Transgenic Expression of the Recombinant Phytase in Rice (*Oryza sativa*)

LIU Qiao-quan[1, #], LI Qian-feng[1, #], JIANG Li[1], ZHANG Da-jiang[1], WANG Hong-mei[1], GU Ming-hong[1], YAO Quan-hong[2]

([1]*Key Laboratory of Plant Functional Genomics of the Ministry of Education, College of Bioscience and Biotechnology, Yangzhou University, Yangzhou 225009 China*; [2]*Biotechnology Center, Shanghai Academy of Agricultural Sciences, Shanghai 201106, China*; [#]*These authors contribute equally to this paper*)

Abstract: In most of the cereal crop, phytic acid is the main storage form of phosphorus, which can decrease the bioavailability of phosphate. Transgenic expression of phytase is regarded as an efficient way to release phosphate from phytate in transgenic plants. In this study, a plant expression vector, containing the recombinant phytase gene driven by the maize ubiquitin (*Ubi*) promoter was constructed and introduced into an elite rice variety via *Agrobacterium*-mediated transformation. During the experiment, a total of 15 independent transgenic rice lines were regenerated. The results of PCR and Southern blot indicated that the target gene was integrated into the genome of transgenic rice plants. Moreover, the RT-PCR analysis of total RNAs extracted from the immature seeds of several transgenic lines showed that the recombinant phytase gene could be normally expressed. The inorganic phosphorus content, both in the mature seeds and the leaf was significantly higher in the transgenic plants than in the untransformed wild type.

Key words: transgenic rice; recombinant phytase; inorganic phosphorus content; nutritional quality

In many plants, phytic acid (phytate, 1, 2, 3, 4, 5, 6-hexakisphosphate) is one of the main storage forms of phosphate. About 80% of phosphorus (P) in cereal plants, including rice is stored as phytic acid [1-2]. P in phytic acid can't be utilized by monogastric animals including human, while it was estimated that only 1/3 of the total P in most of the vegetal feedstuff could be efficiently utilized by the livestock. Therefore, for animal feed with P supplementation is expected to meet the dietary requirements. However, such supplementation not only increases the cost of the feeds, but also causes the shortage of phosphorus in nature. Moreover, in the regions with intense animal production, the large amount of undigested and excreted phosphate contributes significantly to the environment pollution. In addition, phytic acid is considered to be an anti-nutritional factor, as it always combines with proteins and cations, thereby decreasing the bioavailability of proteins and some important minerals such as iron, zinc, magnesium and calcium [3].

Phytase [myo-inositol- (1, 2, 3, 4, 5, 6)-hexakis-phosphate phosphohydrolase] is considered to eliminate the anti-nutritional effect of phytate and improve the bioavailability of nutritive substances through catalyzing the hydrolysis of phytic acid to orthophosphate and myo-inositol [4-6]. The microbial phytase, which mainly produced by yeast fermentation had widely used as a feed additive [7-10]. But the cost of phytase production is relatively expensive. Hereby, introduction and expression of heterogenous phytase in plants is regarded as an efficient way to eliminate this limitation as the expressed phytase could hydrolyze phytic acid and then improve the bioavailability of phosphorus as well as other nutritive elements. Such kind of genetic engineering is also helpful in reducing the stockbreeding cost and protecting the environment. Moreover, transgenic expression of phytase in major crops, such as rice, could improve the assimilation and utilization of iron and some other essential nutritive compounds. Previously, many experiments have been conducted on different plants, for examples, tobacco, soybean, wheat, etc [11-12]. Rice (*Oryza sativa* L.), one of the

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Corresponding author: LIU Qiao-quan (qqliu@yzu.edu.cn)

leading crops and the staple food of over half of the world's population, especially in China, is a relatively cheaper source of energy and protein. The expression of the recombinant phytase in the high-yield rice varieties is getting common to enhance the nutritive value of rice as well as its by-products. In present study, to improve the bioavailability of the nutritive elements in rice, the recombinant phytase gene of *Aspergillus niger*, driven by the maize ubiquitin (*Ubi*) promoter, was successfully introduced into a high-yield rice cultivar via *Agrobacterium*-mediated transformation. In response to the expression of phytase, the inorganic phosphorus content in transgenic rice plants was increased.

