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RESEARCH ARTICLE

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Improvement of organic phosphate acquisition in transgenic tobacco plants by overexpression of a soybean phytase gene *Sphy1*

Abstract Due to the huge amount in the soil, phytate is an important potential source for providing the plants with available phosphorus (Pi) by the involved catalytic reaction of phytase. In this study, a construct fusing the open reading frame (ORF) of *Sphy1* into corresponding positions in the fragment of binary expression vector pBI121 was created and used to transform tobacco. Molecular identification by PCR and RT-PCR indicated the target gene *Sphy1* in the transgenic tobacco plants was transcribed under the regulation of an upstream promoter. Compared with the control plants, the phytase activities in all the transgenic plants were increased, with the increased range consistent with the expression levels in the transgenic plants. Under the growth conditions with phytate as the sole phosphorus source, the transgenic line 1 plants displayed a high expression level of *Sphy1* and shows notable improved growth performance, such as higher fresh weight and dry weight, as well as higher total P content and more accumulative P amount per plant than CK. This clearly indicated that overexpression of *Sphy1* could improve the phosphorus acquisition by the extruded Sphy1 phytase in the rhizosphere, where this enzyme could catalyze the degradation of the phytate and release the available Pi for plants. The *Sphy1* gene seemed to have a potential value in the creation of new crop cultivars with high phosphorus use efficiency.

Keywords *Sphy1* gene, overexpression, phosphorus utilization, transgenic tobacco

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1 Introduction

As one of the inorganic macronutrients, phosphorus is indispensable for the normal growth and development in plants (Raghothama et al., 1999; Vance et al., 2003; Kavanová et al., 2006). Generally, most of the soil phosphorus and the fertilizer P applied in the soil exist as an insoluble form and non-utilizable for the plants, due to its easy-absorption by the soil particles and organic compounds (Anderson, 1980; Von Uexku and Mutert, 1995). Therefore, the limitation resulted from phosphorus starvation frequently occurs in crop production. It is estimated that forty percent and two-thirds of arable lands around the world and in China, respectively, are short of the available phosphorus (Vance et al., 2003).

About 30% to 80% of the total phosphorus in the soil exists as organic compounds, of which nearly half are phytate (Bieleski, 1973; Dalal, 1977). Thus, it would play a vital role in crop production if the available inorganic phosphorus (Pi) from the organic compounds, such as the phytate in the soil, could be released.

Previously, we cloned a novel soybean phytase gene (*Sphy1*) by screening a cDNA library constructed from a germinated cotyledon (cv. Kefeng 6) (Guo et al., 2008). The expression of *Sphy1* in the cotyledon shows an increasing pattern with the progress of germination and seedling growth, in accordance with the changes of the phytase activity in the cotyledon, suggesting that *Sphy1* played an important role in the degradation of phytate, the major phosphorus-deposited compound in the soybean cotyledon. In this study, we have generated the transgenic tobacco plants in which the open reading frame (ORF) of *Sphy1* was integrated, aiming at the further investigation of the gene function and its potential value in breeding crop varieties with an improved ability to utilize the phosphorus in the soil by degrading the phytate and its derivatives in the rhizosphere.

2 Materials and methods

2.1 Construction of binary expression vector fusing the open reading frame (ORF) of *Sphy1*

Based on the cDNA sequence of *Sphy1* (Guo et al., 2008), the specific primers for amplification of the *Sphy1* ORF were synthesized (Sangon Biotechnology Ltd, Shanghai, China) as follows: the forward primer and reverse primer were 5′-CTGGATCCATGGCGTCAATTACTTTT-3′ (*Bam*HI) and 5′-TCGAGCTCTTATATCGAAGCAATGCA-3′ (*Sac* I), respectively. The restriction digestion sites *Bam*HI and *Sac*I were introduced in the forward primer and reverse primer, respectively, for further subcloning. The PCR reaction was performed in a 20-μL volume containing 1.6 mmol·L^{-1} dNTP mix, 20 ng plasmid DNA harboring the whole *Sphy1* cDNA, 0.5 μmol·L^{-1} forward primer and reverse primer each, and 1 unit of high fidelity Taq DNA polymerase (TaKaRa). Five colonies with ampicillin (50 mg·L^{-1}) resistance were cultured in LB liquid medium overnight for the isolation of recombinant plasmids. The target fragment from one correctly sequenced recombinant plasmid was recovered after double restricted digestion by *Bam*HI and *Sac*I. In the meantime, the binary expression vector plasmids of pBI121 were also double digested with the same restriction enzymes. The large segment separated from the plasmid after double digestion containing all the elements for regulating the downstream gene expression was also recovered. The DNA of *Sphy1* ORF and pBI121 large segment were isolated separately by UNIQ-10 column DNA recovery kit (TaKaRa) and ligated by T4 DNA ligase. The diagram of the construct fusing the *Sphy1* ORF in the binary expression vector was described in Fig. 1.

