-2} s^{-1}]; JAAS45 – JAAS45 pollen line; MV – methyl viologen; OAA – oxaloacetate; PCR – polymerase chain reaction; PC – PEPC transgenic rice; PEPC – phospho*enol*pyruvate carboxylase.

total DNA was extracted using the improved method of SDS dram extraction. Through the software of GENTYX on the basis of the analysis of maize C_4-specific PEPC genome di-sequence, two specific primers (SP1: 5'-CAT CTG CTG GCT TCT GGA GTT TCT-3'; SP2: 5'-ATC GTC ACA AAG CAA TGG GCA TG-3') were designed with the expected product size of 1 190 base pairs. The PCR amplification was carried out in a 35 mm^3 reaction mixture containing 2.0 mm^3 of 1 mM of each primer set (about 50 ng) of DNA as a template, 2.0 mm^3 of 2 mM each of dNTP, 3.5 mm^3 of 10×PCR buffer, 0.2 mm^3 of Taq DNA polymerase (5 U per mm^3) (*TaKaRa*, China), and 16.3 mm^3 of distilled H_2O. PCR amplification was performed with a *Gene Amp PCR System 9600* (*Perkin Elmer*, USA). The cycling parameters for PCR were (*a*) 94 ºC for 10 min (initial de-naturation), (*b*) 94 ºC for 60 s (de-naturation), (*c*) 60 ºC for 30 s (annealing), (*d*) 72 ºC for 60 s (extension), and (*e*) 72 ºC for 10 min (final extension). The steps (*b*) to (*d*) were repeated for 30 cycles. PCR products were electrophoresed in a 1.4 % agarose gel.

PEPC activity was assayed according to the method of Gonzalez *et al*. (1984) and Ku *et al*. (1999). About 0.25 g of leaf tissue was harvested from newly mature leaves from each plant in the light and quickly ground in 1.5 cm^3 extraction buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2 % (m/v) insoluble polyvinylpolypyrrolidone (PVP), and 10 % glycerol. After total maceration, the crude extract was centrifuged at 13 000×*g* for 10 min at 4 ºC, and the supernatant was used immediately for PEPC assay. PEPC was assayed spectrophotometrically at room temperature in a mixture containing 50 mM *Hepes*-KOH, pH 8.0, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 1.5 units of NAD-malate dehydrogenase (MDH), 0.2 mM NADH, and 20–50 mm^3 of enzyme extract. Adding PEP to a final concentration of 2 mM started the reaction. The change in NADH was monitored at 340 nm using a spectrophotometer. Then, P_N of attached leaves under high irradiance of 1 200 μmol m^{-2} s^{-1} in air was measured using a portable photosynthetic gas analyzer (model *TPS-1*, *PP Systems*, UK). In addition, intact rice leaves were cut during the booting stage and immediately inserted into 200 μM oxaloacetate (OAA) as experimental variant, while other ones were put into distilled water as control (CK) variant, all this under photon flux density (PFD) of 1 200 μmol m^{-2} s^{-1} for 30 min (Ji *et al*. 2004). Then P_N of rice leaves was measured under the given PFD.

The method of Lin *et al*. (1999) was adopted to produce photo-oxidative stress in leaves. The upper surface of intact flag leaves were smeared with an oxidative reagent containing 1.5 mM methylviologen (MV) and 1 % (v/v) *Tween-80*. Distilled water containing 1 % v/v *Tween-80* was used as a control. After a 3-h irradiation by 1 400 μmol(photon) m^{-2} s^{-1}, PS2 photochemical efficiency were measured with an *FMS-2* fluorescence meter (*Hansatech*, UK), and calculated according to Genty *et al*. (1989).

Using the maize genome as positive control, all materials were amplified by PCR according to the characteristic prime of maize C_4-specific PEPC genome. The band of 1 190 base pairs was observed in three materials including maize, PEPC transgenic rice, and JAAS45, but not in 9516 (Fig. 1). The transgenic rice expressing the maize C_4-specific PEPC gene could be precisely selected through PCR, and maize C_4-specific PEPC gene was introduced into JAAS45 with high-level of expression.