MATERIALS AND METHODS

Construction of plant expression vector

The plasmid pYF7035 contained a recombinant phytase gene *PRSPhyI*, which was synthesized according to the coding sequence of phytase gene of *Aspergillus niger* and had a broad thermal and acid stable character [13]. The 5' end of the recombinant gene had a secretory signal peptide encoding sequence of tobacco *PRs*（Pathogenesis-related proteins）gene, while its 3' end was linked with the microsome orientation encoding sequence of four amino acids of KDEL [14-15]. The whole size of the gene was 1419-bp long with a *Bam*H I site at the 5' end and a *Sac* I site at the 3'end (GenBank accession number AY182955). The *Bam*H I/*Sac* I fragment of intact phytase gene was sub-cloned into the same sites between the maize *Ubi* promoter and the *A. tumeficiens* nopaline synthase (*NOS*) transcriptional terminator of the binary vector pYH582 [16]. Plasmid pYH582 was derived from the binary vector pCAMIBA1300 (provided by Prof. Jefferson, CAMBIA, Australia), which contained the 2.0-kb maize ubiquitin (*Ubi*) promoter and first intron, used for the performance of the constitutive expression of foreign gene in rice. The resulting plasmid was named pYU159 (Fig.1), and mobilized into *Agrobacterium tumefacien* strain EHA105 for rice transformation as described by Hofgen et al [17]. The standard procedures for molecular cloning were done according to Sambrook et al [18]. Enzymes such as restriction endonuclease and T_4 DNA ligase were bought from Roche Company.

Rice transformation

An elite japonica rice (*Oryza sativa* L.) variety Guanglinxiangjin (GLXJ) (provided by Prof. Tang Su-zhou, Agricultural College of Yangzhou University) was used as the explants. *Agrobacterium*-mediated rice transformation, selection, and plant generation were done as described by Liu et al [19]. The stable transformed plants were screened for hygromycin resistance and then transgenic rice plants were transferred into soil in the genetically modified rice growing field.

PCR and Southern blot analyses of transgenic plants

Total genomic DNAs were extracted from the leaves of transgenic rice plants according to the method of Murray and Thompson [20]. Two sets of primer pairs, Phy-5 (5'-GAACTTCTTGAAATCTTT CCC-3') and Phy-3 (5'-GCAAAGCATTCAGCCCA ATC-3') for *PRSPhyI* gene, and HP1 (5'-GCTGTTAT GCGGCCATTGTC-3') and HP2 (5'-GACGTCTGT CGAGAAGTTTG-3') for hygromycin resistance gene (*Hyg*) were synthesized by Shanghai Sangon Company for PCR analyses, and the PCR products will be 1.4-kb and 0.63-kb in size, respectively. The PCR amplification was carried out in the conditions of

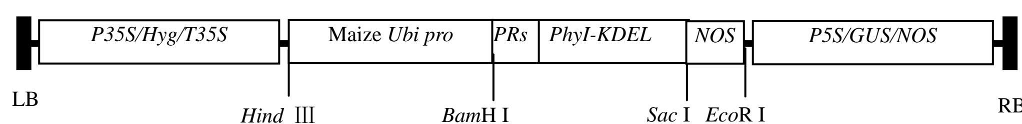

Fig. 1. T-DNA structure of binary vector pYU159 containing the recombinant phytase gene *PRSPhyI*.

PRs, Signal peptide encoding sequences of tobacco pathogenesis-related proteins; *PhyI*, Recombinant phytase gene; *P35S* and *T35S*, Promoter and terminator of CaMV35S gene, respectively; *NOS*, Terminator of napoline synthase gene; *Hyg*, Hygromycin resistance gene; RB and LB, Right and left borders of T-DNA region, respectively.

one cycle of complete denaturizing at 95℃ for 5 min, 30 cycles at 94℃ for 50 s, 55℃ for 50 s, and 72℃ for 60 s, followed by 1 cycle of 72℃ for 10 min. PCR products were analyzed by loading samples onto a 1% agarose gel containing ethidium bromide (EB), followed by electrophoresis and visualization via ultraviolet transillumination. In Southern blot, 10 μg of total genomic DNAs, used after digestion by restriction endonuclease *Hin*d III (Roche), were separated in a 0.8% agarose gel, and transferred onto a Hybond-N^+ nylon membrane (Roche) according to the procedure by Sambrook et al [18]. The membrane was hybridized with Digoxigenin (Roche) labeled *PRSPhyI* gene coding sequence as a probe. Hybridization and signal detection were carried out according to the manual of Roche Company.

Reverse transcription (RT)-PCR analysis of total RNAs

Total RNAs were isolated from developed rice seeds 12-15 days after flowering with the cold-phenol method [21]. An aliquot of 0.5 μg total RNAs, after treatment with RNase-free DNase I, was used for RT-PCR by using the One Step RNA PCR Kit (TaKaRa). The phytase gene specific primers Phy-5 and Phy-3 were used and the products of RT-PCR were separated and identified in a 1% agarose gel. At the same time, 1 μg total RNAs of each sample was separated in an agarose/formaldehyde gel containing EB and observed under UV illumination, which was taken as inner reference.

