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# Improving organic phosphate utilization in transgenic white clover by overexpression of *Aspergillus niger PhyA* gene

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**Abstract** Using the cotyledon of white clover as explants, the transgenic white clover lines ectopic expression of the *PhyA* gene were established based on *Agrobacterium tumefaciens*-mediated transformation method. It was found that the tested transgenic lines were all Polymerase Chain Reaction (PCR) positive. The transgenic lines 1 to 4 were used for further Southern blot and Northern blot analysis. The lines 1 and 3 with higher level of *PhyA* expression were used to assay the phytase activities in root and its intercellular space. When the phytate was the sole phosphorus source, the phytase activities in root in lines 1 and 3 were 31.43% and 44.76% higher than those in control (CK), respectively. Meanwhile, the phytase activities in the root intercellular space in lines 1 and 3 were 3.3-fold and 5.12-fold higher than those in CK, respectively. The phosphorus concentration of plants, the accumulative P amount per plant, plant fresh weight, and plant dry weight were all much higher in lines 1 and 3 than in CK. Thus, it is clearly shown that ectopic expression of *Aspergillus niger PhyA* gene could significantly increase the ability for white clover to utilize organic phosphate under inorganic phosphate ( $P_i$ )-deficient condition.

**Keywords** white clover (*Trifolium repens* L.), genetic transformation, *PhyA* gene, organic phosphate utilization

## 1 Introduction

Organic phosphorus generally makes up 30%–80% of the total P in the surface layer of the soil, and about half of the organic P is phytic acid and its derivatives (Dalal, 1977).

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Therefore, the improvement of soil organic P utilization by plants will play an important role in promoting the phosphorus resource sustainability around the world. Phytic acid and its derivatives can be hydrolyzed to inorganic phosphate ( $P_i$ ) and myo-inositol through the action of phytase enzymes (Mudge et al., 2003). Recently, some phytases have been used for feed additives to speed up the degradation of organic P in the feed and increase the P utilization of monogastric animals (Pagano et al., 2007; Vohra and Satyanarayana, 2003). The phytase isolated from *Aspergillus niger* by fermentation technique has been widely used in animal feed products (Brinch-Pedersen et al., 2002). In a study of the transgenic *Arabidopsis* lines ectopic fungal phytase gene, it was found that the *PhyA* transgenic products could be secreted into the culture medium from roots when driven by the signal peptide, causing  $P_i$  to be efficiently released from the phytase, the sole P source in the medium. At the same time, the plant growth and the accumulative P amount in plant tissues were all obviously improved (Richardson et al., 2000a). These results indicated that the phytase genes play an important role in improving the soil organic P utilization in plants. However, there have been no reports for the evaluation of transgene in herbage ectopic phytase gene in utilizing organic P until now.

With the properties of elite quality, affluent nutrition and excellent feed palatability, white clover (*Trifolium repens* L.) is becoming an important forage legume for monogastric animals and being planted worldwide (Hu et al., 1994; Zhang et al., 2002). Meanwhile, it also plays an important role in the protection of agricultural ecology and the environment, such as improving the soil fertility, increasing the cover degree of soil layer, controlling weed, and alleviating soil erosion (He and Zhu, 2003). In this study, a genetic transformation system of white clover with high efficiency mediated by *Agrobacterium tumefaciens* was established, in which the cotyledons of white clover (line Irrigation) were used to be the explants. The results showed that the transgene products of *PhyA* in transgenic white clover lines could secrete into the intercellular space of roots and rhizosphere. Under the condition of phytate as the sole P source in the growth medium, the accumulative phosphorus (P) amount, fresh weight and dry

weight of the transgenic lines were all much increased compared with those of the control (CK). Thus, this work provides guidance for the establishment of transgenic white clover line (cultivar) with high P efficiency based on genetic engineering technology.

