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Biolistic transformation of cotton (*Gossypium hirsutum* L.) with the *phyA* gene from *Aspergillus ficuum*

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Abstract Transgenic cotton with an increased level of phytase activity was generated from cotton (*Gossypium hirsutum* L.) cv. ND94-7 by subjecting shoot-apex explants to particle bombardment. These tissues were transformed with plasmid pC-KSA2300 carrying a selectable marker (for kanamycin) and a target gene (phytase, or *phyA*, from *Aspergillus ficuum*). Primary plants were regenerated in a medium containing 75 mg l^{-1} kanamycin. Of 1,534 shoot apices, 52 (3.4%) survived on this selection medium. Southern and Northern blot analyses confirmed that *phyA* was stably integrated and expressed in those primary transgenics. The progenies of the primary transgenic plants were found to have a 3.1- to 3.2-fold increase in root extracellular phytase activity, resulting in improved phosphorus (P) nutrition. Growth also was enhanced when they were supplied with phytate, and their P content was equivalent to that of wildtype plants supplied with inorganic phosphate. These results demonstrate that the expression of *phyA* in cotton plants improves their ability to utilize organic P in response to a deficiency.

Keywords Cotton · Phosphorus acquisition · Phytase gene · Phytate · Transgenic plants

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Introduction

Cotton (*Gossypium hirsutum* L.) is one of the most economically important crops in the world. After nitrogen (N), phosphorus (P) is the most limiting nutrient in cotton production (Bronson et al. 2001), being essential for vigorous root and shoot growth, the storage and transfer of energy, early boll development, and the hastening of maturity. Thus, if a trait for high-P accumulation could be introduced into those plants, cotton production could be increased in regions where soil-P contents are low. Achieving this goal is now feasible because of rapid advances in genetic engineering technology. Transgenic cotton plants are now available with desirable traits, including resistance to herbicides (Stalker et al. 1996; Keller et al. 1997), insects (Kumar et al. 2009), and diseases (Emani et al. 2003). However, only a few studies have focused on enhancing the acquisition of P by cotton through transgenic means.

Phytases have been identified in roots (Hayes et al. 1999; Richardson et al. 2000). Nevertheless, the activities of these enzymes are insufficient for the effective utilization of organic P (Brinch-Pedersen et al. 2002). Manipulating the expression of phytase to improve P acquisition is an attractive strategy in terms of improving plant cultivation. For example, a study by Richardson et al. (2001) on the expression of a fungal phytase gene in *Arabidopsis* revealed shown that phytase excreted from the roots facilitates plant growth on organic phosphates, thereby indicating that this microbial phytase could be functionally expressed to enhance plant development. Likewise, the overexpression of a phytase gene in roots was found to promote the uptake of P from the soil (Mudge et al. 2003; Xiao et al. 2005). Therefore, it is possible that the introduction of such genes into cotton roots could also improve the ability of such plants to utilize P from phytate, leading to better growth in soils with a low P content.

To test this strategy, we fused the pyk10 promoter (from a root-specific myrosinase gene, *pyk10*, cloned from *Arabidopsis thaliana*) to the *Aspergillus ficuum* phytase gene (*phyA*). To the vector we added the leader sequence from the carrot (*Daucus*) extensin gene to ensure that the phytase would be secreted into the extracellular environment. Transgenic cotton plants were then evaluated for their capacity to utilize phytate as a phosphorus source.

Materials and methods

Plant materials

Seeds of cotton (*Gossypium hirsutum* L.) cv. ND94-7 were surface sterilized with 0.1% $HgCl_2$ (10 min), followed by three washes in sterile distilled water. They were then germinated aseptically in 90×15-mm petri dishes (10 seeds each) that contained a MS-based medium (Murashige and Skoog 1962) supplemented with 20 g l^{-1} sucrose and 1.0% (w/v) agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min.