Measurement of inorganic phosphorus content

The dehulled mature seeds and fresh dried leaves during flowering period were milled into fine powder, and 1 g of dry powder of each sample was soaked in 50 mL 0.5 mol/L $NaHCO_3$ and shaked for 3-4 h. The suspension was performed to filtrate for 2-3 times to get the transparent extract solution. The 5 mL of the extracts was heated in a boiling water bath for 30 min and filtrated again for 2-3 times under consistent temperature till the elimination of denatured proteins. The inorganic phosphorus content was measured according to the colorimetric determination method [22] with some modification. The standard solution, containing 1 mg/mL inorganic phosphorus, was prepared by accurate weighing of 0.4390 g KH_2PO_4, which was oven-dried to a consistent weight at 105℃ and then put in a desiccators at room temperature for cooling and then dissolved in 100 mL ultra pure water.

RESULTS

Generation of transgenic rice plants

The target gene was introduced into the primary callus derived from immature embryos of rice variety GLXJ according to the transformation procedure developed by our laboratory [19]. The resistant calli were selected from hygromycin medium after three continuous selection processes. The well-grown resistant calli were transferred into a regenerating medium, and 2-3 weeks later green spots and little shoots emerged from some of them. Finally, a total of 15 independent transgenic rice lines (denoted from YU159-1 to YU159-15), each contained 1-5 individual plants were regenerated. Most of them were transplanted into a greenhouse and no significant morphological change was observed compared to non-transformed rice plants during the whole period. Moreover, from each individual transgenic line, one to three transgenic plants were selected for subsequent analysis.

Molecular confirmation of transgenic rice

The integration of the target genes into the genome of transgenic rice was firstly detected by PCR technique with primer sets specific for the phytase or hygromycin resistance gene. The specific PCR products could be amplified in all of the independent transgenic lines analyzed (Fig. 2-A, B), while it was consistently absent in non-transformed wild-type plant. However, several transgenic plants, which had been confirmed by PCR amplification, were further performed for Southern blot analysis. After hybridization with the DIG-labeled *PRSPhyI* probe, the hybridization bands were revealed in all of the transgenic plants, as shown in Fig.2-C, while the absence of any signal in the wild-type, demonstrating that the recombinant phytase gene was integrated into the genome of transgenic rice plants. Since there was only one *Hin*dIII restriction site in T-DNA region of

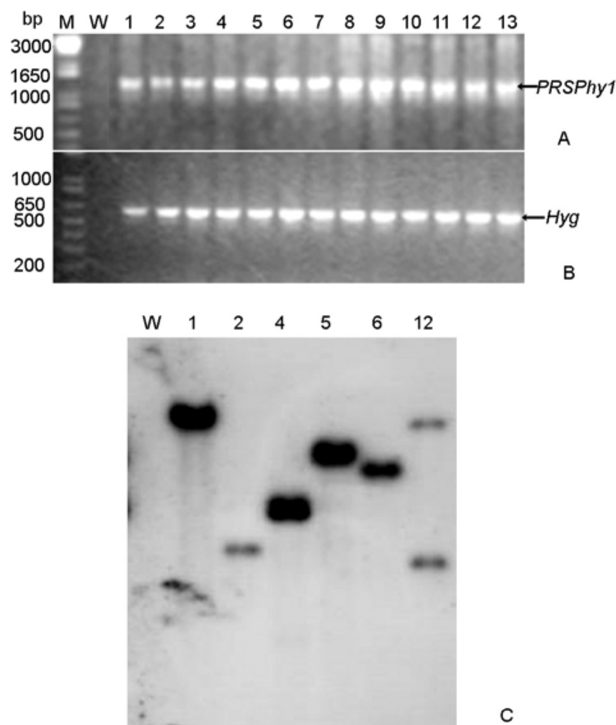

Fig. 2. PCR and Southern blot analyses of the total DNA from the primary transgenic rice plants.

A, PCR analysis for the phytase gene; B, PCR analysis for the hygromycin resistance gene; C, Southern blot analysis. Lanes 1 to 13, Transgenic rice plants; W, Untransformed wild-type plant; M, 1 kb plus DNA marker (Invitrogen).

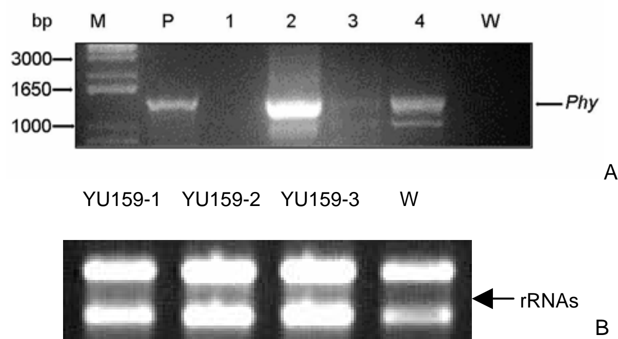

Fig. 3. RT-PCR analysis of total RNAs from the developing seeds of transgenic rice plants.