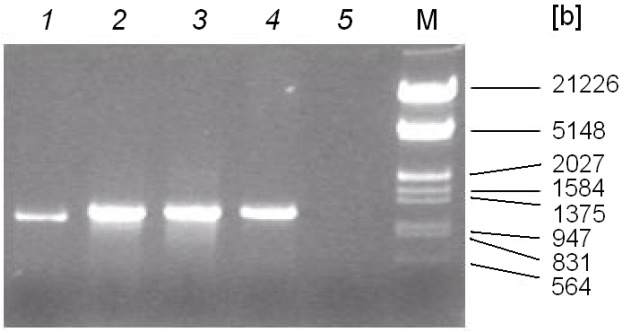

Fig. 1. PCR analysis of JAAS45 pollen line (lane *3*), parent cv. 9516 (lane *5*), PEPC transgenic rice (lane *2*), H137 (lane *4*), and maize (lane *1*). M = markers.

The PEPC activity of JAAS45 pollen line was markedly higher than that in 9516, but it was lower than that of PEPC transgenic rice (Fig. 2*A*). A similar trend was obtained in P_N under high irradiance of 1 200 μmol m^{-2} s^{-1} (Fig. 2*B*). During photoinhibition under high irradiance of 1 400 μmol m^{-2} s^{-1}, the PS2 photochemical efficiency (F_v/F_m) of JAAS45 was closer to that of the male parent by lying between the values of both parents (Fig. 2*C*). After 3-h treatment with MV, this trend was much more evident. The above-mentioned results indicated that the photosynthetic characteristics of male parent were transferred into the hybrid progenies. In addition, when the leaves of the parents and hybrid progenies-JAAS45 were treated with exogenous OAA, P_N of male parent and JAAS45 were significantly increased as compared with CK leaves (Fig. 2*D*), indicating that PC and JAAS45 pollen line possessed a limiting C_4 cycle and the inheritance of physiological characteristics of parents was related to the limiting C_4 cycle.

In conclusion, we found that the PEPC gene from male parent was inherited into JAAS45, and the PEPC activity of the hybrid JAAS45 was similar to that of the male transgenic parent. Moreover, the photosynthetic characteristics and the tolerance to photoinhibition and photo-oxidation in JAAS45 were similar to those of the male parent. On the other hand, we also found that JAAS45, similar to PEPC transgenic rice, possesses a limiting C_4-like cycle with primitive CO_2 concentrating mechanism (Jiao *et al*. 2003). All this suggests that the PEPC transgenic rice can transfer over-expression of

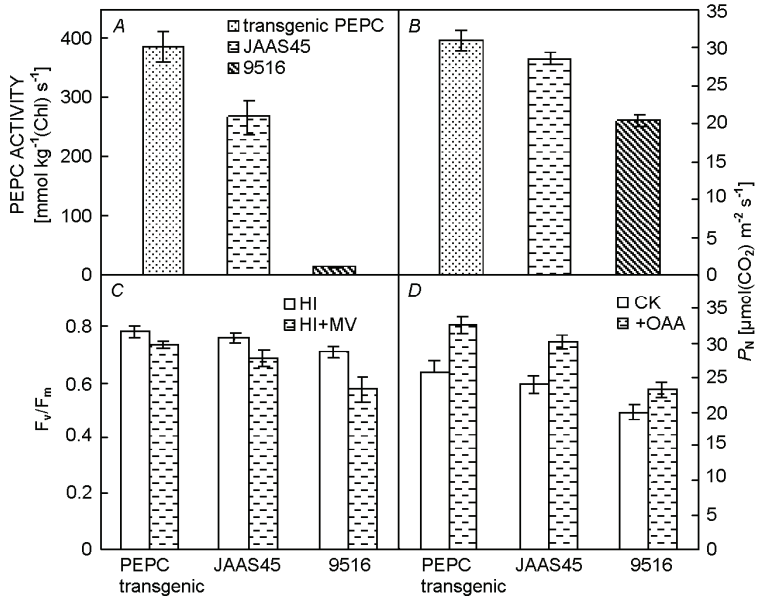

Fig. 2. The PEPC activities (*A*), net photosynthetic rate, P_N (*B*), changes in F_v/F_m after 3-h photoinhibition and photo-oxidative treatments (*C*), and effect of oxaloacetate (OAA) on P_N with distilled water as control, CK in leaves of PC, JAAS45, and 9516 (*D*). Means ± SE from 5 replicates of measurement.