Vector construction

The construct used for cotton transformation was made by inserting the phytase gene from *Aspergillus ficuum* into the expression cassette region of vector pC-KSA2300. Expression of this cassette is driven by a root-specific promoter (pyk10) and terminated by nopaline synthase. This vector also carried an extensin leader signaling peptide from carrot (Lung et al. 2005) and the kanamycin gene as a selectable marker (Fig. 1).
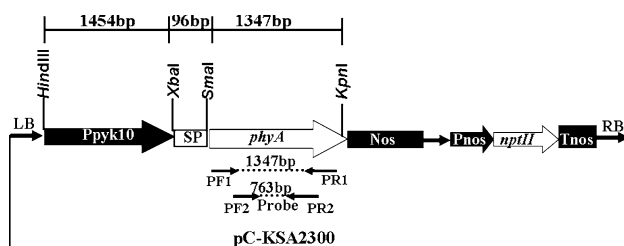

Fig. 1 Schematic representation of the pC-KSA2300 plasmid construction used in the transformation experiments. Vector pC-KSA2300, carrying the *Aspergillus ficuum* phytase gene (*phyA*) and neomycin phosphotransferase II (*nptII*; coding for kanamycin resistance), was driven by the Ppyk10 and nopaline synthase (Pnos) promoters, respectively. *LB* Left border, *RB* right border, *SP* extracellular targeting peptide sequence from carrot, *Hin*dIII, *Xba*I, *Sma*I, *Kpn*I restriction enzymes, *Tnos* nopaline synthase terminator. Primers *PF1* and *PR1* were used in PCR screening; primers *PF2* and *PR2* were used in probe production. The amplification strategy and restriction maps are shown in scale

Shoot-apex isolation

Shoot apices (Fig. 2a) were isolated under a dissecting microscope and excised from the hypocotyls by cutting at the base of the apex, below the attachment of the largest unexpanded leaf. Part of the remaining cotyledon was removed, leaving behind 2–3 mm of meristem and cotyledon. These explants were cultured on an agar-solidified MS medium (Fig. 2b) containing 3 mg l^{-1} of benzylaminopurine (BAP), 0.037 mg l^{-1} of naphthaleneacetic acid (NAA), 1.68 mg l^{-1} of thiamine-HC1, 1.38 g l^{-1} of proline, and 3% sucrose.

Optimization of kanamycin concentration for selection and confirmation process

To develop a selective growth system for genetically transformed cotton, we first studied the effects of various concentrations of kanamycin on wildtype plants (ND94-7). Shoot-tip explants were pre-cultured on MS for 15 days, transferred to the same medium but now containing 0, 25, 50, 75 and 100 mg l^{-1} kanamycin, respectively, and cultured for a further 2 weeks, at which time the rate of shoot regeneration was examined. The concentration of kanamycin chosen as the selection concentration in subsequent experiments was that at which 50% of the regenerated shoots survived.

DNA bombardment

Gold particles were coated with the pC-KSA2300 DNA according to the calcium chloride/spermidine method (Finer and McMullen 1990), and the DNA/particle suspension was bombarded twice into the shoot apices using a PDS-1000/He system (Bio-Rad, Hercules, CA). The explants were then washed with sterile distilled water, blotted dry, and transferred to a shoot induction medium containing 0.1 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 400 mg l^{-1} of cefotaxime, and 75 mg l^{-1} of kanamycin (Satyavathi et al. 2002). This concentration of kanamycin was also chosen based on results of the preliminary trials. The regenerated primary plants were designated as the T_0 generation and then self-pollinated to yield, ultimately, T_3 transgenic lines for further analysis. The seeds were germinated in a greenhouse at 28/20°C (day/night) and under a 16/8-h (light/dark) photoperiod.

