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Overexpression of Rice Phosphate Transporter Gene *OsPT2* Enhances Tolerance to Low Phosphorus Stress in Soybean

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ABSTRACT

Low phosphorous (P) availability in soils limits production of soybean [*Glycine max* (L.) Merr.] around the world. This study was conducted to determine whether exogenous expression of the rice (*Oryza sativa* L.) phosphates transporter gene *OsPT2* would increase inorganic phosphates (Pi) acquisition and improve yield in transgenic soybean. Cotyledonary-node explants of the soybean were inoculated with the *Agrobacterium tumefaciens* strain EHA105 harboring the vector pCAMBIA3301-*OsPT2*, which contained *OsPT2*, *gus* and *bar* genes. Ten fertile T_0 transgenic plants were obtained and semi-quantitative RT-PCR of progenies demonstrated that *OsPT2* gene was overexpressing in the T_2 generation. Three T_2 transgenic lines overexpressing *OsPT2* were selected and subjected to testing for tolerance to low concentrations of Pi (low-Pi; 20 µM Pi) by hydroponic culture using modified Hoagland's nutrient solution. The total P contents in the leaves, stems, roots, and seeds of the transgenic plants significantly increased under the concentrations of low-Pi and 1,000 µM Pi of standard Hoagland's nutrient solution. Under low-Pi stress, the yields of the transgenic lines were significantly higher than those of the wild type. Taken together, our data suggest that the overexpression of *OsPT2* in transgenic soybean lines improves Pi acquisition and seed yield, and *OsPT2* may serve as one of the promising target genes that can be manipulated in crop improvement for minor use of Pi fertilizers.

Keywords: *Glycine max*, Transgenic plants.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.], one of the most widely grown leguminous crops, is one of the principal food sources for human and livestock (Herridge *et al.*, 2008). Soybean oil is the most common vegetable oil and is considered as a promising bio-diesel (Chapotin and Wolt, 2007). The global production of soybean has doubled over the past 20 years (http://faostat.fao.org), and reached 83.18 million metric tons in 2011 (http://soystats.com/2012/Default-frames.htm). Nonetheless, low phosphorus (P) availability in soils limits soybean production (Vance, 2001). To obtain maximal crop yields, nearly 30 million tons of P fertilizer are applied to agricultural fields each year, up to 80% of which is lost (López-Bucio *et al.*, 2000). Phosphorous becomes immobile and unavailable for plant uptake (Qin *et al.*, 2012), leading to accelerated soil degradation and environmental problems. Excessive application of phosphates to soils also wastes phosphorite resources, which may be depleted by the end of the 21st century (Hata *et al.*, 2010).

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Tropical, subtropical, and temperate regions are the main area for soybean production, however, these regions are low in P due to weathering, intensive erosion, and strong P fixation by free ion and aluminum oxides (Wang *et al.*, 2009). Especially in the acid soils in southern parts of China, the concentrations of inorganic phosphates (Pi) are lower than 6 μM, and the rate of soil Pi diffusion is much lower than the rate of absorption by plant roots (Li *et al*., 2011; Vance *et al.*, 2003). As a key element in metabolism and an essential building block of cell components (Rouached *et al.*, 2010), P is considered to be the most important nutrient for soybean (Malik *et al.*, 2006). Soybean plants require more P compared with other nutrients, due to the root nodules responsible for nitrogen fixation and the seeds containing more oil and proteins (Qin *et al.*, 2012; Win *et al.*, 2010). Lack of P in soybean can cause serious effects, one of which may prevent other nutrients from being absorbed by soybean plants (Win *et al.*, 2010). In order to increase the yield and improve the nutritive quality of the soybean seed, enhancing the efficiency of Pi acquisition is necessary to achieve high yields of soybean seeds (Shah *et al.*, 2001).

In the past decade, increasing attention has focused on the mechanisms involved in improving Pi acquisition and metabolism in plants (Ai *et al.*, 2009; Guo *et al.*, 2013; Vance *et al.*, 2003; Wu *et al.*, 2013). Transport systems are essential for the uptake of Pi and for its internal redistribution within plants (Smith *et al.,* 2003). Rice (*Oryza sativa* L.) phosphate transporter *OsPT2* is thought to function in Pi translocation processes in plants (Ai *et al.*, 2009; Liu *et al.*, 2010). Knockdown of *OsPT2* expression by RNA interference decreases both the uptake and long-distance transport of Pi from roots to shoots, and overexpression of *OsPT2* can cause over-accumulation of Pi in rice shoots and, thus, a Pi toxicity phenotype (Ai *et al.*, 2009; Liu *et al.*, 2010). These data suggest that *OsPT2* has potential to be used for crop improvement in low P stress via genetic engineering.

Recently, several crops have been improved in the Pi utilization efficiency by introducing phosphate transporter genes, such as rice, wheat, and tobacco (Guo *et al.*, 2013; Jia *et al.*, 2011; Sun *et al.*, 2012; Wu *et al.*, 2013). However, very limited work has been done to improve Pi acquisition in soybean. In this study, we generated fertile T_0 *OsPT2* transgenic soybean plants and their progeny, and tested if ectopic expression of *OsPT2* could confer soybean stable transformants (T_2 transgenic lines) tolerance to low-Pi.

MATERIALS AND METHODS

Plant Material and Seed Germination

The soybean cultivar 'Xinliaoxian' (provided by Nanjing Lüling Seed Company, Nanjing, China) was used as the experimental host, which was based on the GUS transient expression frequencies of sixteen soybean cultivars (Liu *et al.*, 2014). Dry seeds were surface sterilized and germinated according to Liu *et al*. (2014).

