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Transgenic soybean with low phytate content constructed by *Agrobacterium* transformation and pollen-tube pathway

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Abstract The expression of a microbial phytase in transgenic plants may create a new biochemical pathway that mobilizes its endogenous phytate and release inorganic phosphate from it, so that more phosphorus is available for plant growth. In this study, transgenic soybean plants were generated via both *Agrobacterium* transformation and pollen tube pathway with the *PhyA* gene of *Aspergillus ficuum*. The optimal concentrations of plant hormones including N_6-benzylaminopurine (BAP), gibberellin (GA_3) and indole-3-butyric acid (IBA) were tested based on their effectiveness on promoting the growth of transgenic explants. Genomic PCR results and Southern blot hybridization analysis showed that transgenic soybean plants selected for resistance to kanamycin contained the *phyA* transgene. The transgenic soybean plants with *phyA* gene integrated in their genome exhibited lower amount of phytate in different soybean tissues including leaf, stem and root, which indicated that engineering crop plants with a higher expression level of heterologous phytase could improve the degradation of phytate and potentially in turn mobilize more inorganic phosphate from phytate and thus reduce phosphate load on agricultural ecosystems.

Keywords Microbial phytase · Phytate · Transgenic soybean · *Agrobacterium* transformation · Pollen tube pathway

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Introduction

Phytate is the primary storage form of phosphate and inositol in plant species through its strong chelating with positively charged proteins, amino acids, and minerals in insoluble complexes (Cheryan 1980). Phytase catalyzes the hydrolysis of phytate and releases minerals, orthophosphate and a series of lower isomers of myo-inositol phosphates (Brinch-Pedersen and Hatzack 2006). As a supplement in diets for monogastric animals to improve phosphate utilization from phytate, phytases have been utilized as animal feed (Lei and Porres 2003; Cao et al. 2007) and human food additive (Da Silva et al. 2005; Greiner and Konietzny 2006), and synthesis of lower inositol phosphates (Dvorakova et al. 2000; Haefner et al. 2005).

Phytases are widely distributed in nature and could be derived from a number of sources including plants, animals, and microorganisms (Fu et al. 2008). Three

distinct classes of phytase (EC 3.1.3.8, EC 3.1.3.26, and EC 3.1.3.72) have been identified and they catalyze the dephosphorylation of phytate at the 3, 6, and 5 positions of the inositol ring, respectively (Gibson and Ullah 1988; Barrientos et al. 1994). Because of the absence or insufficient amount of phytase in monogastric animal gastrointestinal tract, phytases are frequently added to their feed to facilitate optimal growth and decrease the supplementation of inorganic phosphate besides consequently reducing the phosphorus pollution in the areas of intensive livestock units (Fu et al. 2008). Phytase A (PhyA) produced by *Aspergillus niger* is the first commercialized phytase (Natuphos^{TM}) currently holds a large share of the world market (Abelson 1999). The expression of a microbacterial phytase in transgenic plants may create a new biochemical pathway that mobilizes inorganic phosphate from phytate, so that improve phosphate bioavailability for plant growth or physiology (Tye et al. 2002).

Soybean [*Glycine max* (L.) Merrill] is an important source of vegetable oil and protein (Cho et al. 1995). As demands for soybean oil and protein increase, the improvement of soybean quality and production through genetic engineering has became an important issue throughout the world. The objective of this study was to explore the feasibility of reducing phytate in soybean via hydrolyzing the endogenous phytate with PhyA from *Aspergillus niger*. The extent of hydrolysis was then evaluated by the measurement of phytic acid content in the transgenic soybeans. Two transformation methods including *Agrobacterium* transformation and pollen-tube pathway were employed and compared for their efficiency. Variables such as plant hormones used in transformation procedures were also investigated for their influences on transformation efficiency.