PCR analysis of the regenerated cotton plants

The genomic DNA from confirmed kanamycin-resistant plants was isolated according to a modified cetyltrimethylammonium bromide method (Reichandt and Rogers
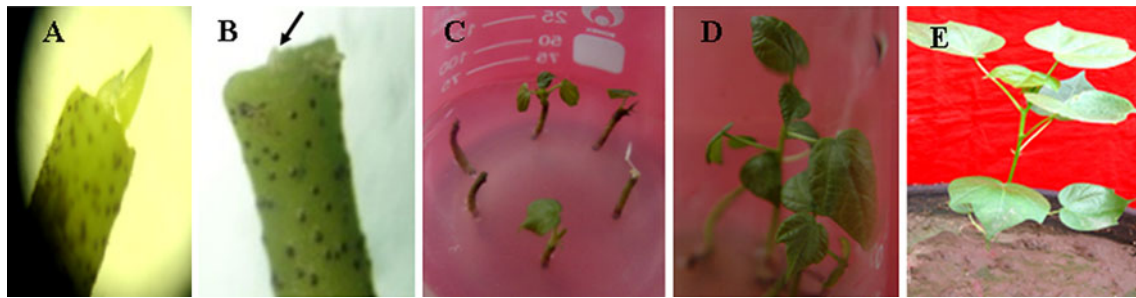
Fig. 2 a Shoot-apex explant tissue from cotton. **b** *Arrow* indicates site for explant isolation. **c** Shoot apices after 3 weeks on selection medium with 75 mg l^{-1} kanamycin, including apices with expanded leaves. **d** Cotton plant after rooting in medium with 75 mg l^{-1} kanamycin. **e** Transformed plantlet established in soil

1994). It was then dissolved in TE and used as template for amplifying the full-length *phyA*. PCR reactions were carried out with primers PF1 (5′-TCAGGGGTATCAATGCT TCTCGG-3′) and PR1 (5′-GCAAAGCTCAAACCCTT CACGAAG-3′). The reaction sample contained 80 ng of genomic DNA, 5 pmol each of primers PF1 and PR1, 1.5 mM MgCl$_2$, 1× PCR buffer, and 1 U of Ex *Taq* polymerase (TaKaRa, Dalian, China) in a final volume of 20 μl. PCR cycling was performed on a TaKaRa PCR machine, and the amplification program consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 30 s at 95°C, 45 s at 55°C, and 90 s at 72°C, with a final elongation step at 72°C for 10 min. Products were separated by electrophoresis on 1% agarose gels in 1× TAE buffer.

Southern and Northern blot analyses

Southern blot analysis was performed according to the procedure described by Sambrook et al. (1989). *Kpn*I-digested genomic DNA (30 μg) was separated on a 1% (w/v) agarose gel, then transferred to Hybond N$^+$ membranes (Amersham Biosciences, Arlington Heights, IL). The 763-bp *phyA* fragment, which was amplified from plasmid pC-KSA2300 with primers PF2 and PR2 (5′-GGGCTTCCA GAGCACTAA-3′ and 5′-ACCAAGACACGGACCAAA-3′, respectively), was used as a probe. Labeling and detection were conducted by following the protocol for the DIG High Prime Labeling and Detection Starter kit (Roche, Basel, Switzerland; http://www.roche.com). Banding patterns were observed by exposing the X-ray film to the membrane for 15 min.

For the Northern blotting, total RNA (25 μg) isolated from root tissues was separated on a 1% agarose–formaldehyde denaturing gel, with 1× MOPS as running buffer, then transferred to a Hybond N$^+$ nylon membrane. Probe-labeling, hybridization, and detection were conducted as described above.

Assay of extracellular phytase

The extracellular phytase activity in homozygous lines (T$_2$ and T$_3$ generations) was assayed at 50°C and pH 6.5 using a modified ammonium molybdate method (Greiner et al. 1997). After the seeds were germinated on MS agar for 10 days, the transgenic seedlings were grown for 15 days in a hydroponic medium [liquid MS medium with 1 mM inorganic phosphate (Pi)]. Enzyme activity was determined by measuring the amount of phytase secreted over 2 days into fresh nutrient solution (without P). In this experiment, 50 μl of solution filtrate was incubated with 100 μl of phytate. Activity was calculated from the amount of Pi released over time and was measured spectrophotometrically at 882 nm using the molybdate blue procedure (Murphy and Riley 1962). One unit (U) of activity was defined as the amount of phytase that released 1 μmol of phosphate per minute under these assay conditions.