Binary Vector and *Agrobacterium* Strain

The binary vector pCAMBIA3301-35S (CAMBIA, Australia) was used for this study, which had been added the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator. To this vector, the ORF of *OsPT2* (Accession number: AF536962), digested with the *Bam*HI and *Sac*I (Promega, USA), was inserted between the CaMV 35S promoter and the NOS terminator. The new construct, pCAMBIA3301-*OsPT2*, was transferred into *Agrobacterium tumefaciens* strain EHA105 as described in our previous report (Liu *et al.*, 2014).

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Production of Transgenic Soybean Plants

Cotyledonary-node explants were prepared from 5-day-old seedlings. The procedure for the genetic transformation was based on the *Agrobacterium*-mediated method described by Liu *et al*. (2013, 2014). The transgenic T_0 plants were confirmed by PCR and southern blot. The T_1 and T_2 seeds harvested from the positive transgenic plants were germinated and identified by PCR and GUS assays. The positive transgenic plants of T_2 generation were used for the low-Pi tolerance evaluation.

GUS Assays and Leaf Painting

Various parts of transgenic plants and seedlings were collected and used for histochemical detection of *gus* expression (Liu *et al*., 2014). Leaf painting was performed to test herbicide tolerance in the transgenic plants according to Liu *et al*. (2014).

Molecular Analysis of Transformed Plants

The genomic DNAs of the transgenic and wild type (WT) plants were extracted, and the *gus* and *bar* genes were amplified (Liu *et al*., 2014). The amplification of *OsPT2* was performed according to Ai *et al*. (2009). PCR reactions were conducted using a Thermal Cycler (TProfessional, Biometra, Germany).

To further characterize the copy number of the integrated foreign genes in the T_0 transgenic plants, southern blot analysis was performed using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Germany). The 944 bp *gus* gene was labeled with digoxigenin-dUTP and used as the probe for hybridization (Liu *et al*., 2013, 2014).

To evaluate the expression level of the *OsPT2* gene in the T_2 transgenic plants, a semi-quantitative RT-PCR was performed according to Yannarelli *et al*. (2006). Total RNA was extracted and the *OsPT2* gene was amplified by PCR using the special primers as described by Ai *et al*. (2009). The housekeeping gene *TefS1* (Encoding the soybean elongation factor EF-1a: X56856) was used as a control. Images of ethidium bromide stained gels were captured and analyzed using Integrated Gel Imaging Systems (GenoSens 1880, Shanghai Clinx Science Instruments, China). The ratio of *OsPT2* mRNA to *TefS1* mRNA was quantified.

Progeny Segregation Analysis

Transgenic plants were allowed to self-pollinate to produce subsequent generations of progeny. The progeny of four independent lines (12PT2-1, 12PT2-2, 12PT2-3, and 12PT2-4) were evaluated for the *gus* expression and tolerance to the herbicide by testing leaves of T_1 plants. The chi-square (χ^2) analysis was performed to test the ratios 3:1 and 15:1 (GUS positive: GUS negative and herbicide tolerant: herbicide sensitive), where significance was determined for those values with a *P*-value greater than 0.05.

Pi Deficiency Treatment

To evaluate low-Pi tolerance under greenhouse conditions, three transgenic seedlings (10-day-old) from independent T_2 lines (12PT2-1, 12PT2-2, and 12PT2-4) and one WT seedling (10-day-old) were grown in modified Hoagland's nutrient solution (substituting some of the $NH_4H_2PO_4$ with NH_4Cl until the final concentration of P was 20 μM; the other nutrients were as follows: 15 mM N, 6 mM K, 4 mM Ca, 2 mM Mg, 2 mM S, 0.98 mM Cl, 140 μM B, 36 μM Mn, 46 μM Zn, 100 μM Cu, 30 μM Fe, and 1 μM Mo; EC= 2.61 dS m^{-1}) and aerated continuously. The nutrient solution was replaced every 3 days, and the pH was

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adjusted to 6.5. Ten plants of each T_2 line were hydroponically grown as replications. The standard Hoagland's nutrient solution (normal-Pi; 1,000 μM Pi) was used for the control. The environmental conditions in the greenhouse were artificially controlled as follows: day/night temperature at 28°C/20°C, light and dark periods of 12 hours each, with light from HPS lamps at a photosynthetic photon flux density (PPFD) of 600 μmol m^{-2} s^{-1}, a relative humidity of 70–80%, and a CO_2 concentration of 400 μM.

Measurement of Growth Parameters and P Concentrations

Thirty days after hydroponic cultivation, five plants of each line were randomly subjected to growth parameter measurements, including plant height and root length. The plants were then harvested and the biomass of shoots and roots was determined according to Chen *et al*. (2011).

Inorganic phosphates and total P concentrations of plant tissues were determined according to Zhou *et al*. (2008). For Pi measurement, a frozen sample was homogenized in 1 mL 10% (w/v) of perchloric acid (PCA), and the homogenate was then diluted 10 times with 5% (w/v) PCA and placed on ice for 30 min. The supernatant was collected from the homogenate by centrifugation at 10,000×g for 10 minutes at 4°C, and used for Pi measurement via the molybdenum blue method. Inorganic phosphates concentration was calculated by normalization of fresh weight. Total P content was analyzed by the molybdenum blue method after digesting with H_2SO_4-H_2O_2 at 300°C, and normalized by dry weight.

Seed Yields of Plants

When the five remaining plants reached maturity at approximately 90 days after low-Pi treatment, the plants were harvested. The number of seeds per individual plant was counted, and the seeds were weighed.

