ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (2): 323-327, 2008
ISSN 1682-296X

Improving Inorganic Phosphorus Content in Maize Seeds by Introduction of Phytase Gene

[1]Yaou Shen, [2]Hongning Wang and [1]Guangtang Pan
[1]Maize Research Institute, Sichuan Agricultural University, Ya' an 625014, Sichuan, China
[2]College of Animal and Technology, Sichuan Agricultural University, Ya'an 625014, Sichuan, China

Abstract: In our research, the plant endosperm special expression vector pCBA with target gene *phyA* was introduced into maize inbred line 18-599 (red) by microprojectile bombardment transformation. The result of PCR assay and Southern hybridization for regenerated plants showed genomes of seven plants were embedded with target gene *phyA*. Of the seven plants, seeds of four plants were collected successfully. The result of phytase activity assay revealed, phytase activities of dry seeds of three plants exhibited distinct increase compared to the control. The top phytase activity of them increased by 60.85% and the according inorganic phosphate content increased by 47.84%. It is concluded that the foreign gene *phyA* was expressed to a certain extent and that transgenic phytase promoted decomposing of phytic acid of seeds, which led to improvement of inorganic phosphorus content in transgenic seeds.

Key words: Maize seeds, phytase gene, inorganic phosphorus

INTRODUCTION

Phosphorus is an essential macronutrient for growth and development of all living organisms. It is a constituent of key molecules such as ATP, nucleic acids, or phospholipids and as phosphate, pyrophosphate, ATP, ADP, or AMP, plays a crucial role in energy transfer, metabolic regulation and protein activation (Rubio *et al*., 2001). Phosphorus is one of the most limiting nutrients for animals because most of phosphorus in plant seeds including feeding plants is in the form of phytic acid (De Boland *et al*., 1975), most of which can not be digested by monogastric animals and become an antinutritional factor hindering the uptake of a range of minerals. Furthermore, high phytic acid content in animal manure by excretion results in elevated phosphorus levels in soil and water and accompanying environmental concerns (Lambrechts and Boze, 1992). While the enzyme phytase is able to release bioavailable phosphorus from phytic acid, consequently improving phosphorus bioavailability and uptake of minerals. On the other hand, the content of phytase in plant itself is too limited to fail to release sufficient inorganic phosphorus (Brinch-Pedersen *et al*., 2002). So it is desirable that transgenic plants themselves express sufficient recombinant phytase to minimize the disadvantage from phytic acid by plants gene engineering.

As the largest part of animal feed, inorganic phosphorus content of maize is very significant to animal. If the transgenic phytase is expressed sufficiently in maize seeds by plant gene engineering, phytic acid in maize seeds will decrease and inorganic phosphorus will increase accordingly.

MATERIALS AND METHODS

The experiment was conducted in Maize Research Institute, Sichuan Agricultural University, China in 2006.

Plasmid constructions: An about 1400 bp fragment encoding the *Aspergillus niger* N_{25} phytase was generated from a *phyA*-containing plasmid pANP-1 by PCR with the upper strand primer (5'-CGTCTAGATGCTGGCAGTCCCCGCCTC-3') and the lower primer (5'-GCGGTACCATCGATCTAAGCAAAA CACTCC-3'). A Xba I site was introduced via the upper primer and a Kpn I site was inserted via the lower strand primer. Following digestion with Xba I and Kpn I, the *phyA* fragment was ligated into the Xba I -Kpn I site of pBPC47 plasmid. The resulting plasmid was named pBA. Then pBA was digested by EcoR I and Hind¢ó and a fragment EGH5-*phyA*-nos was taken out. The fragment was inserted between the EcoR I and Hind III site of the vector pCAMBIA1300, the plant endosperm special expression vector named pCBA was constructed successfully (Fig. 1).