Materials and methods

Vector construction

A full-length phytase gene (GenBank accession no. AF537344) was amplified from the strain *Aspergillus ficuum* 3.4322 by PCR. Phytase gene (*PhyA*) open reading frame was generated using the 5′ end primer 5′-atgcccgggctgcgcagtccccgcctcgaga-3′ (underlined *Sma*I site added) and the 3′ end primer 5′-ctaggt accctaagcaaaacactccgcccaatc-3′ (underlined *Kpn*I site added). PCR amplification products were separated on 1% agarose gel and then purified using the QIAEX II agarose gel extraction kit (Qiagen, Beijing, China). *PhyA* fragment was first subcloned into the pMD18-T cloning vector (TaKaRa, Dalian, China) and then introduced into the expression construct pBin438 where the *PhyA* gene was positioned between a double CaMV35S promoter and nos terminator, and the resulting vector was designated as pBin438-*PhyA* (Fig. 1). This construct was mobilized into the *Agrobacterium* strain LBA4404 harboring the helper vector pAL4404 through electroporation method.

Plant materials

Soybean [*Glycine max* (L.) Merrill, Chinese soybean cultivar genotypes Jidou12] seeds were used in these experiments. For *Agrobacterium* transformation, mature, dry seeds were sterilized with chlorine gas for 8–10 h, as previously described (Di et al. 1996). The seeds were then soaked in distilled water for 24 h in the light at 28°C. After the seeds germinated for 5–14 days, seed coat and most of the hypocotyledonary axis were removed and 1–2 cm long acropetal segment of hypocotyls as explants were produced. After excision of the apical and lateral meristem

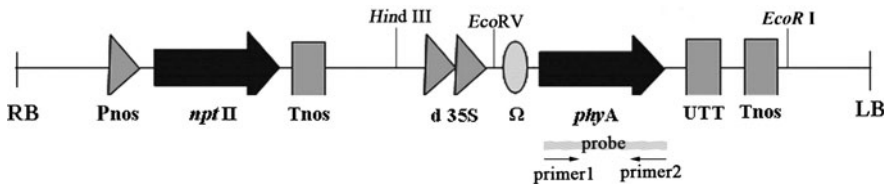

Fig. 1 Schematic map of the linear construct used in this study for soybean transformation experiments. The vector contains the double CaMV 35S promoter, Ω enhancer of TMV, *Aspergillus ficuum PhyA* gene, and nos terminator within T-DNA left and right borders (LB and RB). Restriction sites for vector construction and the positions of PCR primer used in transformant screening and probe production are also marked

explant embryonic tips, the intact cotyledon was wounded by scalpel and ready for *Agrobacterium* transformation.

For pollen pathway transformation, all experiments were performed according to previously reported method (Gao et al. 2007) on fresh flowers with the corolla height greater than calyx. Two wing petals and one keel petal were removed to expose the flower stigmas. The style and the top of the ovary were cut off, and the exposed ovary was dipped with DNA solution for transformation.

Plant transformation

A single bacterial colony was transferred to liquid LB medium with 50 mg l^{-1} kanamycin and 50 mg l^{-1} streptomycin and grew overnight at 28°C. When the culture reached an OD_{600} of about 0.6–0.8, bacterial cells were harvested and resuspended with fresh MS liquid medium (Murashige and Skoog medium) with 3% sucrose, 100 μM acetosyringone (AS), 1.7 mg l^{-1} N_6-benzylaminopurine (BAP) at pH 5.8. Bacteria cell density was adjusted to OD_{600} of 0.4–0.5 before infection of hypocotyls. Explants were first placed on MS solidified media with 3% sucrose with acropetal tissue upwards for 3 days in the dark. They were then inoculated with *A. tumefaciens* for 30 min at room temperature, and transferred to inoculation media (MS solidified medium with 3% sucrose, 1.0–2.0 mg l^{-1} BAP, 0.8% agar, pH 5.8) and incubated in the dark for 3 days. The explants were subsequently rinsed with water and moved to selection medium (MS medium with 250 mg l^{-1} ceftaxime, 100 mg l^{-1} kanamycin, 1.0–2.0 mg l^{-1} BAP, 30 g l^{-1} sucrose and 8% agar) and cultured with an 14/10 h light/dark regime at 25°C. Explants were subcultured onto the same fresh medium at 2-week intervals. Resistant shoots (2–3 cm high) were excised from the hypocotyl segments and transferred to shoot elongation medium (MS salts, 30 g l^{-1} sucrose, 0–2 mg l^{-1} gibberellic $acid_3$, pH 5.8, 0.8% agar, 250 mg l^{-1} ceftaxime, 100 mg l^{-1} kanamycin) for 2 weeks. When the shoots reached 2 cm long, they were transferred to rooting medium (MS salts, 30 g l^{-1} sucrose, 0–1 mg l^{-1} IBA, pH 5.8, 0.8% agar) and rooted seedlings were transplanted to a mixture of vermiculite and peat (1:1, v/v) in pots and grown to maturity in a greenhouse.