Statistical Analysis

The data were subjected to normality test, analysis of variance (ANOVA), and levels of significance were determined by the least significant difference (LSD) test using SAS software (SAS Institute, Cary, NC, USA). Graphs were drawn using Origin 8.5 software (Microcal, USA).

RESULTS

Generation of Transgenic Soybean Plants

The plant binary vector pCAMBIA3301-*OsPT2* was constructed. The schematic diagram of the T-DNA region is presented in Figure 1. The cotyledonary-node transformation method using bialaphos for the selection of the transformed shoots is illustrated in Figure 2. In three independent experiments, a total of 673 explants were

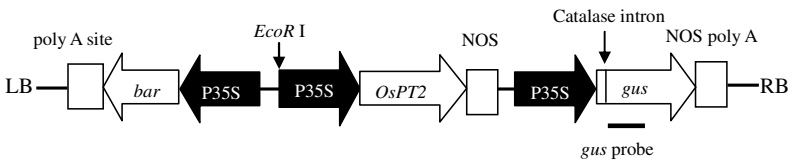

Figure 1. Schematic representation of the T-DNA region of pCAMBIA3301-*OsPT2*. The exogenous genes were driven by the CaMV 35S promoter. The T-DNA left border (LB) and right border (RB) and the positions of enzyme sites are indicated.

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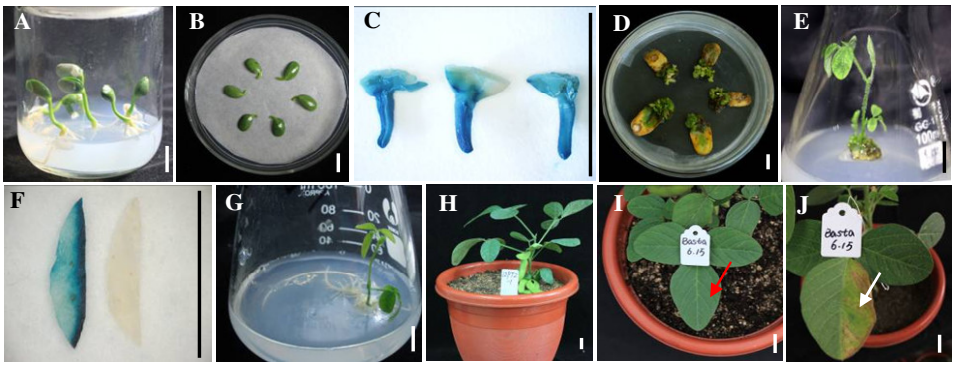

Figure 2. *Agrobacterium*-mediated cotyledonary-node soybean transformation method using the selective agent bialaphos. (A) Five-day-old aseptically grown seedlings; (B) Co-cultivation; (C) Transient expression of GUS in cotyledonary-node region after co-cultivation for 4 days; (D) Shoot induction for 4 weeks; (E) The elongated shoots reached 3–4 cm in height; (F) GUS detection; (G) Rooting; (H) Maturity; (I) and (J) Basta® resistance test, red arrow indicates the Basta® tolerant response, and white arrow indicates the Basta® susceptible response, following leaf painting with a 0.5% Basta® solution. Bar= 1.0 cm.

inoculated by *A. tumefaciens* and fourteen GUS positive shoots were obtained via the half-leaf GUS assay method (Table 1). After rooting, ten plantlets grew in pots in the greenhouse and matured. These ten putative transgenic plants were numbered 12PT2-1 to 12PT2-10. The transformation efficiency ranged from 1.43 to 1.64% with an average of 1.48% (Table 1).

Basta® Resistance and GUS Expression are Evident in Transgenic Plants

We examined the expression of *bar* in the T_0 transgenic plants. Painted with 0.5% Basta® solution for 7 days, the transgenic plants showed resistance to Basta® (Figure 2-I), while the WT plants exhibited necrosis (Figure 2-J). This result verified the functional expression of *bar* in the transgenic plants.

As shown in Figure 3, stable expression of *gus* gene was detected in various organs of T_0 transgenic plants, including stems, leaflets, flowers, and pods. In addition, the integrated *gus* gene was inherited by the progeny, as stable GUS expression was detected in germinating T_1 [Figure 3 (F and G)] and T_2 (Figure 3-H) seeds. Finally, fifty GUS positive T_1 transgenic plants of four lines (12PT2-1 to 12PT2-4) were obtained (Table 2), and twenty positive transgenic plants from each T_2 line of 12PT2-1, 12PT2-2, and 12PT2-4 were used for hydroponic cultivation.

Table 1. Transformation efficiency of soybean cv. 'Xinliaoxian' using the *Agrobacterium*-mediated cotyledonary-node method.

Experiment no.	No. of explants infected (A)	No. of explants with resistant shoots	GUS$^+$ shoots^a (B)	Early transformation efficiency (B/A, %)	Southern$^+$ plants^b (C)	Final transformation efficiency (C/A, %)
1	244	152	5	2.05	4	1.64
2	219	141	5	2.28	3	1.37
3	210	139	4	1.90	3	1.43

^{a} GUS$^+$ shoots were identified based on the half-leaf GUS assay method, ^{b} Southern$^+$ plants were identified based on the analysis of T_0 plants exhibiting pod-setting.