Maize transformations: The ears of maize inbred lines 18-599 (red) were got from the plants on the 11th day after pollination. Then the bracts were stripped one by one

Corresponding Author: Guangtang Pan, Maize Research Institute, Sichuan Agricultural University, Ya' an 625014, Sichuan, China

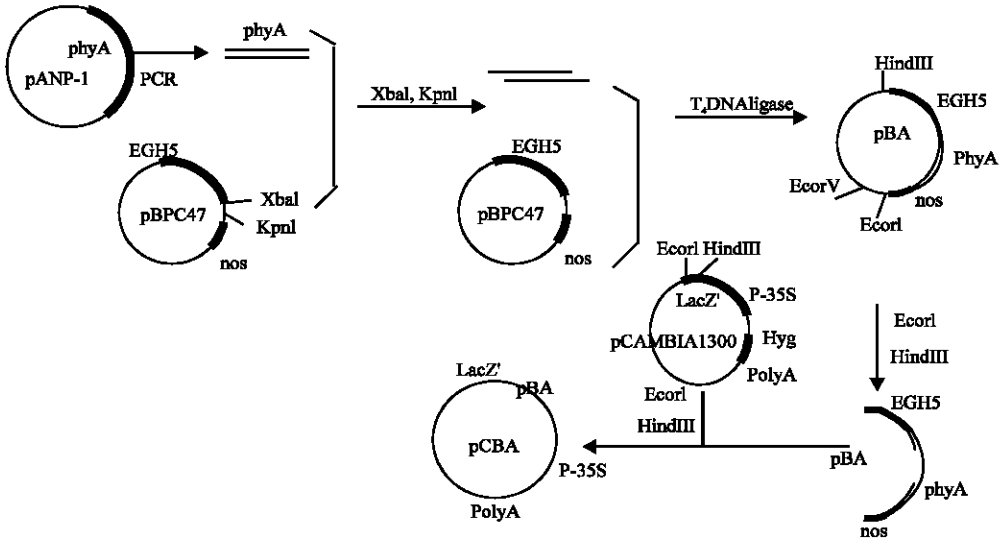

Fig. 1: Recombination of plant expression vector

until the last layer next to the seeds appeared, subsequently they was polished with the cotton ball moistened by 75% alcohol. Under sterile condition the last layer was stripped and part of endosperm on the ears was cut off. Then the immature embryos were taken out by scalpel and placed on a modified N_6 solid medium with the scutellum side up.

After 40 days of callus induction and succession at 27°C, aphoticly, the embryonic callus were transferred to medium containing 0.2 M mannitol for 4 h before microprojectile bombardment (Wu *et al*., 1995). After bombardment, the callus were placed on the mannitol medium for 12 h. For selection of transformed cells, they were subsequently transferred to modified N6 solid medium supplemented with 8 mg L^{-1} hygromycin with the bombarded side in contact with the medium.

After two weeks of culture the callus were divided into 2 ~ 3 fragments and subjected to another four rounds of bi-weekly selection steps until rapidly proliferating cell lines had been generated. Lines originating from the same embryonic callus were considered as having arisen from the same transformation event.

For regeneration of plants, Hygromycin-resistant callus were transferred to modified N6 solid medium lacking 2,4-D and hygromycin. When plantlets had reached 8-12 cm in height and roots had developed, they were planted in soil and cultured in a greenhouse at 25°C, 14 h light period.

Identification of transgenic plants by PCR: PCR amplification for detection of the gene *phyA* was achieved by using the same primers (the upper strand primer 5'-CGTCTAGATGCTGGCAGTCCCCGCCTC-3' and the lower strand primer 5'-GCGGTACCATCGATCTAAGC AAAACACTCC-3'). PCR was performed under the condition of 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec and 56°C for 30 and 72°C for 1 min, subsequently the last step of 72°C for 5 min.

Southern hybridization analysis: Genomic DNA was extracted from young leaves of transgenic and wild-type plants and was subsequently digested by HindIII (there is no HindIII site in the gene *phyA*). The digested DNA was separated in a 7% agarose gel and transferred to a nylon membrane and subjected to Southern blotting as described by Sambrook and Russell (2001). Blots were hybridized with *phyA*, isolated as a Kpn I -Xba I fragment of pCBA. Then the fragment was marked with DIG-High Primer. The performance was directed by the DIG-High Primer Kit (from TaKaRa).

Determination of phytase activity in transgenic dry seeds: Dry seeds of the same ear were milled. One gram seeds of them were grinded to homogenate with acetic acid buffer solution and centrifugalized at 8000 rpm at room temperature for 30 min. The supernatant was collected and subjected to determination of phytase activity. Dry seeds of wild-type plants was taken as the control. Assay of phytase activity was performed as described by Ames (1966).

Assay of inorganic phosphorus content in transgenic dry seeds: Transgenic dry seeds were grinded down and sieved by sieve of 40 mesh to remove bigger fragments.