## 2 Materials and methods

### 2.1 Experimental materials

The white clover (*Trifolium repens* L.) line used was Irrigation provided by Wang Zengyu (the Samuel Roberts' Noble Foundation)

### 2.2 Construction of expression vector fused *PhyA*

The binary expression vector pCAMBIA3301 was used to fuse the target gene *PhyA*. A specific primer pair for *Aspergillus niger PhyA* gene including forward primer 5'-AGATCTATGGTGGCAGTCCCCGCCTC (the *Bgl*III restriction site was introduced and the translation start codon ATG was blackened) and the reverse primer 5'-CACGTGCTAAGCGAAACTCCCC (the *Pml*I restriction site was introduced) were synthesized by Sangon Corp. (Sangon, Shanghai, China). The opening reading frame of *PhyA* was then amplified based on the technique of Polymerase Chain Reaction (PCR) with the DNA of *Aspergillus niger* as the template. The PCR amplification condition was as follows: 94°C for 5 min, then with 30 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 2 min. After the cycles there was another 7 min extension at 72°C (TaKaRa). The PCR products were ligated with PUC-m T cloning vector and transformed DH5 $\alpha$ . The plasmid of a positive recombinant was then isolated and digested by *Bgl*III and *Pml*I. The target gene segment was recovered, and linked with binary expression vector pCAMBIA3301 digested with the above restriction enzymes to get the plasmid pCAMBIA3301-*PhyA* in which the ORF of *PhyA* was fused. In the meantime, the patatin signal peptide of potato was PCR amplified with the potato DNA and one specific primer pair, including forward primer 5'-GCCATGGCAACTACTAAATCTTT (*Nco*I restriction site was introduced) and reverse primer 5'-GAGATCTCGTAGCACATGTTGAACT (*Bgl*III restriction site was introduced). The digested segment of patatin signal peptide by *Nco*I and *Bgl*III was then inserted in the front of the translation start codon ATG of *PhyA* of the plasmid pCAMBIA3301-*PhyA*. This way, a signal peptide secreting the transgene products into intercellular space and rhizosphere was fused at the upstream of the target gene *PhyA* (Li et al., 1997). The constructed pCAMBIA3301-sp-*PhyA* was used to genetically transform *Agrobacterium tumefaciens* strain C58. A positive clone identified was then grown at liquid yeast extract broth (YEB) medium (Agrobacterial medium) containing 50 mg/L kanamycin for 14 h, under the temperature of 28°C with a shaking rate of 200 r/min. After that, 2 mL culture medium was transferred to 18 mL novel liquid YEB medium without

antibiotic and grown to the OD<sub>600</sub> to 0.3 for transforming the explants.

### 2.3 Genetic transformation of white clover explants mediated by *Agrobacterium tumefaciens*

The procedure of explants transformation of white clover followed Gu et al. (2007). First, the seeds of white clover (Irrigation) were soaked at 70% ethanol for 3 min, then sterilized at 3% calcium hypochlorite (Ca(ClO)<sub>2</sub>) for 40 min with stirring. The seeds were then stored in ddH<sub>2</sub>O overnight at 15°C. Before genetic transformation, the seed coat was removed with a sterilized needle, and the cotyledon was parted into two pieces before being transformed with *Agrobacterium tumefaciens* C58 harboring the construct pCAMBIA3301-sp-*PhyA*. The explants after 40 min of transformation were transferred to the co-cultivation medium (MS medium, pH 5.8, supplemented BAP 4.4  $\mu$ mol/L, NAA 0.5  $\mu$ mol/L, sucrose 30 g/L and agar 8 g/L) with the culture condition of 16-h photoperiod and a temperature of 25°C /20°C (day/night) for three days. Next, the explants were then transferred to the selection MS medium (same as the co-cultivation medium except adding 250 mg/L Cefotaxime and 5 mg/L Glufosinate ammonium (PPT)). The transformed explants were regenerated every two weeks by using new culture media. The four-week-old buds generated from the explants were transferred to root induction medium (1/2 MS medium, pH 5.8, supplemented IBA 1  $\mu$ mol/L, Cefotaxime 250 mg/L, sucrose 10 g/L and agar 8 g/L) to induce root formation. About two weeks later, the rooted young seedlings were grown in pots in a greenhouse for analysis.

### 2.4 Polymerase Chain Reaction and Southern blot analysis of the transgenic plants

The genome DNA of transgenic white clover lines and the control (non-transformed, CK) were extracted based on CTAB method (Murray and Thompson, 1980). The specific primer pair for *PhyA* cloning from the *Aspergillus niger* was used for transgene PCR analysis of the control and the transgenic lines. The Southern blot analysis of the control and the transgenic lines was performed according to Xiao et al. (2006). The PCR amplified fragment was labelled with  $\alpha$ -<sup>32</sup>P-dCTP as the probe.

### 2.5 Northern blot analysis

The plasmid pCAMBIA3301-sp-*PhyA* was used as the template, and the *PhyA* segment was PCR amplified and  $\alpha$ -<sup>32</sup>P-dCTP labelled to be the probe for Northern blot analysis. The procedure of Northern blot analysis was carried out according to Xiao et al. (2005).