For pollen pathway transformation, the exposed ovary was dipped with 5 ml of the gene construct DNA solution (300 μg ml^{-1}). Treated flowers were tagged and untreated flowers and buds at the same node were removed. The pods that developed from the treated flowers were harvested individually (Gao et al. 2007).

PCR and Southern blot analyses

Genomic DNA was isolated from leaf samples of the putative transformants and untransformed control plants by CTAB method (Sanghai-Maroof et al. 1984). PCR analyses were carried out using *PhyA* specific primers with DNA extracted from untransformed plants used as a negative control while the pBin438-*PhyA* vector used as a positive control. Southern blot analysis was carried out on transformants using 15 μg genomic DNA digested with *Eco*RI or *Eco*RI plus *Hin*dIII. The digested DNA was resolved on 0.8% agarose gel and transferred to positively charged nylon membranes (Amersham, USA). The coding sequences of *PhyA* were radiolabelled with α-^{32}P dCTP using random primer labeling beads (Amersham, USA) and used as probes.

For dot blot analysis, 10 μg DNA of putative transformants and untransformed control plants were denatured for 10 min at room temperature in 0.25 M NaOH, chilled on ice, diluted in 0.125 M NaOH with 0.125 M SSC and applied onto the same kind membrane as the Southern blots. The membranes were air-dried at room temperature and hybridized in the same way as the Southern blots.

Phytate measurements

The colorimetric assay was used to quantify phytate content in soybean plants. About 200 mg soybean leaf, stem and root tissues were ground in 10 ml 2% HCl·10% Na_2SO_4 for 2 h with stirring. After centrifugation at 4,000 rpm for 30 min, 2.5 ml supernatant was mixed with 2.5 ml 15% trichloroacetic acid (TCA) and left at 4°C for 2 h, then centrifuged for 30 min at 4,000 rpm. The pH of 2.5 ml supernatant was then calibrated to 6.0–6.5 with 0.75 M NaOH before mixing with ddH_2O to 10 ml volume, then 2 ml diluted supernatant was mixed with 1 ml 0.3% 5-sulfosalycyclic acid·0.03% $FeCl_3 \cdot 6H_2O$ and measured OD_{500} on a photospectrometer. Phytate quantitative standard curve was prepared with phytate dodecasodium salt from corn (Sigma P-8810, USA)

and stock solution was prepared in the concentration range from 5 to 50 mg l^{-1}.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical differences among mean values were determined by one-way analysis of variance and Student's *t* test with SPSS 11.0. *P* value less than 0.05 was considered statistically significant.

Results and discussion

The present study describes results dealing with the establishment of transgenic soybean plants with low phytate content by the introduction of beneficial alien genes into the soybean genome using both *Agrobacterium* transformation and the pollen tube pathway.