Whereas the fine powder was subjected to assay of phosphate content. Three gram of the seeds powder was put in a triangular flask and followed 12 mL chloroform and 12 mL Sodium Sulfate in it. Then the mixture was oscillated for 30 min at 25°C and subsequently filtered. The filter liquor was collected in a volumetric flask and metered volume to 50 mL. The resultant liquid was called leaching liquor of seeds and subjected to assay of phosphate content to follow. The method was just as described by Chen *et al*. (2003).

RESULTS

Generation of embryonic callus: The relationship between induction efficiency of embryonic callus and the size of immature embryo was illuminated in our research as follow. To immature embryos less than 1.4 mm, the induction efficiency of embryonic callus was about 10% only. And the induction efficiency of immature embryo more than 2.2 mm was approximate 30% yet. Furthermore, the coleoptile and embryonic root were like to germinate for embryos of length more than 2.2 mm. In this study the embryonic callus induction efficiency of the embryonic callus in size of 1.4 to 2.2 mm. When the length of immature embryo was from 1.4 to 2.0 mm, the induction efficiency trended to increase with the rise of embryo size. Whereas the induction efficiency showed to decrease with the improving of embryo for the embryo more than 2.0 mm (Fig. 2). With inoculation convenience and efficient induction of embryonic callus considered, the immature embryos in length of 1.7 to 2.1 mm were thought to be the best alternative for culture.

Generation and molecular identification of transgenic maize: The plasmid pCBA was introduced into embryonic callus by particle bombardment. Fifty three hygromycin-resistant callus were screened, from which 31 plants regenerated. By PCR seven plants appeared to contain the *phyA* gene (Fig. 3). The number of transgene integrations was evaluated by Southern blot analysis. Genomic DNA was digested with HindIII. The restricted DNA was blotted and hybridized with Kpn I -Xba I *phyA* gene fragment derived from the plasmid pCBA. According to the following figure (Fig. 4), 1 ~ 4 copies were, respectively inserted to the seven transgenic plants and no signal could be detected in the control plant.

Phytase activity in transgenic dry seeds: Under same condition, phytase activity was almost invariant. Accordingly the phytase content in dry seeds was exactly reflected by phytase activity in per kilogram seeds. Through the whole process of determining phytase

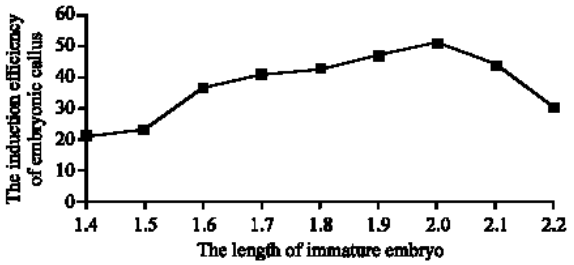

Fig. 2: The relationship between the size of immature embryo and induction efficiency of embryonic calli for inbred line 18-599 (red)

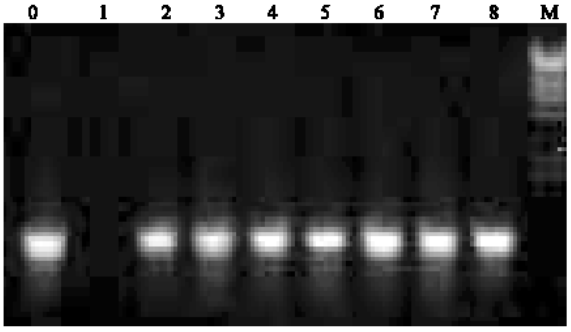

Fig. 3: PCR analysis of maize transformants. M. Marker DNA (λ\HindIII); 0. Positive control (plasmid pCBA); 1: Negative control; 2-8: Transgenic plants