### 2.6 Assay of the phytase activities in root and root intercellular space

Uniformly growing plants of transgenic lines and control (CK) were selected and transferred into plastic pots (15 cm in

diameter) filled with 500 g of medium (vermiculite:sands was 1:1) containing 10 g phytate- $\text{Na}_2$  (myo-inositol hexaphosphoric acid dodecasodium salt from rice, Sigma, St. Louis, MO). The plants were then irrigated with Hogland solution free of  $\text{P}_i$  once every two days. After 20 days of growth, the phytase activities in the root of control (CK) and transgenic lines were assayed according to Richardson et al. (2000b). In the meantime, other root samples were taken and the saps in the intercellular space of root were isolated according to Yu et al. (1999). The phytase activities of the saps were assayed also according to Richardson et al. (2000b) mentioned above.

### 2.7 Measurement of P concentration and P accumulative amount

The P concentration and P accumulative amount in control and transgenic lines were assayed according to the description of Ames (1966).

### 2.8 Measurement of fresh weight and dry weight of plants

Before the assays of phytase activities in root and root intercellular space, the fresh weights of control and transgenic lines were measured. For the measure of dry weight of plants, the samples were first dried at 100°C for 30 min, and then at 80°C for 24 h to a constant weight.

## 3 Results

### 3.1 Construction of binary expression vector fused *PhyA*

Using the genome DNA of *Aspergillus niger* and potato, and gene-specific primer pairs, the open reading frame (ORF) of *PhyA* and signal peptide of patatin (sp) were PCR amplified, respectively. Based on that, the binary expression vector fused the *PhyA* and sp located in front of *PhyA* without frame shift was constructed (Fig. 1).

### 3.2 Analysis of genetic transformation efficiency of white clover

After genetic transformation mediated by *Agrobacterium tumefaciens*, the explants of white clover could effectively differentiate the young buds in the bud induction culture medium. The differentiated buds per explant changed from

2.38 to 3.23 among the replicates. The buds could also regenerate plants efficiently in the selection culture medium. In this study, the amount of regenerated plants per explant was 1.32–1.53 among the replicates. The percentages of induced buds in the bud induction culture medium to regenerated plants in the selection culture medium were 47.37%–55.46%. Therefore, the system of genetic transformation of explants mediated by *Agrobacterium tumefaciens* and tissue culture in this study were efficient for generation of transgenic white clover lines (Table 1).

**Table 1** Induced bud numbers, generated plants from buds per explant and plant generation rate of induced buds

Replicate	No. of explant	Shoot No./explant	Generated plant No./explant	Percentage of plant regenerated /%
I	125	2.38	1.32	55.46
II	108	3.23	1.53	47.37
III	136	2.56	1.35	52.73
Average	123	2.72	1.40	51.85

### 3.3 Polymerase Chain Reaction identification of the transgenic plants

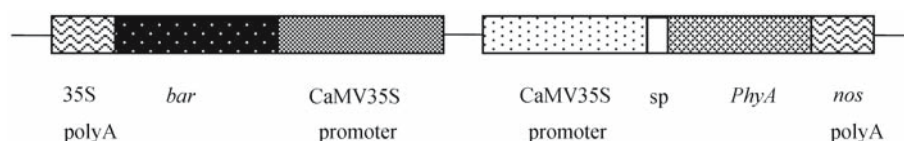
The DNA of untransformed plants (CK) and ten randomly selected transgenic lines were extracted. The PCR analysis was carried out to identify whether the target gene *PhyA* was integrated into the genome of white clover in the transgenic lines. The results indicated that all the transgenic lines were PCR positive and the length of PCR products was the same as expected (Fig. 2).

### 3.4 Southern and Northern blot analysis of *PhyA* in transgenic plants

Transgenic lines 1 to 4 were selected for Southern blot analysis. It was found that there were low copies of *PhyA* in the tested transgenic lines (Fig. 3(A)). Among the four lines, lines 1, 3 and 4 had the *PhyA* transcripts with strong signals in lines 1 and 3 by Northern blot analysis. The *PhyA* gene in transgenic line 2 was silenced (Fig. 3(B)). These results showed that the *PhyA* could be expressed efficiently in most of the transgenic lines.