Variables affecting the soybean transformation efficiency

Agrobacterium-mediated transformation is the most widely used method for achieving plant genetic transformation. In soybean, acropetal segment of hypocotyl explants were used efficiently for in vitro propagation and genetic transformation. In this study, we firstly tested the competence of acropetal segment of hypocotyl explants for transformation in terms of seedling age (days after seed germination). We found that at 5 D seedling age, the hypocotyl explants are most prone to generate adventitious shoots (data not shown). For *A. tumefaciens* mediated soybean transformation, adventitious shoots were directly induced from acropetal segments of hypocotyls. To obtain more adventitious shoots to increase the transformation efficiency, we added 1.0–2.0 mg l^{-1} BAP to the shoots induction medium and recorded the average shoot number on each hypocotyl explant (Table 1). It has shown that 1.7 mg l^{-1} BAP could induce highest number of adventitious shoots. GA_3 has been reported to enhance the elongation of adventitious shoots on explants (Emery et al. 2001; Hamano et al. 2002), so we added different levels of GA_3 to the shoot elongation medium and measured the resulting average length of shoots and survival rate after replanting. Our results showed that GA_3 did have obvious effect on the elongation of adventitious shoots (Table 1). In addition, the level of GA_3 was also observed to affect the coloration of shoots. At concentration of 1.5 mg l^{-1}, the adventitious shoots reached the maximum average length, however, the shoots were phenotypically thin and weak due to their excessive elongation, so we used 1.0 mg l^{-1} GA_3 in this study and at this concentration, the adventitious shoots were medium-sized, robust and survived well after replanting. IBA has been reported to be able to promote rooting of adventitious shoots and enhance the growth of roots (Ercisli et al. 2004; Balestri and Lardicci 2006; Sharma et al. 2006). We divided the adventitious shoots on hypocotyl explants into single one, then planted them on rooting medium with different levels of IBA and the results showed that at 0.4 mg l^{-1} IBA, the adventitious shoots exhibited highest rooting rate as 73% (Table 1).

Table 1 Effects of BAP, GA_3 and IBA on the transgenic soybean plants regeneration system from cotyledonary nodes

Hormones	Effect index	Hormone levels (mg l^{-1})				
		1.0	1.2	1.5	1.7	2.0
BAP	No. of regenerated shoots per explant	3.1 ± 0.8^a	3.8 ± 0.7^a	4.7 ± 0.9^b	5.8 ± 1.2^c	5.0 ± 1.0^b
		0	0.5	1.0	1.5	2.0
GA_3	Shoot height (cm)	1.2 ± 0.3^a	4.1 ± 0.5^c	4.2 ± 0.4^c	4.7 ± 0.4^c	3.4 ± 0.3^b
	Leaf color	Yellow green	Green	Green	Dark green	Dark green
	Transplanting survival rate (%)	37.6 ± 6.5^b	85.2 ± 6.2^c	72.6 ± 8.5^c	41.4 ± 4.2^b	16.7 ± 2.3^a
		0	0.2	0.4	0.7	1.0
IBA	Rooting rate (%)	23.3 ± 3.7^a	66.7 ± 7.8^d	73.3 ± 5.9^d	53.3 ± 4.2^c	36.7 ± 4.7^b

Notes: Mean values (±standard deviations) with different superscript letters (a, b, c and d) indicate significant different ($P < 0.05$)

Table 2 Summary of genomic PCR and Southern analysis results of pollen-tube pathway soybean transformation system using different transgenetic DNA preparations

Injected materials	Circular plasmid DNA	Linear plasmid DNA	Circular plasmid DNA with *Agro.*	Circular plasmid DNA with *Agro.* protein
No. of obtained seeds	811	262	163	179
Seedling generation rate (%)	64	62	62	59
PCR-positive plants	33	10	6	7
Southern-positive plants	17	5	3	3
Transformation frequency (%)	3.3	3.1	3.0	2.9

For pollen tube pathway, we compared the transformation efficiency of circular plasmid DNA, restriction enzyme digested linear plasmid DNA, undigested plasmid DNA with *A. tumefaciens* and undigested plasmid DNA with *A. tumefaciens* protein extract and found that although intact plasmid DNA was easier to manipulate and had slightly higher transformation efficiency (Table 2), linear DNA fragments also resulted in successful transformation. This indicated that linear DNA fragment (composed of the target gene, flanking regulation elements and T-DNA border repeats) could be introduced into plants directly through pollen tube pathway to generate marker-free transgenic plants.