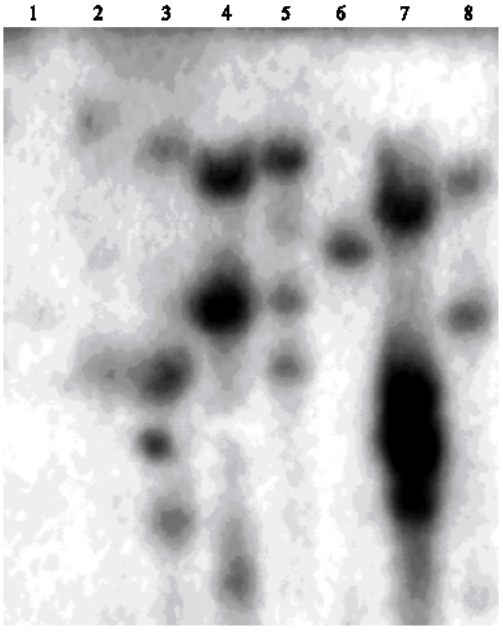

Fig. 4: Southern hybridization for maize transformants. 1: Negative control; 2-8: Transgenic plants

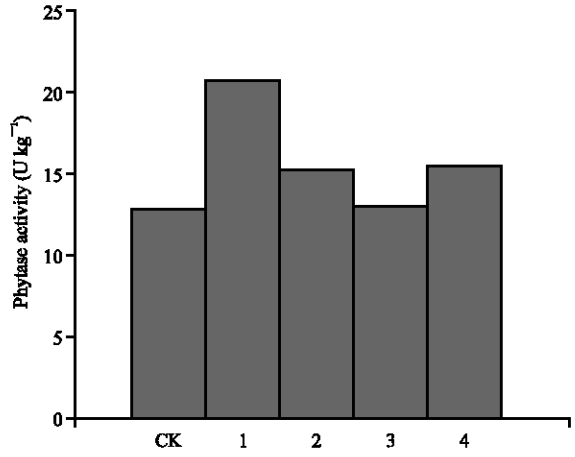

Fig. 5: Phytase activity of transgenic dry seeds

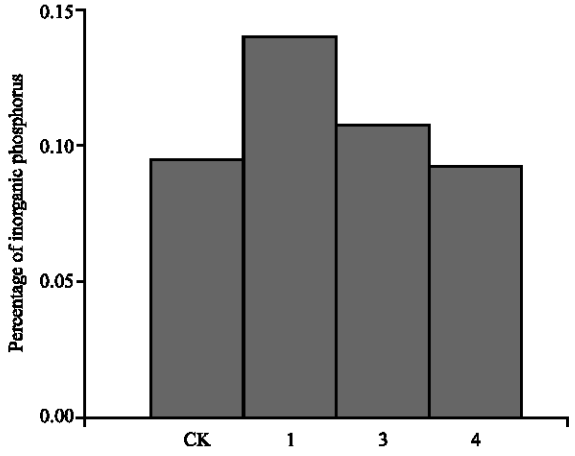

Fig. 6: Inorganic phosphorus content of transgenic dry seeds

activity, the manipulation of each sample was exerted to be controlled in the same condition. As there were four transgenic plants bearing fruits normally, only the phytase activities of the four transgenic plants and the control were determined in our research. The results were exhibited by the following figure (Fig. 5). For the control plant, the phytase activity was about 12.85 U kg^{-1} and phytase activity of transgenic line 01, line 02, line 03 and line 04 was 20.67, 15.25, 13.01 and 15.41 U kg^{-1}, respectively. Analysis of variance indicated phytase activity of each trangenic line show significant deviation ($p<0.01$) to the control. Multiple comparison revealed that there was significant deviation ($p<0.01$) between 01 and line 02, also 01 and line 04. Notably phytase activity of line 01 rose by 60.85% to the control.

Inorganic phosphate content in transgenic dry seeds: Phytase can release inorganic phosphorus from phytic acid, so it could be concluded whether the transgenic phytase decomposed effectively phytic acid by assay of inorganic phosphorus in seeds. Inorganic phosphorus content in transgenic dry seeds was displayed by Fig. 6. From the results of analysis of variance and multiple comparison, there was no significant deviation for line 03, 04 and the control. However, inorganic phosphate content of line 02 rose by 47.84% to the control ($p<0.01$).