Evaluation of cotton seedling growth with exogenous phytate

Sterilized seeds from transgenic homozygous lines and wild-type plants were germinated on MS agar for 15 days. Seedlings were then grown in pots filled with sterilized vermiculite but lacking any nutrient elements. Hoagland's liquid solution, supplemented with 0.2 mM phytate instead of soluble P, was supplied for 30 days, following which shoot and root tissues were collected and oven-dried at 80°C for 72 h to measure dry weights. The shoot samples were subsequently ashed at 550–600°C for 16 h, and the residues were dissolved in 0.1 M HCl. The Pi concentration of the acid solution was determined in a molybdenum blue reaction (Murphy and Riley 1962).

Statistical analysis

Data were analyzed by one-way analysis of variance on Origin software ver. 7.5 (OriginLab, Northampton, MA).

Values at $P < 0.05$ or $P < 0.01$ were considered significant for all experiments. Treatment means were compared by least significant differences tests.

Results

Production of transgenic plants

Shoot-tip explants were pre-cultured on MS for 15 days before being transferred to the same medium but containing 0, 25, 50, 75 and 100 mg l^{-1} kanamycin (Fig. 3). After 2 weeks, shoot regeneration was nearly 100% at 0 or 25 mg l^{-1} kanamycin, 75% at 50 mg l^{-1} kanamycin and 50% at 75 mg l^{-1} kanamycin. All apices died when exposed to 100 mg l^{-1} kanamycin. We therefore chose 75 mg l^{-1} as the selection concentration. When exposed to that level, kanamycin-sensitive explants turned yellow after 1 week while resistant explants remained green (Fig. 2c). Of 1,534 shoot apices cultured on this selection medium, 52 (3.4%) survived. When those resistant shoots were 3–4 cm tall, they were transferred to a rooting medium where they developed normally and produced healthy roots (Fig. 2d, e). These confirmed transformants were used for further analysis of phyA expression.

From those 52 surviving shoots, 45 well-developed plantlets were transferred to vermiculite, and samples of their DNA were used in PCR analyses to detect phyA. All 45 lines gave the expected product sizes when primers PF1/PR1 were used (Fig. 4a). Genomic DNA from eight randomly selected PCR-positive plants was analyzed by Southern blots after digestion with KpnI, which did not cut the phyA cassette. Of these, four presented simple integration patterns, with hybridization revealing at least one band from each (Fig. 4b). Lines L6 and L9 exhibited two bands, suggesting that they had two loci for the transgene. Line L12 had a single band (Fig. 5a, b), indicating a single locus, while line L15 had three bands, i.e., three loci. The number of insertions and their sizes differed among plants, confirming the independent origin of these transgenic lines.

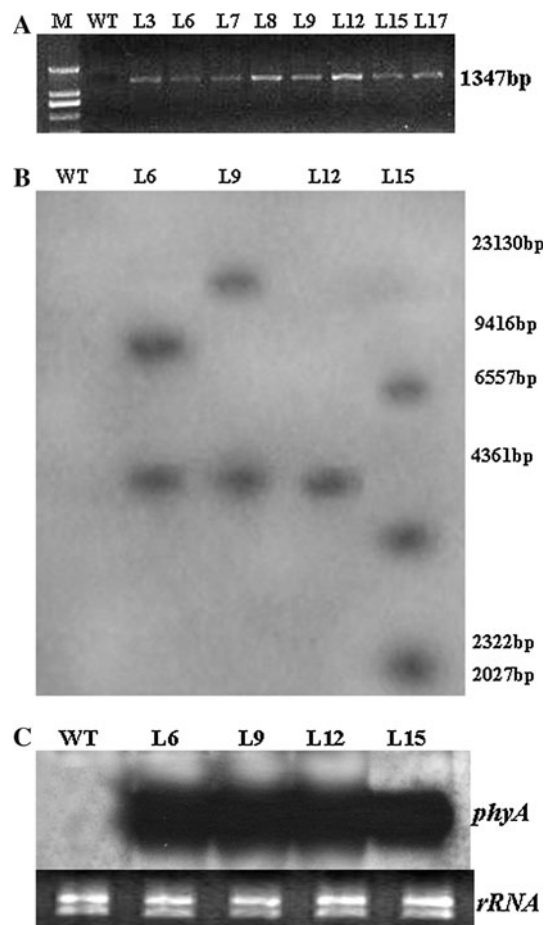The expression of phyA in positive plants that had been verified by Southern blots was verified by the results