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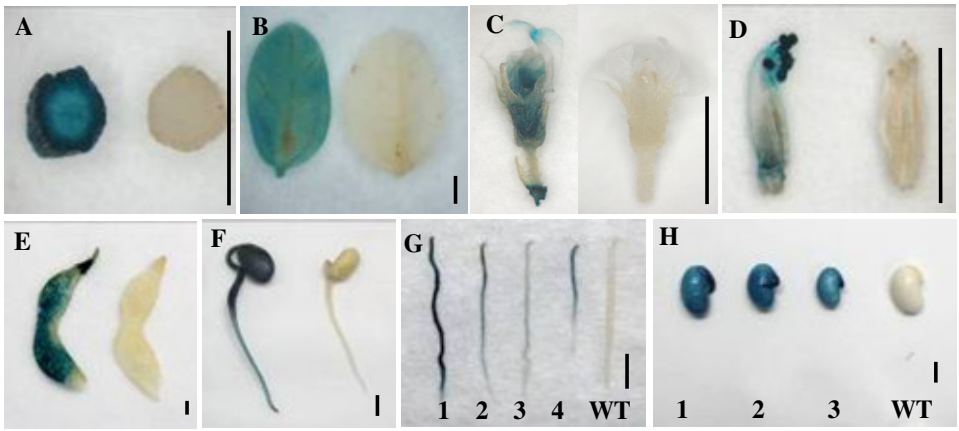

Figure 3. Stable expression of *gus* gene in various organs of transgenic plants (left) in contrast to non-transformed plants (right), (A–E) From T_0, (F) and (G) From T_1, (H) From T_2. (A) Transverse section of stems; (B) Leaflets; (C) Flowers; (D) Anthers; (E) Pods; (F) Three-day-old seedlings from T_1 generation; (G) Lateral roots from 10-day old seedlings of T_1 generation (1, 2, 3 and 4 represent T_1 lines of 12PT2-1, 12PT2-2, 12PT2-3 and 12PT2-4, respectively), and (H) Seeds after germination for 24 hours from T_2 generation (1, 2 and 3 represent T_2 lines of 12PT2-1, 12PT2-2 and 12PT2-4, respectively). Bar= 0.5 cm.

Molecular Analyses of Transgenic Plants

We performed PCR analysis of the T_0 transgenic plants to confirm the integration of the exogenous genes into the genome (Figure 4). The expected 944 bp band (for *gus*, Figure 4A-a), 413 bp band (for *bar*, Figure 4A-b), and 1587 bp band (for *OsPT2*, Figure 4A-c) were detected in the soybean genome. Sixty T_1 transgenic plants of four lines (12PT2-1 to 12PT2-4; Table 2) were identified by PCR analysis, and expected bands were observed in fifty GUS positive plants. These PCR results confirmed that *gus*, *bar*, and *OsPT2* were successfully introduced into the genomes of the transgenic plants.

To confirm the copy number of the exogenous genes in the transgenic soybean genome, we performed Southern blot analysis (Figure 4-B). Hybridization of the *gus* probe to total genomic DNA digested with *Eco*RI was used to identify DNA fragments unique to individual integration events. Analysis of genomic DNA from ten T_0 transgenic plants revealed at least one transgene-hybridizing DNA fragment per plant, with six plants harboring a single copy (Figure 4-B, Lanes 1, 2, 4, 8–10), two plants harboring two copies (Figure 4-B, Lanes 5 and 6), and the remaining two plants harboring three transgenic loci (Figure 4-B,

Table 2. Segregation analysis of T_1 transgenic lines.

T_0 line	No. of T_1 plants[a]				Segregation ratio	χ^2 value	*P*-value[b]
	GUS^+	GUS^-	Bar^+	Bar^-			
12PT2-1	12	5	12	5	3:1	0.02	0.89
12PT2-2	14	3	14	3	3:1	0.18	0.67
12PT2-3	13	0	13	0	15:1	0.13	0.72
12PT2-4	11	2	11	2	3:1	0.23	0.63

[a] Data were based on the GUS histochemical assay for the *gus* gene and the Basta®-resistant analysis for the *bar* gene; [b] A single degree of freedom was used to obtain *P*-values. $\chi^2_{0.05,1}$= 3.84.

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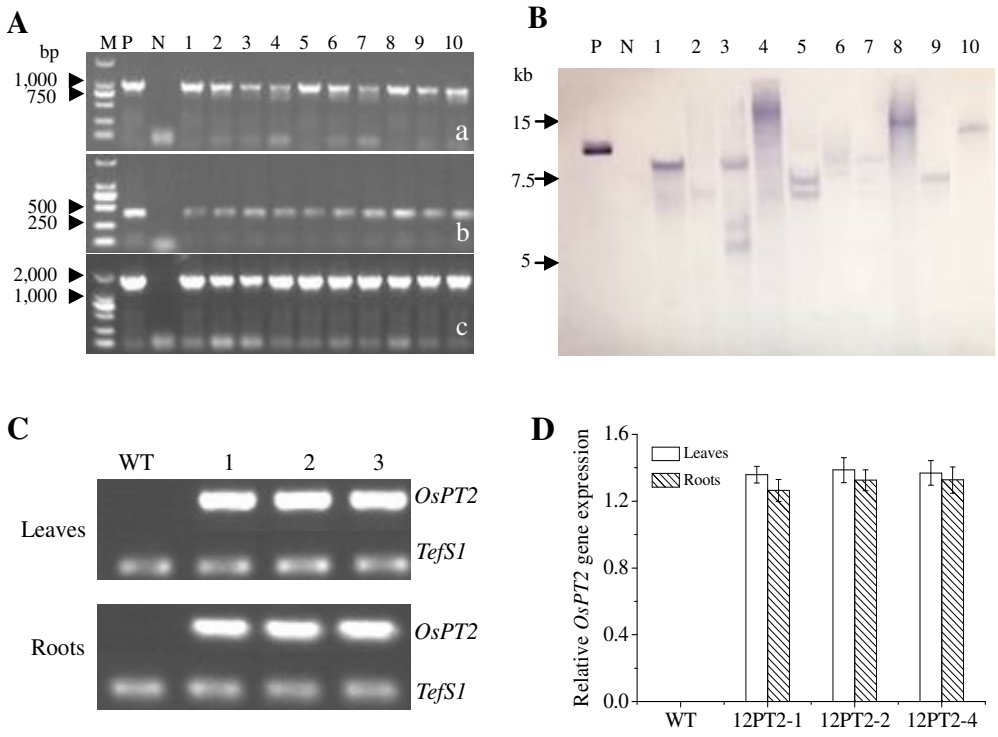