2.2 Genetic transformation of *Agrobacterium tumefaciens* strain EHA 105

The ligated DNA of *Sphy1* ORF fragment and pBI121 large segment was firstly transformed competent *E. coli* strain DH5α. Single colonies with kanamycin (50 mg·L^{-1}) resistance were selected and grown in LB liquid medium at 37°C overnight. After the identification by double restriction digestion and sequencing, the correct recombinant DNA was used to transform *Agrobacterium tumefaciens* strain EHA105 according to the description of Hood et al. (1993). A positive colony was selected after PCR identification and used for tobacco transformation.

2.3 Generation of transgenic tobacco plants

The tobacco cultivar *N. tabacum* cv. Wisconsin 38 was used for genetic transformation of *Sphy1*. The fully-expanded leaves from the 30-day-old seedlings growing in the growth chamber were selected as explants. The genetic transformation was performed following the *Agrobacterium tumefaciens*-mediated approach as described by Zhang et al. (2008).

2.4 Molecular identification of the transgenic tobacco plants

Ten generated transgenic tobacco plants and two control plants (transformed the empty binary expression vector EHA105, CK) were selected for molecular identification analysis, including the PCR and the expression level detected by RT-PCR. As for PCR analysis, the DNAs of the samples were first extracted using the CTAB method (Manfield et al., 2005). The PCR reaction components, volume and procedure were all the same by the *Sphy1* ORF PCR as mentioned previously. The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to detect the expression levels of the target gene in the transgenic plants, with the control plants as CK. The total RNA of the sixth-leaf for all the transgenic plants and the control plants was isolated with the TRIzol reagent (Invitrogen). The total RNA was then reverse-transcribed into cDNA with the AMV reverse transcriptase kit (TaKaRa) by following the instructions of the manufacturer. Except for the fact that the reverse transcribed cDNA was used as the template, the PCR procedure in the RT-PCR process was also the same as that of *Sphy1* ORF amplification. The consistent RT-PCR results in the three replicates in each sample were obtained and used in this study.

2.5 Assay of the phytase activities

In parallel with the expression level analysis in the transgenic plants and the CK plants, the phytase activities for the plants were assayed, using the fifth-leaf as the sample. The phytase activities were measured following the description of Xiao et al. (2005).

2.6 Plant fresh weight, dry weight, total phosphorus content, and accumulated phosphorus amount

After the expression analysis, a transgenic line (line 1) with the highest *sphy1* transcripts and a control plant (CK1)

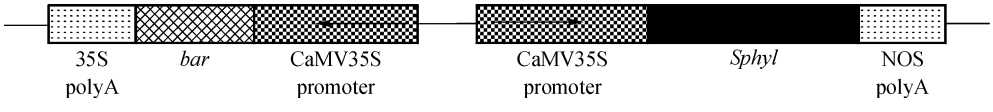

Fig. 1 The diagram of binary expression vector fusing the open reading frame (ORF) of *Sphy1*

were grown further to maturity. Then, the seeds from the transgenic line 1 and CK1 were separately sown in pots filled with vermiculite and grown in a growth chamber under the following growth conditions: at 25°C and 20°C (day and night), in 16 h of photoperiod, and at 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ photo intensity. During the seeds germination and young seedling growth stage, MS nutrient solution was irrigated twice per week.

At the fifth-leaf stage, the seedlings were then transferred into the pots filled with vermiculite, but supplemented with 1% phytate (phytic acid dodecasodium salt from rice, Sigma), and irrigated with the modified MS nutrient solution free of inorganic phosphate (Pi) instead of the normal MS nutrient solution. The growth condition and nutrient solution irrigation frequency were all the same as in the germination and young seedling growth stage. After 30 d of treatment, the transgenic plants and the control plants were sampled for the measurement of plant fresh weight, dry weight and total phosphorus content. The measurement of fresh weight was conducted by putting six representative plants together and weighing them on an electronic balance. The plants were then transferred into a pre-set 100°C oven for 30 min, followed by another 24 h at 85°C for drying. The dried samples were then weighed by using an electronic balance. The total phosphorus content for the transgenic plants and the control plants was assayed with the dried samples according to the method described by Murphy and Riley (1962). In the meantime, the traits of the plants in the phytate treatment were compared with those grown under the normal growth condition treated with MS nutrient solution.