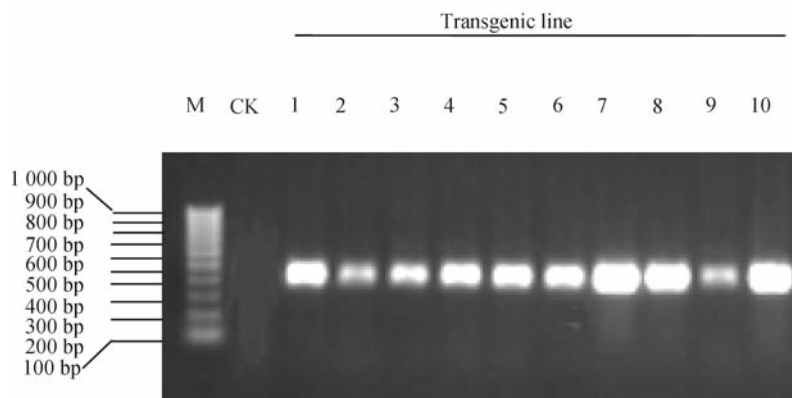
### 3.5 Phytase activities in root and root intercellular space in the transgenic lines

After 20 days of growth in the medium with phytate as the sole P source, the phytase activities of control and lines 1 and

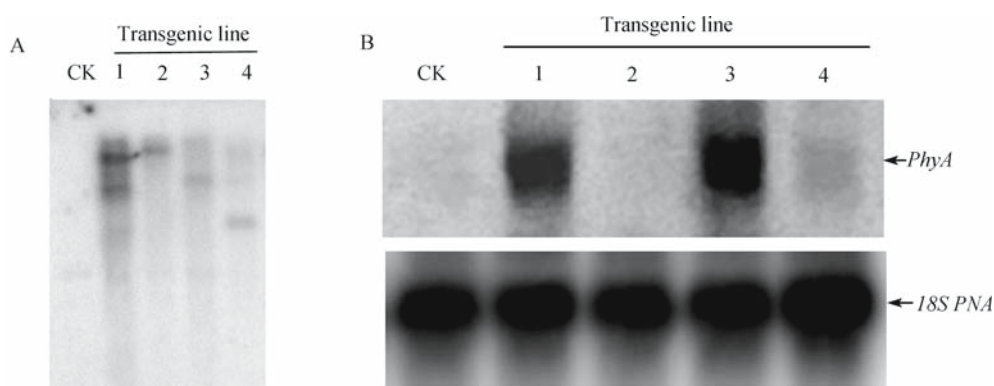


Note: 35S stands for the tailing sequence of CaMV35S gene, bar for PPT acetyltransferase gene, CaMV35S promoter for CaMV35S gene promoter, and sp, *PhyA* and nos polyA for signal peptide from potato patatin, *Aspergillus niger PhyA* gene and tailing sequence of nopaline synthase gene, respectively.

**Fig. 1** Diagram of binary expression vector fusing patatin signal peptide (sp) of potato and *PhyA* gene



**Fig. 2** PCR identification of different transgenic white clover lines and non-transgenic control plants (CK)



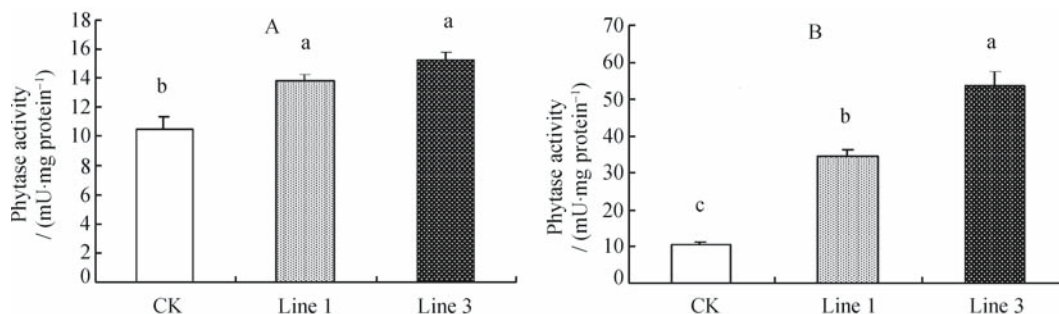
Note: (A) stands for Southern blot analysis and (B) for Northern blot analysis.