Figure 4. Molecular analysis of transgenic plants. (A) PCR detection of transgenic soybean; (a) 944 bp fragment of the *gus* gene; (b) 413 bp fragment of the *bar* gene; (c) 1587 bp fragment of the *OsPT2* gene; Lane M: Marker (DL 2000, TaKaRa); Lane P: Positive control (plasmid pCAMBIA3301-*OsPT2*); Lane N: Negative control (WT), Lanes 1–10: Independent T_0 transgenic plants from 12PT2-1 to 12PT2-10. (B) Southern blot of T_0 transgenic plants probed with the 944 bp *gus* gene probe; Lane P: Positive control (plasmid pCAMBIA3301-*OsPT2*; 200 pg); Lane N: Negative control (WT); Lanes 1–10: independent T_0 transgenic plants from 12PT2-1 to 12PT2-10; Molecular weights were estimated based on the DL 15,000 DNA Marker (TaKaRa). (C) RT-PCR analysis of *OsPT2* expression in T_2 transgenic lines; the upper and lower panels indicate gene expressions in leaves and roots, respectively; *TefS1* was used as an internal control; Lanes 1–3: *OsPT2* T_2 transgenic lines 12PT2-1, 12PT2-2, and 12PT2-4, respectively; Lane WT: Wild-type. (D) Relative *OsPT2* gene transcript expression compared to the housekeeping gene *TefS1*; values are the mean of five independent plants; bars indicate SD.

Lanes 3 and 7). No hybridization signal was observed in the WT sample (Figure 4-B, Lane N). These results indicated that the ten T_0 transgenic plants were derived from independent transformation events.

We detected the expression levels of *OsPT2* in five plants from each T_2 line (including 12PT2-1, 12PT2-2 and 12PT2-4) and the WT by semi-quantitative RT-PCR (Figure 4-C). Using gene-specific primers, the *OsPT2* transcripts were detectable in both shoots and roots. Transcript levels of *OsPT2* were higher than those of the housekeeping gene *TefS1* (Figure 4-C), which was uniformly expressed in all samples examined, suggesting that *OsPT2* was overexpressed in all the three transgenic lines examined (Figure 4-D).

Phenotypic Analysis of T_1 Segregating Progenies

Segregation analyses of *gus* and *bar* genes were conducted on the progenies (T_1 lines) of four independent T_0 plants (12PT2-1, 12PT2-2, 12PT2-3, and 12PT2-4) (Table 2). The chi-

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squared (χ^2) analysis indicated that three T_1 generations (12PT2-1, 12PT2-2, and 12PT2-4) segregated in a 3:1 ratio, and one T_1 generation (12PT2-3) in a 15:1 ratio (Table 2). These results suggested that 12PT2-1, 12PT2-2 and 12PT2-4 contained a single copy of the transgene whereas 12PT2-3 had two or more copies integrated into two different chromosomes, consistent with the Southern blot data.

P Contents and Yields of Transgenic Plants under Low-Pi and Normal-Pi Conditions

The concentrations of Pi and total P in leaves, stems, and roots of plants under hydroponic culture for 30 days are shown in Figures 5-A and 5-B. The Pi and total P contents in the leaves, stems, and roots of transgenic plants under low-Pi and normal-Pi conditions were significantly higher than those of the WT plants. As shown in Figures 5-C and 5-D, the total P contents in the fallen leaves and seeds of transgenic plants significantly increased compared to the WT plants under low-Pi and normal-Pi conditions.

As shown in Table 3, the plant height and the biomass of shoots and roots of the T_2 transgenic lines (12PT2-1, 12PT2-2, and 12PT2-4) were significantly higher than those of the WT after 30 days of low-Pi treatment. The roots of 12PT2-2 and 12PT2-4 were significantly longer than those of the WT. Under normal-Pi conditions, there were no significant differences in the parameters of vegetative organs between the transgenic and WT plants (Table 3). Figure 6 shows the symptoms of P deficiency that occurred in the WT plants at 10 days after low-Pi treatment, appearing as scattered small necrotic spots on the leaf surfaces (Figure 6-C). At 45 days after treatment, necrosis appeared in the leaf veins of the WT (Figure 6-F), and at 75 days after treatment, early abscission of the leaves was observed in the WT (Figure 6-G).