3 Results

3.1 Construction of binary expression vector pBI121-*Sphy1* which fused *Sphy1* ORF

The open reading frame (ORF) of *Sphy1* was PCR amplified using the recombinant plasmids as the template, fusing whole *Sphy1* cDNA and the *Sphy1* specific primers. The PCR result is listed in Fig. 2(a). The positive DH5α colony fused by the ligated *Sphy1* ORF subcloning plasmid was grown in MS liquid medium for the isolation of plasmids. The results, after plasmids were restrictedly double digested by *Sac*I and *Bam*HI, are listed in Fig. 2(b). For the integration of the *Sphy1* ORF, the binary expression vector pBI121 plasmid was isolated and also double digested by *SacI* and *Bam*HI (Fig. 2(c)). The DNA of *Sphy1* ORF fragment and pBI121 large segment were

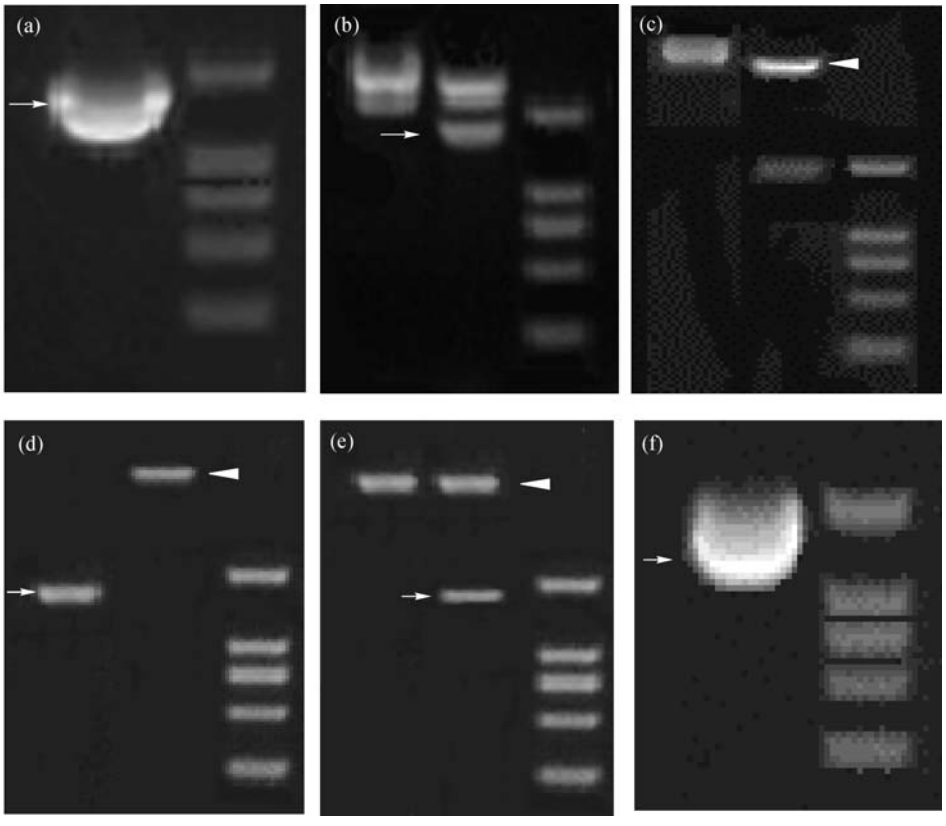

Fig. 2 PCR amplification of open reading frame (ORF) of *Sphy1* (a), double restricted digestion of recombinants (b) and pBI121 vector plasmid (c), isolation of the target fragments (d), double restricted digestion of pBI121-Sphy1 (e), and PCR identification of the construct (f)

Note: The arrows and triangles represent the ORF fragment of *Sphy1* and target pBI121 large segment, respectively.