PEPC activity and the C_4 photosynthetic characteristics into conventional rice. Therefore, the technology combining the conventional breeding with biological technology may be an effective approach to physiological breeding.

At present, Chinese super-hybrid rice achieves high yield mainly because of the increase of "sink", *e.g.* it reaches maximum number of grains on the basis of good architecture. However, in major hybrid rice combinations used so far, the panicles are big, but the seed-empty rate is high as well. To further increase the yield, the emphasis should be logically shifted to the increase of "source". In this article, showing the combination of the conventional breeding with biotechnology, the C_4 gene was rapidly and stably introduced into JAAS45. So, we can apply such strategy to the "super-hybrid" rice with high efficiency and good architecture. That means to introduce C_4 enzymes into parental lines of "super-hybrid" rice, and to integrate the two improved traits.

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Govindjee, Beatty, J.T., Gest, H., Allen, J.F. (ed.): **Discoveries in Photosynthesis.** – Springer, Dordrecht 2005. ISBN-10 1-4020-3323-0. XXXVIII+1304 pp., € 315.65.

History of research is very important and much time of young researchers would be spared if they would know what had been done, in some cases many years ago. Govindjee as Historical Corner Editor of the journal Photosynthesis Research persuaded many scientists to write their historical recollections or prepare review papers, collected this material, and did edition of it, often completing the reviews with his own photographs of scientists (and he takes pictures often). But all this material has already been published in voluminous issues of the mentioned journal in the years 2002–2004. Now all these 112 review articles were published once more in one, very thick book. On the one hand, the book brings all this material together, but on the other hand, nothing new is presented and only in very few cases some new references were added. And the old imbalance remains, *e.g*. there is little information on the physiology and ecology of photosynthesis of higher plants or algae and their relations to photosynthetic production, the US research is usually preferred to the European one, *etc*. It is also not easy to find the citations of the original review papers, because they do not accompany the reprinted chapters.

Unfortunately, also the quality of print is much worst in the book when compared with the original journal version. The print is more gray-toned than black and on some photographs it is very difficult to recognize the faces of people one has never met. Thus the reader often doubts whether it was necessary to publish photographs of such low quality (see pp. 266, 365, 395 top, 440 top, 653, 924 and many other ones). In other cases it is not clear why the respective pictures were published, *e.g*. greeting card on p. 544. I also do not understand why the (generally fine) painting of T. Ogawa was chosen for book cover design.

Another problem is with the name index. Only a selection of names is presented here and this does not help the reader. For example, I know the name of Maria Ghirardi. The index gives p. 682, where one of her papers is cited, but why do I have not the opportunity to easily find her picture that is on p. 72? Why it is so difficult to find a fine picture of my teacher Ivan Šetlík that is on p. 939? The names of many researchers that are shown on the photographs are not given in the index. The book will also not serve as an often needed source of references to original research papers, because the names of their authors are not given in the index. I know that this would extremely increase the size of the index, but nowadays this could have been solved by presenting a name index on CD as a welcome supplement or by hanging the full index on the net.

I regret also that more attention has not been given to methods used in photosynthesis research. For example, our book—Šesták, Z., Čatský, J., Jarvis, P.G. (ed.): Plant Photosynthetic Production. Manual of Methods. – Dr W. Junk Publ., The Haag 1971—is not presented in the list of important books, and I know that it has been cited more than 1 000 times. Similarly, there is no mention on the main photosynthesis journals and their history, even if Govindjee co-authored an article on this topic (Photosynthetica **40**: 1-11, 2002).

To sum up, it was certainly easy to reprint the huge material that has already been published in Photosynthesis Research, but the incompleteness of this material remained in the book version. The quality of printing is lower than that in the journal. There is not a good name index. Hence I wonder if those who own the journal would like to buy this book. For the other scientists interested in photosynthesis this volume is certainly an important material to have on the book shelf.

Z. ŠESTÁK (*Praha*)