Molecular characterization of transformants

For *A. tumefaciens* transformation, 15 out of 50 kanamycin-resistant soybean seedlings turned out to be positive in genomic DNA PCR and gave out a ~1.4 kb amplification products of transgene with *PhyA* primers (Fig. 2a), which was similar to that of the positive control pBin438-*PhyA* vector. No such band was observed in the untransformed controls under similar conditions. This observation clearly indicated the presence of the transgenes in the genomes of putative transformants.

Southern blot analysis of *Eco*RI digested genomic DNA of PCR-positive transformants disclosed clear hybridization bands in four plants indicating the integration of intact *PhyA* expression cassette into their genomes (Fig. 2b). Three of four transformants showed the presence of a single band, implying single copy integration of the transgene in the soybean genome. When two restriction enzymes (*Hin*dIII plus *Eco*RI) flanking the expression cassette were used, one single hybridization band of 2.2 kb was observed (Fig. 2c). It was reported that single copy integration was essential for achieving predictable inheritance pattern and for avoiding gene silencing in transgenic plants (Finnegan and McElroy 1994).

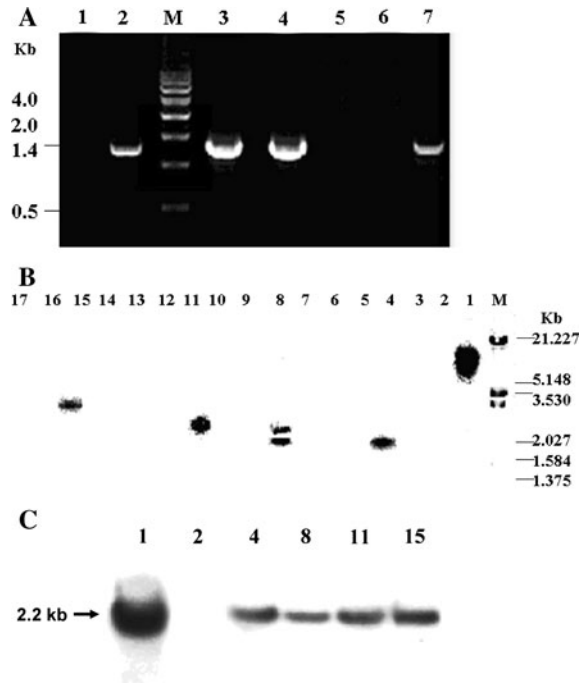

Fig. 2 Molecular Characterization of soybean plants transformed by *Agrobacterium* transformation. **a** PCR analysis on genomic DNA of *Agrobacterium* transformed soybean plants. *Lane 1*: Untransformed plant control; *lane 2*: plasmid of pBin438-phyA; *lane 3–7*: independent transformed plants; *lane M*: 500 bp DNA ladder. **b** Southern blot analysis of genomic DNA digested by *Eco*RI of 15 *Agrobacterium* transformed plants. *Lane 1*: Plasmid of pBin438-phyA; *lane 2*: untransformed plant control; *lane 3–17*: independent transformed plants; *lane M*: λ-DNA marker (*Hin*dIII + *Eco*RI). **c** Southern blot analysis of genomic DNA digested by *Hin*dIII + *Eco*RI of four selected *Agrobacterium* transformed plants. *Lane 1*: Plasmid of pBin438-phyA; *lane 2*: untransformed plant control; *lane 4, 8, 11 and 15*: transformed plants 4, 8, 11 and 15 in **b**