isolated from the agarose gel after electrophoresis (Fig. 2 (d)). The two parts were then ligated by T4 DNA ligase and transformed to the competent DH5α strain. The plasmid from a positive clone was isolated and double restrictedly digested by *Sac*I and *Bam*HI for the demonstration of correct ligation. Two fragments at expected sizes were separated after the double digestion shown in Fig. 2(e), indicating that the recombinant plasmid was accurate and could be used to transform the *Agrobacterium tumefaciens* strain EHA105. The PCR amplification of *Sphy1* ORF with the isolated plasmid from EHA105 positive colony as the template and the *Sphy1* ORF specific primers is shown in Fig. 2(f). The positive EHA105 colony with specific PCR amplification of *Sphy1* ORF was used for further tobacco genetic transformation.

3.2 Establishment of transgenic tobacco lines which were genetic transformed of *Sphy1*

The generation process of the transgenic tobacco plants are shown in Fig. 3. The calli appeared around the leaf explants one week after the infection of EHA105 harboring the pBI121-*Sphy1* recombinant (Fig. 3(a)). The new buds were differentiated from the calli under the phosphinothricin (PPT)-selection growth medium (containing 5 mg·L^{-1} PPT) with the progression of time. At the 30th day after transformation, the buds deriving from the calli were about 2 cm in length (Fig. 3(b)), which were then cut with a sterilized scissors and transferred into rooting medium where the new seedlings were generated 15 d later (Fig. 3(c)). In this study, 6–8 seedlings were generated from one explant after the whole generation process, suggesting that the preparation of the explants, transformation condition, and the generation process were all feasible and effective.

3.3 Molecular identification of the transgenic tobacco plants

The genome DNA of ten transgenic tobacco plants and two control plants (transformed the empty binary expression vector pBI121, in parallel with the pBI121-*Sphy1* transformation) were extracted using the CTAB approach and shown in Fig. 4(a). The PCR amplification of the target gene *Sphy1* ORF for the transgenic plants and the control plants were performed and the products went through agarose electrophoresis identification. It was found that all of the transgenic plants were PCR positive, whereas no PCR products were amplified in CK plants (Fig. 4(b)). For the evaluation of the *Sphy1* expression level in the transgenic plants, the total RNA were isolated (Fig. 4(c)). The RT-PCR analysis was performed with the transcribed cDNA and the specific primers. The results were in accordance with those in PCR. No transcripts were found in the two control plants. The transcripts were detected in all ten transgenic plants, but the expression levels varied greatly (Fig. 4(d)). This indicated that the target gene *Sphy1* in transgenic plants transcribed under the regulation of upstream promoter.

3.4 The phytase activities in the transgenic tobacco plants

The phytase activities of the ten transgenic tobacco plants and two control plants are listed in Fig. 5. Compared with the control plants, all the ten transgenic plants were all increased in phytase activity, with the increased range consistent with the expression levels in the transgenic plants. The transgenic Plant 1 and 2 had much more *Sphy1* transcripts, and also higher phytase activities. Meanwhile, the transgenic Plant 4, with barely some *Sphy1* transcripts, shows a much lower phytase activity than transgenic Plant 1 and 2. Therefore, it was speculated that the increase of the phytase activity in transgenic Plant 1 and 2 was mainly due to their more *Sphy1* transcripts, suggesting that *Sphy1* played a vital role in the degradation of the phytate and its derivatives.

3.5 Dry weight, total P content and accumulated P amount in the transgenic plants

The plant fresh weight, dry weight, total P content and accumulated P amount in the transgenic line 1 plants (the

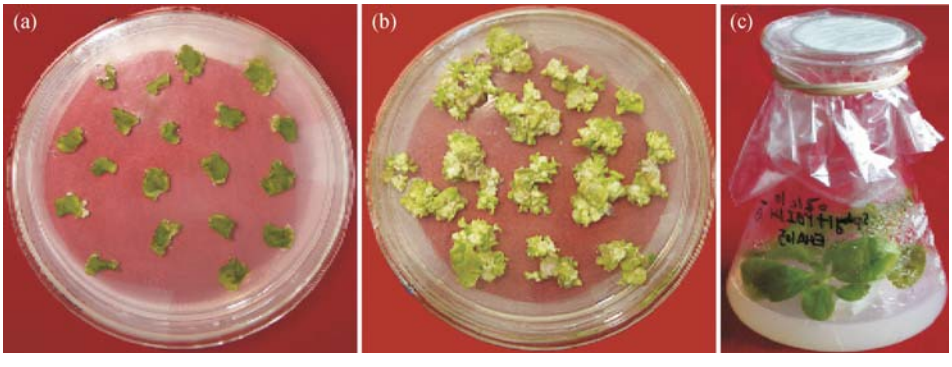

Fig. 3 The regeneration process of transgenic tobacco plants which were fused *Sphy1*

Note: In (a), the calli around the explant were appeared one week after *Agrobacterium tumafeciens* infection. In (b), the buds were differentiated from the calli one month after the infection. In (c), the young seedlings were generated after 15 d growth in rooting growth medium.