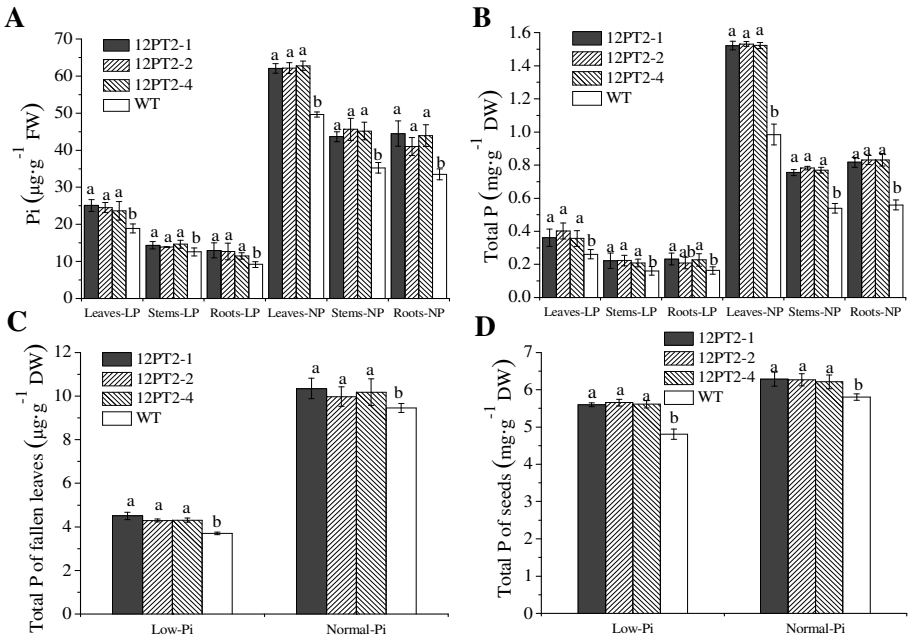

Figure 5. Phosphorus accumulation in different organs of the hydroponically-grown plants under low-Pi (LP) and normal-Pi (NP) conditions. (A) Pi concentrations in leaves, stems and roots at 30 days after hydroponic culture; (B) Total P concentrations in leaves, stems and roots at 30 days after hydroponic culture; (C) Total P concentrations in fallen leaves, (D) Total P concentrations in seeds. Five plants per line were measureds. Error bars indicate SD (n= 5). The columns indicated by different letters indicate significant differences between different lines by LSD test at $P< 0.05$.

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Table 3. Parameters of vegetative organs of hydroponically-grown plants.[a]

Pi level	Plant line	Plant height (cm plant^{-1})	Root length (cm plant^{-1})	Shoot matter (g plant^{-1})		Root matter (g plant^{-1})	
				Fresh	Dry	Fresh	Dry
Low-Pi	12PT2-1	31.24±2.06 a	92.40±2.79 ab	58.03±6.78 a	7.67±0.69 a	25.30±2.52 a	2.37±0.31 a
	12PT2-2	30.62±1.52 a	93.86±5.66 a	58.71±3.70 a	7.71±0.33 a	22.05±3.13 a	2.15±0.16 a
	12PT2-4	31.14±2.29 a	95.04±4.79 a	54.00±3.70 a	7.57±0.40 a	23.16±2.38 a	2.21±0.19 a
	WT	26.58±1.84 b	85.56±7.79 b	44.11±3.05 b	5.93±0.16 b	17.70±1.28 b	1.77±0.08 b
Normal-Pi	12PT2-1	39.90±0.91 a	82.70±2.65 a	65.30±3.17 a	10.23±0.49 a	21.07±1.36 a	2.35±0.28 a
	12PT2-2	40.04±1.27 a	83.10±2.81 a	65.39±3.18 a	10.49±1.16 a	21.48±1.99 a	2.32±0.33 a
	12PT2-4	39.26±1.42 a	82.46±2.05 a	63.16±2.27 a	10.40±1.47 a	21.67±2.09 a	2.50±0.38 a
	WT	40.74±0.56 a	83.40±2.51 a	65.57±1.82 a	10.95±1.22 a	22.39±2.57 a	2.57±0.29 a

[a] Parameters were measured after the plants were cultured for 30 days under low-Pi conditions, and data are Mean±SD; five plants per plant line were measured; different letters indicate significant differences between plant lines by LSD test at $P< 0.05$.