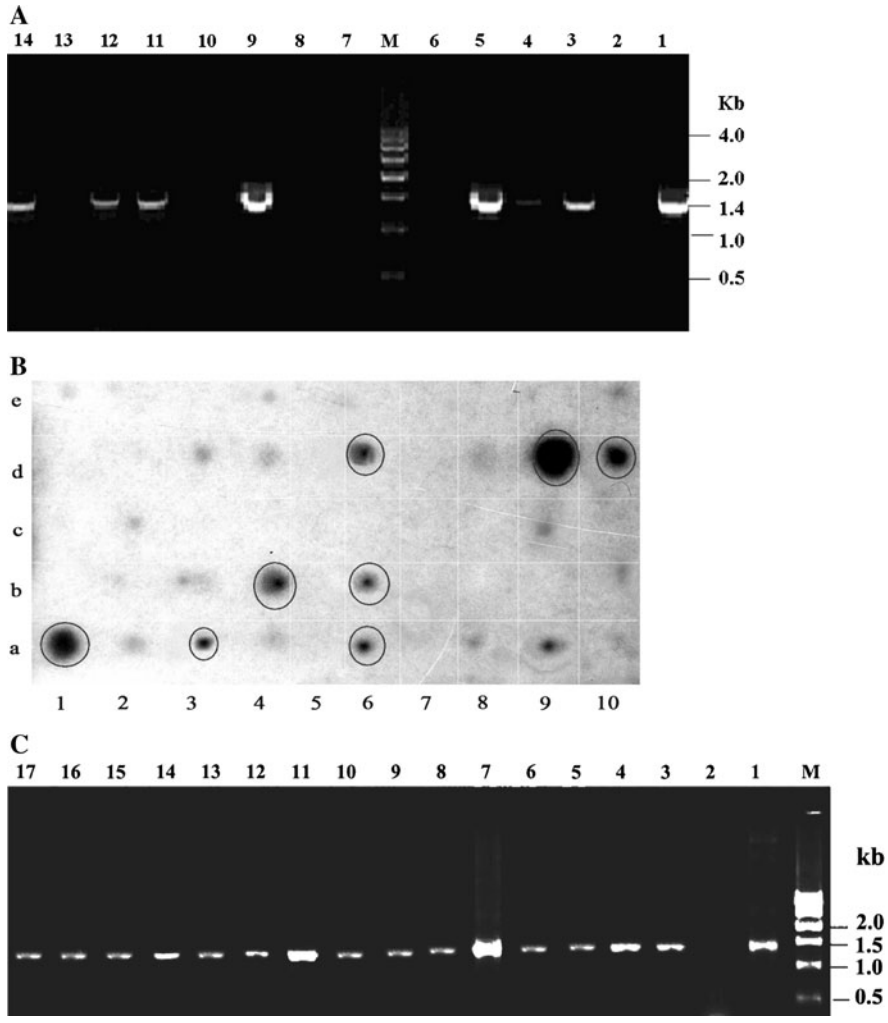

Fig. 3 Molecular characterization of transformed soybean plants via pollen-tube pathway. **a** PCR analysis on genomic DNA of pollen-tube pathway transformed T_1 soybean plants. *Lane 1*: Plasmid of pBin438-phyA; *lane 2*: untransformed plant control; *lane 3–14*: transgenic plants; *lane M*: 500 bp DNA ladder. **b** Southern dot blot analysis of pollen-tube pathway transformed soybean plants. *Dot a-1*: plasmid of pBin438-phyA; *Dot b-1*: untransformed plant control; *other dots*: transformed plants. **c** Genomic DNA PCR analysis on Southern dot blot positive plants. *Lane 1*: plasmid of pBin438-phyA; *lane 2*: untransformed plant control; *lane 3–17*: transgenic plants; *lane M*: 500 bp DNA ladder

For pollen tube pathway transformed soybean plants, all collected seeds were germinated in a greenhouse. The selection process was first conducted by PCR analysis on the genomic DNA pool of multiple potential transgenic plants, then screening was conducted in the positive pool to identify the individual transformant. A total of 56 out of 888 soybean plants contained the gene cassette as evidenced by specific PCR amplifications of the 1.4 kb fragments (Fig. 3a). PCR-positive seedlings were further confirmed by Southern dot blot and resulted in 28 positive transformants (Fig. 3b). PCR analysis on their genomic DNA was then carried out and the results indicated exogenous *PhyA* gene has integrated in all the 28 transformants (Fig. 3c).