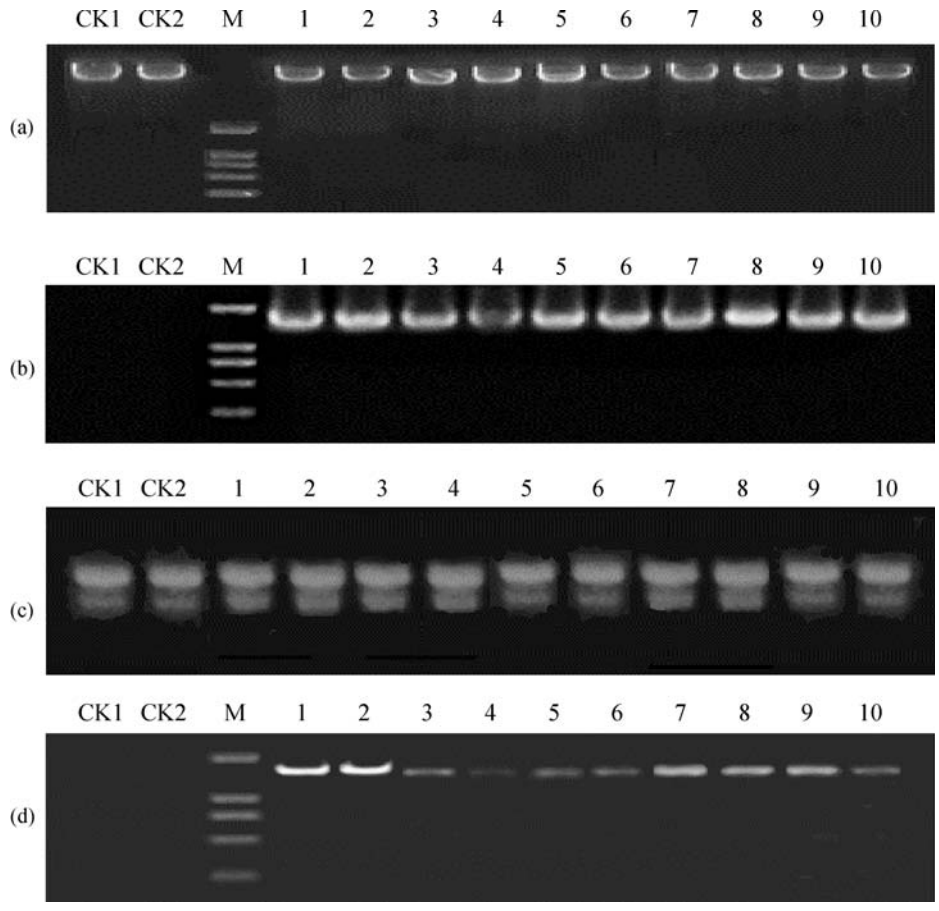

Fig. 4 The extracted genome DNA (a), PCR amplification of *Sphy1* ORF (b), isolated total RNA (c) and RT-PCR results (d) in tobacco plants transformed by *Sphy1* and empty binary vector PBI121

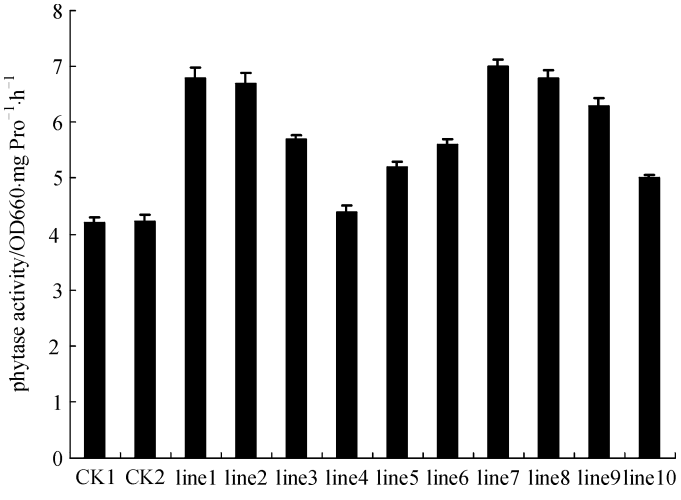

Fig. 5 The phytase activities in tobacco plants transformed by *Sphy1* and empty binary vector pBI121