DISCUSSION

From present study, there are some problems yet for plants expressing transgenic phytase, such as heat stability and does of transgenic phytase (Verwoerd *et al*., 1995). In this research phytase activity of the transgenic plant seeds rose by 60.85% only compared to the control. To regret, we did not determinate enzyme activity of leaves. *Aspergillus niger* phytase is known to be a secreted protein (Wodzinski and Ullah, 1999). However, there was not a signal sequence at the upstream of the promoter that could promote phytase expressed in other issues to transfer to seeds. On the contrary, an endosperm specific expression promoter EGH5 was used to construct the expression vector in our study. Even though the promoter EGH5 might avoid harming to development of transgenic plants because of phytase excessive expression in other issues as leaves, stem and roots etc. (Yip *et al*., 2003). On the other hand, the promoter EGH5 limited the time and the does of expression of transgenic phytase accordingly. In brief, the use of the endosperm specific expression promoter and the absence of a signal sequence might be the predominant reasons that led to low efficiency of transgenic phytase. In addition, it is well known that there is code preference during the process of translation for different organism (Fan *et al*., 2004). So no modification of *phyA* gene coden is possibly another reason that the dose of transgenic phytase was small. Of course there were other factors such as transgene silence caused by multi-copies.

Although the expression of transgenic phytase in maize seeds was not as expected, transgenic phytase of seeds was confirmed to be effective by the result of inorganic phosphorus content assay. Remarkably, phytase activity of line 01 was the highest among the transgenic plants and the inorganic phosphate content of it was the largest accordingly. Which was as we had expected. As to the reason that expression of transgenic phytase in line 04 did not lead to rise of its inorganic phosphorus content, we will continue paying close attention to it.

CONCLUSION

Previous studies about plant transgenic phytase are mostly concentrated on some mode plants as tobacco, *Arabidopsis thaliana*, alfalfa and other crops as wheat, soybean and rape etc. Compared to other crops, maize is the largest part of animal feed and accordingly offers the main phosphorus source. Consequently, improving inorganic phosphorus content of maize is effective measure to improve available phosphorus content of feed. In all, high-phytase maize did not yet be got, but we gained one high-inorganic phosphorus line by the research. In additional days we are going to set about optimizing expression vector by using secretory signal sequence and maize consistent promoter Ubi (Xu *et al*., 2004), expecting to get the lines containing high phytase and inorganic phosphorus content.

ACKNOWLEDGMENTS

This research was supported by Program for Scholars and Innovative Research Team in University of China (Grant No. IRT-0453) and supported by Hi-Tech Research and Development Program of China (Grant No. 2006AA100103) and International Science and Technology Exchange and Cooperation Program of Sichuan Province.

REFERENCES

Ames, B.N., 1966. Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol., 8: 115-118.

Brinch-Pedersen, H., L.D. Sørensen and P.B. Holm, 2002. Engineering crop plants: Getting a handle on phosphate. Trends Plant Sci., 7: 118-125.

Chen, H.X., Z.N. Yuan and M. Fu, 2003. The analytical method for phytic acid. Food Sci. Technol., 2: 88-89.

De Boland, A.R., G.B. Garner and B.L. O'Dekk, 1975. Identification and properties of phytate in cereal grains and oilseed products. J. Agric. Food Chem., 23: 1186-1189.

Fan, S.H., A.G. Guo, W.L. Shan and X.P. Hu, 2004. Analysis of genetic code preference in *Arabidopsis thaliana*. Prog. Biochem. Biophys., 30: 221-225.

Lambrechts, C. and H. Boze, 1992. Utilization of phytate by some yeast. Biotechnol. Lett., 14: 61-66.

Rubio, V., F. Linhares, R. Solano, A.C. Martin, J. Iglesias, A. Leyva and J. Paz-Ares, 2001. A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev., 15: 2122-2133.

Sambrook, J. and D.W. Russell, 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, America.

Verwoerd, T.C., P.A. Van Paridon, A.J.J. Van Ooyen and J.W.M. Van Lent, 1995. Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves. Plant Physiol., 109: 1199-1205.

Wodzinski, R.J. and A.H.J. Ullah, 1999. Phytase. Adv. Applied Microbiol., 42: 263-302.

Wu, Y.C., J.M. Widholm and P.G. Lemaux, 1995. Type I Callus as a bombardment target for generating fertile transgenic maize (*Zea mays* L.). Planta, 196: 7-14.

Xu, Z.Q., L.G. Gong and X. Huang, 2004. Transgenic maize plants with low copy number of foreign genes were produced with maize ubi-1 promoter. Chin. J. Biotechnol., 20: 120-125.

Yip, W., L.J. Wang, C.W. Cheng and W.L. Wu, 2003. The introduction of a phytase gene from *Bacillus subtilis* improved the growth performance of transgenic tobacco. Biochem. Biophys. Res. Commun., 310: 1148-1154.