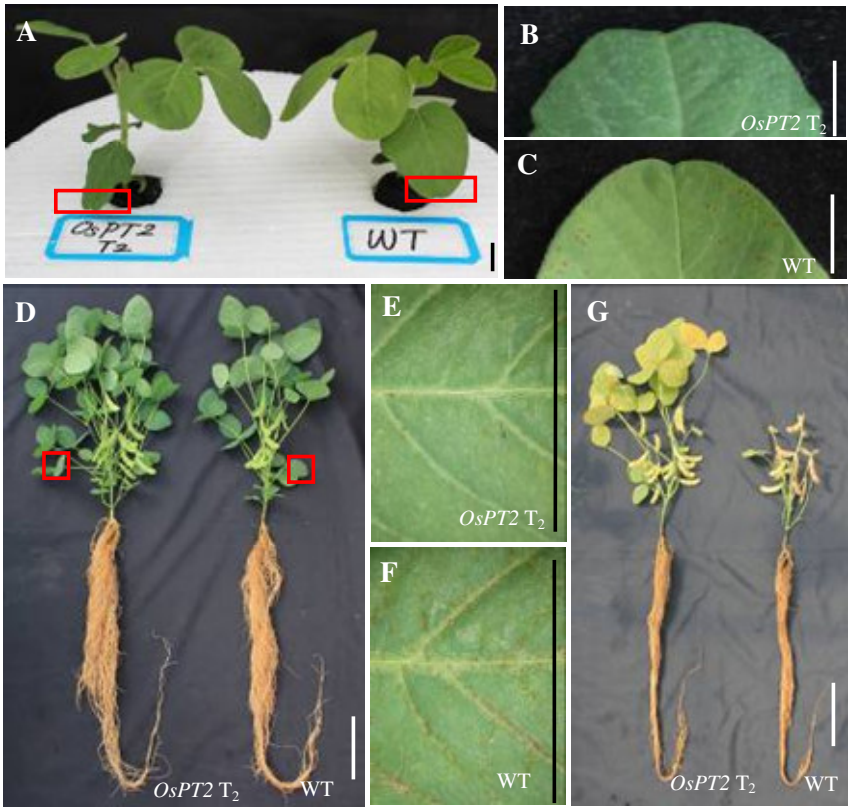

Figure 6. Growth performance of hydroponically-grown plants under low-Pi conditions. (A–C) 10 days after low-Pi treatment; (D–F) 45 days after low-Pi treatment, (G) 75 days after low-Pi treatment. *OsPT2* T_2= T_2 transgenic plants; WT= Wild-type. Bar= 15 cm in A, D, and G; 1 cm in B, C, E, and F.

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Under low-Pi conditions, the number of flowers, pods, seeds, and seed yield per plant was significantly higher in the T_2 transgenic lines (12PT2-1, 12PT2-2, and 12PT2-4) than in the WT (Table 4). However, under normal-Pi conditions these parameters did not show significant difference between the transgenic and WT plants (Table 4).

DISCUSSION

The ten T_0 transgenic plants produced in the current study using *Agrobacterium*-mediated transformation contained one to three integrated copies of the *gus* gene, and six of them possessed a single copy (Figure 4-B). This result is consistent with previous reports (Subramanyam *et al.*, 2012; Zhang *et al.*, 1999). Furthermore, the results of GUS analysis and molecular analyses including PCR, Southern blot, and semi-quantitative RT-PCR suggest that the transgene cassette was stably integrated into the soybean genome of the T_0 transgenic plants [Figure 3 (A–E); Figure 4 (A and B)] and inherited by the T_1 and T_2 progeny [Figure 3 (F–H); Figure 4-C]. These low copy number and stable integration of the transgenes into the plant genome, as well as consistent expression of the transgenes, are the features which the *Agrobacterium*-mediated transformation system possesses, compared with other transformation systems (Liu *et al.*, 2014).

Half-leaf GUS assay method (Figure 2-F) was used for early detection of transformants, which shortened the culture period for the early identification of transformed shoots and reduced the risk of retaining resistant shoots that represented "escapes" (Liu *et al.*, 2014). In this study, using the 2–4 mg L^{-1} bialaphos selection pressure combined with the half-leaf GUS assay method, it took only 3 to 6 months to identify GUS-positive shoots, which was in accordance with our previous reports (Liu *et al.*, 2013, 2014). As shown in Table 1, a total of ten GUS-positive shoots successfully rooted, survived, and produced T_1 seeds.

The PCR and Southern data confirmed the integration of the transgenes in the soybean genome from ten independent transgenic plants (Figure 4). Six plants showed one single loci integration, and the remaining four plants contained the integrative structure of multiple transgenic loci (Figure 4-B). As the first batch of seed harvest, T_1 fertile and viable seeds were successfully harvested from four T_0 transformed plants (12PT2-1, 12PT2-2, 12PT2-3, and 12PT2-4). The results of GUS assay and Basta® painting indicated that the introduced alien genes were inherited to the T_1 generation [Figure 3 (F and G)]. Expression of *gus* and *bar* in the transformed plants demonstrated

Table 4. Parameters of reproductive organs of hydroponically-grown plants.[a]

Pi level	Plant line	No. of flowers (flower plant^{-1})	No. of pods (pod plant^{-1})	No. of seeds (seed plant^{-1})	Seed yield (g plant^{-1})
Low-Pi	12PT2-1	25.40±2.72 a	18.4±1.07 a	40.80±4.34 a	10.96±1.27 a
	12PT2-2	25.40±1.71 a	18.2±0.79 a	36.20±4.73 ab	10.42±0.65 a
	12PT2-4	25.20±2.53 a	17.8±3.29 a	34.20±2.62 b	9.91±1.26 a
	WT	18.80±1.40 b	11.6±0.84 b	23.20±1.23 c	6.11±0.62 b
Normal-Pi	12PT2-1	30.60±1.07 a	24.00±0.67 a	50.20±1.03 a	14.09±0.72 a
	12PT2-2	30.80±1.93 a	24.60±1.58 a	50.80±0.79 a	14.43±0.41 a
	12PT2-4	30.60±2.55 a	24.00±2.11 a	50.60±1.71 a	14.28±0.99 a
	WT	31.80±1.23 a	25.10±1.58 a	51.00±1.49 a	14.85±0.67 a

[a] Data are Mean±SD; five plants per plant line were measured; different letters indicate significant differences between plant lines by LSD test at $P< 0.05$.

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3:1 segregation consistent with Mendelian inheritance of a single dominant locus (Olhoft *et al.*, 2003; Olhoft and Somers, 2001) in three independent lines, 12PT2-1, 12PT2-2, and 12PT2-4 (Table 2; Figure 4-B). The other line, 12PT2-3, showed a 15:1 segregation ratio for *gus* and *bar* expression consistent with the presence of multiple genetic loci (Table 2; Figure 4-B) (Olhoft *et al.*, 2003; Olhoft and Somers, 2001). GUS assay and RT-PCR showed that the alien genes were inherited to the T_2 generation (Figures 3-H and 4-C).