line with the highest expression level among the transgenic plants) and the control plants (CK) are shown in Fig. 6(a)–(d). No obvious differences could be found in the above traits between line 1 and CK in the BT (before treatments). Under the sufficient-P supply (Normal P treatment, with normal MS irrigation.) condition, the plant growth performance and phosphorus acquisition were all better than those in the deficient-P condition (Phytate treatment, the phytate to be the sole phosphorus source). Under the growth condition with the phytate as the sole phosphorus

source, line 1 plants show notable improved growth performances, including higher fresh weight and dry weight, as well as higher total P content and accumulated P amount per plant than CK 30 d after the treatment. This clearly indicated that the overexpression of *Sphy1* could improve the phosphorus acquisition by the extruded Sphy1 phytase in the rhizosphere, where this enzyme could catalyze the degradation of the phytate and release the available Pi for plants. The plant fresh weight, dry weight and accumulated P amount in line 1 under the sufficient-P condition were a little better than those of CK, but the total P contents displayed little difference. Therefore, the plant performance and phosphorus level could also be improved to some extent under the normal P condition (Fig. 6).

4 Discussion

The application of phosphorus fertilizer is an effective way to supplement the available phosphorus in the soil lacking phosphorus, so as to realize high yield in crop production. However, the phosphorus resource is non-renewable and it is estimated that the whole phosphorus resource around the world will be exhausted in 60–90 years at the current speed (Abelson, 1999).

In the past several years, more attention has been paid to increase the utilization rate of phytate and its derivatives owing to their huge amount in the soil. Some reports have shown that the phytase extruded into the rhizosphere by overexpressing the exogenous phytate genes can markedly improve the plant phosphorus utilization capability because of the phytate degradation resulting from the action of phytase. Richardson et al. (2001) generated the transgenic Arabidopsis plants, in which the phytase gene (*Phy*A) ORF from *Aspergillus niger* was integrated. Compared with the untransformed control plants, the total phosphorus contents and accumulated phosphorus amount per plant in the transgenic plants with high expression level of the target gene were all significantly elevated under the growth condition with the phytate as the sole phosphorus source. In the meantime, the plant growth traits, such as fresh weight and dry weight were also dramatically improved. Similarly, overexpression of a synthetic phytase gene in Arabidopsis also obviously enhanced the phosphorus acquisition capacity in the growth medium with the phytate as the sole phosphorus nutrient (Zimmermann et al., 2003). In our studies, the transgenic Arabidopsis plants with overexpression of a phytase gene from *M. truncatula* and the transgenic white clover (*Trifolium repens* L.) plants with the overexpression of *PhyA* from *Aspergillus niger* could both significantly increase the phosphorus acquisition amount and improve the plant growth under the similar growth condition to Richardson's (Xiao et al., 2005; Han et al., 2007). These