In comparison, pollen tube pathway in this study gave out a 3.2% average transformation frequency in the seed population, and the highest frequency of method using circular plasmid DNA reached 3.3%. It is lower than the 8% of *Agrobacterium* transformation in this study but similar to another previously reported 3.8% using the same pathway (Yang et al. 2002). Their slight differences (3.3% in this study compared to the reported 3.8%) could be due to that different soybean genotypes were used as starting materials for transformation.

Change of phytate content in the transgenic soybeans

Engineering crop plants to produce heterologous phytase has been reported in several studies with the aims to reduce phosphate load on agricultural ecosystems and improve phosphate bioavailability. For

Table 3 Phytate content in leaf, stem and root tissues of wild-type and transgenic soybean plants

Plants	Leaves		Stems		Roots	
	Phytate content (mg g^{-1})	Percent decrease (%)	Phytate content (mg g^{-1})	Percent decrease (%)	Phytate content (mg g^{-1})	Percent decrease (%)
Control	12.5 ± 0.4	–	13.3 ± 0.6	–	6.7 ± 0.1	–
Agro.	11.3 ± 0.8*	9.9	11.9 ± 0.6*	10.7	6.1 ± 0.5*	9.9
Pollen	11.2 ± 0.3*	10.6	11.2 ± 0.3*	10.0	6.0 ± 0.3*	11.3

Notes: Mean values (±standard deviations) with * indicate significant different ($P < 0.05$)

example, when a *B. subtilis* phytase was introduced into tobacco cytoplasm, it resulted in equilibrium shift of inositol biosynthesis pathway, thereby making more phosphate available for primary metabolism (Yip et al. 2003; Fu et al. 2008). Meanwhile, transgenic tobacco and *Arabidopsis* plants over-expressing *B. subtilis* phytase have been reported to be capable of utilizing exogenous phytate (Lung et al. 2005), which may potentially mobilize soil phytate into inorganic phosphate for plant uptake. In our current study, transgenic soybean plants harboring the *PhyA* gene were supposed to constitutively express the target gene and the increased phytase activity should result in a decreased level of phytate. Therefore, we measured the content of phytate in two types of *PhyA* transgenic plants, which included four *Agrobacterium* transformed and 28 pollen tube pathway transformed soybean plants, along with untransformed controls. The results showed that a small decrease (~10%) in phytate content was detected for transgenic soybean plant samples (Table 3). No obvious difference was detected between *Agrobacterium* transformed and pollen tube pathway transformed soybean plants regarding the degree of decrease in the phytate content (Table 3). In literature, the phytate content in soybean seed has been reported to decrease by 8% using seed-specific promoter (Chiera et al. 2004), which was close to our results in vegetative parts of soybean. The range of phytate reduction previously reported for soybean mutants generated by ethyl methane sulfonate treatment was 29–55% (Wilcox et al. 2000). However, the seed germination rate could be seriously affected since phytate played important physiological functions during seed germination (Urbano et al. 2000). In this study, despite that only a moderate decrease in phytate content was achieved, no obvious phenotypic abnormalities have been observed in our transgenic soybean plants. More importantly, our results further verified the feasibility of decreasing the phytate content by inducing its effective hydrolysis in important crop species through transgenic engineering pathway.

Conclusion

This study intended to hydrolyze the endogenous phytate by introducing a microbacterial phytase gene into economically important plant species, soybean, with the aim to increase the phosphate bioavailability for plant growth. Two transformation procedures including *Agrobacterium* transformation and pollen tube pathway were employed in parallel to compare their easiness and transformation efficiency. The transgenic identity of the genetically modified soybeans had been confirmed at the DNA levels and the expression of foreign phytase gene in soybean T_1 generation led to a ~10% decrease in phytate content. Present investigation could be regarded as the groundwork to study enzymatic degradation of phytate by exogenous phytase in transgenic plant, especially those plant species used widely in feed, to improve mineral and protein utilization, and simultaneously reducing excessive phosphorus accumulation in the environment.

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