**Fig. 3** Southern blot analysis and Northern blot analysis of *PhyA* in different transgenic white clover lines and non-transgenic control plants (CK)

3 with high expression levels of *PhyA* were assayed. It was found that the phytase activities in root in lines 1 and 3 were 31.43% and 44.76% higher than the control, respectively (Fig. 4(A)). But compared with the control, the phytase activities in root intercellular space in lines 1 and 3 were increased by 2.3-fold and 4.12-fold, respectively (Fig. 4(B)). Therefore, the transgene products in the transgenic lines could be secreted from the cytoplasm to intercellular space by the guidance of the signal peptide of patatin.

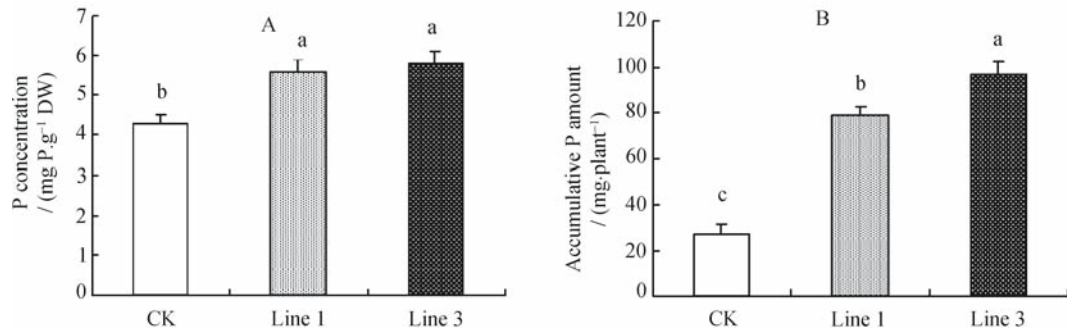
### 3.6 P concentration and accumulative P amount in the transgenic plants

After 20 days of growth in the medium with phytate as the sole P source, the P concentrations in lines 1 and 3 were 30.23% and 34.88% higher than the control, respectively (Fig. 5(A)). In the meantime, the accumulative P amounts in lines 1 and 3 were 1.90-fold and 2.54-fold higher than the control, respectively (Fig. 5(B)). Compared to P



Note: Data with different superscripts labeled with small letters have significant differences at  $P < 0.05$  level.

**Fig. 4** Phytase activity in whole roots (A) and root apoplasts (B) in transgenic white clover lines and non-transgenic control plants (CK)



Note: Data with different superscripts labeled with small letters have significant differences at  $P < 0.05$  level.

**Fig. 5** Phosphorus concentration (A) and accumulative phosphorus amount (B) in plants in transgenic white clover lines and non-transformed control plants (CK)

concentration, the accumulative P amounts in lines 1 and 3 had much higher increase than the control, resulting from the much more dry matter accumulation than P in the transgenic lines (Fig. 6(B)).

### 3.7 Fresh weight and dry weight in the transgenic plants

Similar to the accumulative P amount mentioned above, the fresh weight and dry weight of lines 1 and 3 were all significantly higher than those of the control. The fresh weights of lines 1 and 3 were 1.25-fold and 1.79-fold higher (Fig. 6(A)), and the dry weight of lines 1 and 3 were 1.22-fold and 1.62-fold higher than those of the control (Fig. 6(B)), respectively, after 20 days of growth in the medium with the phytate as the sole P source. Thus, the transgenic white clover ectopic *PhyA* could dramatically improve the plant growth under the condition with phytate as the main P source by secreting phytase into rhizosphere and releasing the P<sub>i</sub> from the organic phosphorus.

## 4 Discussion

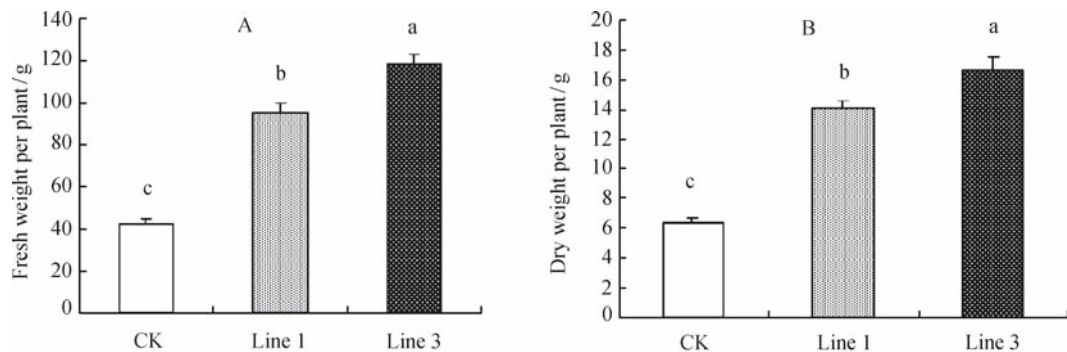
Phosphorus in soil easily becomes immobilized, resulting in a low use efficiency of P fertilizers for crops (von Uexküll and Mutert, 1995; Whitelaw, 2000). In crop production, large amounts of P fertilizers applied to cropland is the chief way