A: RT-PCR analysis. Lane P, Positive control (using total DNAs from transgenic plant as template); Lanes 1 and 2, Total RNAs from transgenic plant YU159-1; Lanes 3 and 4, Total RNAs from transgenic plants YU159-2 and YU159-3, respectively; Lane W, Total RNAs from untransformed plant; The reverse transcriptase was added in lanes 2 to 4, while not in lane 1. Lane M, 1 kb plus DNA marker. B: The stained rRNA was also showed in the bottom under UV illumination after electrophoresis.

binary vector pYU159 (Fig.1), the copy number and the integrated site of the foreign genes in the transgenic plants could be estimated by the hybridization bands pattern. According to the hybridization signals shown in Fig.2-C, the size of the hybridization bands varied from each other in all of the transgenic plants analyzed, indicating that the target gene was integrated into different sites of the rice genomes. Moreover, the number of hybridization bands was also different in these transgenic plants, all at a range of one to three bands, suggesting that the most of the transgenic plants generated were integrated by one to three sites of the phytase gene.

Expression of phytase gene in transgenic rice

To test the expression of the integrated phytase gene in transgenic rice, RT-PCR was performed by using the phytase gene-specific primers and the total RNAs, isolated from the developing seeds of T_0 transgenic plants, as template. The results showed that the 1.4-kb specific RT-PCR product could be amplified in some of the independent transgenic lines (such as YU159-1 and YU159-3), same as that of control, by using the plasmid DNA of pYU159 as the template, while no PCR product in both of the transgenic lines without adding of the reverse transcriptase in PCR reaction and the wild type (Fig. 3), indicating that the recombinant phytase gene could be transcripted normally under the control of maize *Ubi* promoter in transgenic rice plants. However, the transcriptional signal was not detected in a small proportion of the transgenic lines, for example YU159-2 in Fig. 3, which might due to transgene silencing.

Increased inorganic phosphorus content in transgenic rice

The inorganic phosphorus content was measured both in mature seeds and leaves of transgenic rice plants (Table 1). The data revealed that the inorganic phosphorus contents in the mature seeds of several primary transformants and the leaves of their positive progeny were significantly higher than those of the untransformed wild type. For example, in transgenic line YU159-3 with high level of phytase expression (Fig. 3), the inorganic phosphorus content was 57% higher in mature seeds and 54% in the leaves, compared with wild type.

Table 1. The content of inorganic phosphorus (mg/g dry weight) in transgenic rice plants.

Transgenic plant	Seed in T_1 generation[a]	Leaf in T_1 generation[b]
YU159-1	NT [c]	0.551*
YU159-2	NT	0.492
YU159-3	0.239*	0.723**
YU159-4	0.210*	0.514
Wild type	0.152	0.470

[a]Mean value among 2-3 transgenic plants; [b]Mean value of 2-3 transgenic plants in T_1 generation; [c]NT represents not detected.

* and ** represent significance at P=0.05 and 0.01, respectively, when compared with the wild type.

DISCUSSION

Transgenic expression of phytase in transgenic seeds is becoming a cheap source to improve the bioavailability of P in food/feed compared with direct supplementation of microbial phytase. In addition, the existence of proper post-translational processes, such as glycosylation and phosphorylation in plant cells can allow foreign enzymes to express the exact and high activity. Rice is one of the most important crops in China, indicating a huge potential of introducing the recombinant phytase gene into the local rice varieties through transgenic approaches. On one hand it can improve the nutritional value of rice, on the other hand the cost of animal husbandry can be reduce by using their byproducts as animal feed. Moreover, it can also help us to limit the use of phosphorus fertilizer in order to protect our environment.

Recently, Chwan et al [23] reported that in rice the phytase gene driven by amylase promoter gene could express at a high level in the germinating transgenic rice seeds, which can further improve the utilization and nutritive value of rice seed during germination process. In present study, the *Ubi* promoter gene in maize has been successfully expressed in cereal by the direct expression of the introduced phytase gene in transgenic rice plant. In addition, the recombinant target gene expressing was not only appeared in seeds but also in other tissues such as leaves and culms. Thus, the regenerated transgenic rice could be used to improve the human food and as livestock feed. However, the expression of phytase in roots could be helpful in secretion of the phytase into rhizosphere, which might be much beneficial for the release and/or absorption of phosphate from soil. Moreover, the recombinant phytase in present study is with broad acid and heat optima, and our preliminary results showed that the phytase expressed in transgenic rice was also stable during acid and heat treatment (data not shown).

The present results showed that in transgenic rice the phytase gene expressed regularly with significantly more (57%) phosphorus content than that in the untransformed wild type. These engineered rice lines has already gone under cultivation, and further experiments will be conducted to study the effect of phytase expression on release and improvement of the nutritive value of rice.

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