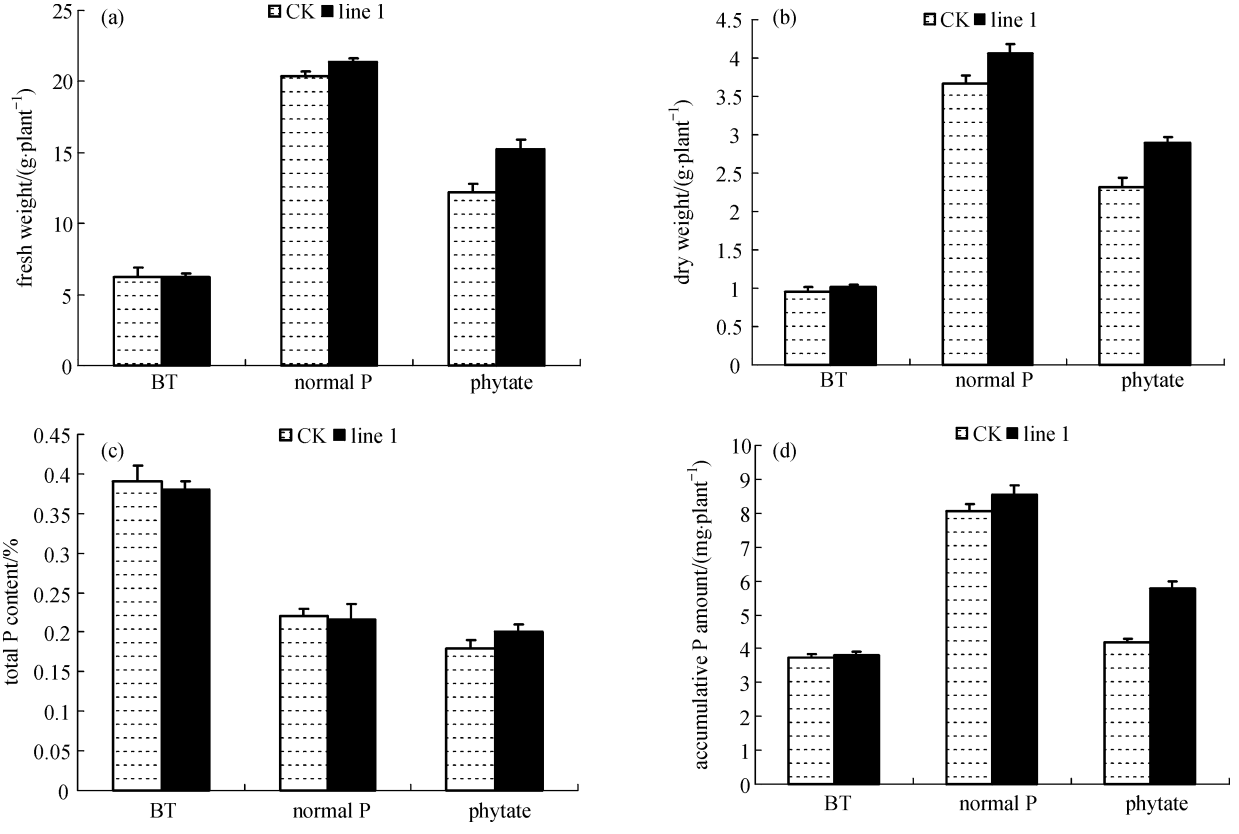

Fig. 6 The plant fresh weight (a), dry weight (b), total P content and accumulated P amount in tobacco plants transformed by *Sphy1* and empty binary vector PBI121

exciting results provide a new approach for the improvement of phosphorus use efficiency in crops in the future.

In this study, the transgenic tobacco plants that fused by a soybean phytase gene *Sphy1*, isolated in our previous research (Guo et al., 2008), were generated, with similarity to the native soybean plants and close correlation between the expression levels of *Sphy1* and the phytase activities in the transgenic tobacco plants. It was suggested that *Sphy1* could also function after the transcription and translation in other plant species. Under the growth condition with the phytate as the sole P source, the transgenic plants with higher *Sphy1* expression (line 1) could significantly improve the growth performance and the phosphorus acquisition capability. In our study, we also found that the line 1 plants could also show a little higher fresh weight, dry weight, and accumulated phosphorus amount under the normal growth condition with the normal MS nutrient solution applied, which was partly due to the shortage of available Pi under the experimental growth condition. Therefore, the *Sphy1* gene was seemingly promising in breeding new crop cultivars with high phosphorus use efficiency in the future.

Proteins in plants are distributed, in the cytoplasm, cell membrane, cell wall and organelles, etc. The translated proteins in the ribosome are transported to distinct positions of the cells 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 lines. The transgenic soybean lines of *PhyA* without the fusion of patatin signal peptide have a drawback of the secretion capabilities of the transgene products (Li et al., 1997). In the present study, the transgenic tobacco plants (line 1) with high expression level of *Sphy1*, could improve the phytate utilization capability, which resulted from its native 27-aa signal peptide located at the N-terminal. This signal peptide could effectively guide the transgene products into the intercellular space and the rhizosphere after the translation and sorting in the ER. Therefore, the *Sphy1* signal peptide could have an important potential application in breeding the transgenic crop cultivars or improving the germplasms in which the tar get gene products are necessary to secrete into the rizhosphere.

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