to supply sufficient P<sub>i</sub> (H<sub>2</sub>PO<sub>4</sub><sup>-</sup> and HPO<sub>4</sub><sup>2-</sup>) for crops. Therefore, the surface layer of soil is becoming a potential reservoir of P for much of the cropland. It is reported that the total P content in soil is several hundred-fold higher than P<sub>i</sub>, the readily utilized form of phosphorus (Zhang, 1995).

Hence, it will play an important role in increasing the utilization efficiency of soil-immobilized P in crop production to promote the phosphorus resource sustainability and alleviate environmental pollution resulting from over application of P in some cropping systems.

About half of the total P in soil is organic P, of which 30%–80% is phytate (inositol hexa- and penta-phosphate), and the compound is poorly utilized by plants (Dalal, 1977). It was reported that the *Aspergillus niger* had a strong ability to hydrolyze the phytate by the action of the phytases from this specie (Wodzinski and Ullah, 1996). As an animal feed additive, the phytases isolated from *Aspergillus niger* could efficiently hydrolyze the phytate in the feed to P<sub>i</sub> and myo-inositol, which would cause the P use efficiency in the feed to be obviously improved by monogastric animals (Swick and Lvey, 1992; Denbow et al., 1995). The products of *PhyA* gene is one of the important components affecting the phytase activities in *Aspergillus niger* (Piddington et al., 1993; van Hartingsveldt et al., 1993).

Li et al. (1997) reported that the recombinant *PhyA* of *Aspergillus niger* could be detected in the soybean cell suspension cultures when the *PhyA* gene was integrated into the soybean genome. Richardson et al. (2000a) established



Note: Data with different superscripts labeled with small letters have significant differences at  $P < 0.05$  level.

**Fig. 6** Fresh weight (A) and dry weight (B) in transgenic white clover lines and non-transformed control plants (CK)

some transgenic *Arabidopsis* lines of the *PhyA* gene from *Aspergillus niger* as the target gene. It has been found that the transgene products of *PhyA* in transgenic lines could be effectively secreted into rhizosphere by a signal peptide. Compared with the control, the accumulative P amount of transgenic plants was significantly higher, and concurrently had an obvious improvement of plant growth, when the phytate was the sole P source in the culture medium. Similarly, Zimmermann et al. (2003) have generated some transgenic potato plants overexpressing a synthetic phytase gene. The utilization efficiency of organic P in the transgenic plants with overexpression of the synthetic phytase gene also dramatically increased than that of the control. In the present study, the transgenic white clover lines with high expression of *PhyA* from *Aspergillus niger* also significantly increased the  $P_i$  concentration, accumulative P amount per plant, and improved plant growth compared with those of the control (CK) when the phytate was the sole P source in the medium. These studies all suggest that genetic engineering technology has a potential application on establishing the novel cultivars of crops or legumes with high efficiency in organic P utilization.

Proteins in plants exist anywhere in the cell, such as the cytoplasm, cell membrane, cell wall and organelles, etc. The translated products in the cytoplasm are transported to distinct positions of the cell by the signal peptides after sorting in the endoplasmic reticulum (ER) (Chen et al., 2004). It has been reported that the patatin signal peptide of potato is indispensable for the secretion of activated fungal phytase from the transgenic soybean suspension lines. The transgenic soybean lines of *PhyA* without the fusion of patatin signal peptide are a drawback on the secretion capabilities of the transgene products (Li et al., 1997). In the present study, the transgenic white clover lines transformed the binary expression vector fusing the patatin signal peptide of potato in front of the target gene *PhyA*, which could significantly enhance the phytase activities of the root intercellular space, indicating that this signal peptide could effectively guide the transgene products into intercellular space and the rhizosphere. Therefore, this signal peptide could have an important potential application in generating the transgenic crop cultivars or germplasm secreting *PhyA* and other useful transgene products.

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