Phosphate uptake in plants is mediated by phosphate transporters using the energy of proton gradient generated by plasma membrane H^+-ATPase (Wu *et al.*, 2011). With a stoichiometry of 2–4 H^+ per $H_2PO_4^-$ transported, Pi absorption is accompanied by H^+ influx (Panigrahy *et al.*, 2009). After uptake into the roots, Pi is rapidly loaded into the xylem, and then transported to shoots with a lone-distance translocation facilitated by phosphate transporters. Previous studies have indicated that overexpression of phosphate transporter genes (*Pht1* family) in plants could enhance Pi acquisition under Pi deficiency conditions (Jia *et al*., 2011; Park *et al*., 2007; Seo *et al*., 2008). In this study, we demonstrated that overexpression of *OsPT2* in the roots and leaves of three soybean T_2 transgenic lines resulted in significant increases in Pi and total P concentrations under low-Pi and normal-Pi conditions (Figure 5). Furthermore, under low-Pi conditions, since P uptake was improved in the T_2 transgenic plants, the growth of both the vegetative and the reproductive organs was better in the transgenic plants than in the WT (Tables 3 and 4), and P deficiency symptoms [Figure 6 (C, F and G)] were observed in the leaves of WT plants but not in the transgenic plants [(Figure 6 (B and E)]. These results suggest that overexpression of *OsPT2* in transgenic plants could facilitate Pi absorption in the roots and Pi transport from roots to shoots. Besides, OsPT2 in transgenic plants could recycle and retranslocate internal Pi under low-Pi conditions (Panigrahy *et al.*, 2009; Park *et al*., 2007;Wu *et al.*, 2013).

Transgenic rice overexpressing *OsPT2* accumulated excess Pi in shoots, resulting in plant Pi toxicity and growth inhibition under Pi-sufficient conditions (Liu *et al.*, 2010). In the present study, the Pi and total P contents of the transgenic soybean were significantly higher than those of the WT plants under low-Pi and normal-Pi conditions (Figure 5). However, under normal-Pi conditions, there was no obvious Pi toxicity phenomenon observed in the transgenic soybean plants and no significant differences in the parameters of plant growth and development were found between the transgenic and WT plants (Tables 3 and 4). The difference in Pi toxicity between the transgenic soybean and transgenic rice under abundant Pi conditions might be due to the reason that soybean plants require more Pi for protein and oil synthesis than rice plants (Fageria *et al.*, 2013).

Recently, Wu *et al*. (2013) have reported that overexpression of *OsPHF1* in rice line 9311, an *indica* restorer line of Super Hybrid Rice, leads to increased tolerance to low-Pi stress and increased grain yield per plant in transgenic rice at a large-scale field test. In the current study, the seed yield per plant of three T_2 transgenic lines, i.e. 12PT2-1, 12PT2-2, and 12PT2-4, was significantly higher than that of WT (Table 4), which may result from improvements in P nutrition (Figure 5) and biomass production (Table 3).

CONCLUSIONS

Using *OsPT2* as the target gene, we produced ten fertile T_0 transgenic soybean plants by *Agrobacterium*-mediated cotyledonary-node transformation. Under low-Pi conditions, the phosphorus concentrations (Pi and total P) and seed yield per plant of the hydroponically-grown T_2 transgenic plants were significantly higher than those of the WT. The overexpression of *OsPT2* led to an increase in seed yield in transgenic soybean.

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اثر فزون بیانی ژن ناقل فسفات *OsPT2* برنج بر افزایش تحمل تنش کمبود فسفر در سویا

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چکیده

کمبود فسفر قابل جذب در خاک تولید سویا [*Glycine max* (L.) Merr.] را در سراسر جهان محدود می کند.هدف پژوهش حاضراین بررسی بود که آیا بیان خارجی ژن ناقل فسفات *OsPT2* برنج می تواند جذب فسفاتهای معدنی(P_i) و در نتیجه تولید سویای تراریخته را افزایش دهد یا خیر. به این منظور، گره های لپه ای جداشده از سویا با ریسه EHA105 اگروباکتریوم تومفاسینس که دارای ناقل pCAMBIA3301-*OsPT2* و *OsPT2*و ژن های گاس و *bar* بود تلقیح شد. در ادامه، با آزمون نیمه-کمّی فرزندان (نتاج) ده گیاه تراریخته بارور T_0مشخص شد که ژن *OsPT2*در نسل T_2 به طور افزونی بیان شده بود. سه رگه تراریخته T_2با فزون بیانی ژن *OsPT2*انتخاب شد و در کشت هیدروپونیک با استفاده از محلول غذایی تغییر یافته هوگلند تحت آزمون تحمل به غلظت کم P_i (برابر با 20 µM P) قرار داده شد. نتایج تجزیه برگ ها،ساقه ها،ریشه ها، و بذرهای گیاهان تراریخته در محلول غذایی استاندارد هوگلند با غلظت کم و زیاد P_i (1000 µM P_i) نشان داد که محتوای فسفر کل در این اندام به طور معنی داری افزایش یافت. در شرایط تنش کمبود P_i، عملکرد گیاهان تراریخته به طور معنی داری بیشتر از سویای وحشی بود. درمجموع، بر اساس نتایج این بررسی می توان گفت که فزون بیانی ژن *OsPT2*در رگه های سویای تراریخته، جذب فسفر معدنی و عملکرد را بهبود بخشید. بنا بر این، برای شرایط مصرف کم کودهای معدنی فسفر، ژن *OsPT2*را می توان به عنوان ژن امید بخشی برای اصلاح ژنتیکی گیاهان در نظر داشت.

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