Fig. 4 Detection of phyA in T_0 transgenic lines (*prefix L*) and wild-type (*WT*) plants. **a** PCR-screening. A 1,347-bp product was amplified from cotton transformed with vector pC-KSA23000 but was not detected in WT plants. *M* Molecular weight marker. **b** Southern blot analysis of four T_0 lines (*L6*, *L9*, *L12* and *L15*) showing simple integration patterns of the phyA gene, compared with the WT. Genomic DNA was digested with KpnI. **c** Northern blot analysis of roots from transgenic plants expressing the phytase gene, compared with the WT

of the Northern blot analysis. Total RNA was isolated from transgenic and WT roots, but transcripts for phyA were detected only in the former (Fig. 4c), indicating that this gene was correctly expressed in our transgenic plants.
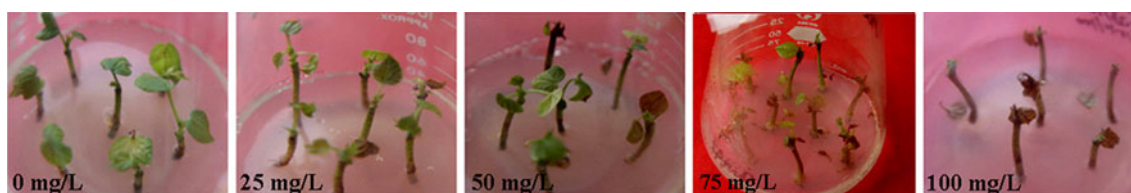

Fig. 3 Effect of different concentrations of kanamycin (0, 25, 50, 75, or 100 mg l^{-1}) on the growth of shoot explants obtained from wild-type plants of cotton (*Gossypium hirsutum* L.) cv. ND94-7

Fig. 5 Comparison of extracellular phytase activities in roots from WT plants and T_2 (**a**) and T_3 transgenic lines (**b**). *L6, L9, L12, L15* Transgenic lines. Data are means ± standard deviation (SD) from three independent measurements. **$P < 0.01$
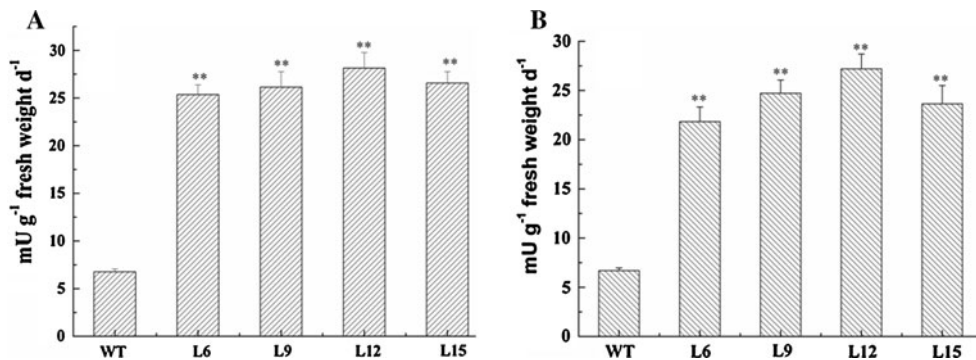
Inheritance of T_1 and T_2 transgenic lines

The heredity of T_1-progeny seedlings was tested by the PCR. All four lines with simple integration patterns were found to contain *phyA*, with the progeny of Lines L6, L9, and L12 segregating in a Mendelian fashion based on our χ^2 tests. The banding patterns for the T_0 plants of these lines (Fig. 4b) also showed that this gene was inherited in a Mendelian fashion. Line L15 was the exception: *phyA* did not segregate in the same manner as in the other three lines (Table 1).

The T_2 populations derived from those four transgenic lines, including L15, segregated as shown in Table 2, generally fitting the Mendelian pattern of inheritance. These results were nearly identical to those found with the T_1 populations.

Table 1 Segregation of *phyA*[a] in the T_1 generation of four transgenic lines of cotton (*Gossypium hirsutum* L.) cv. ND94-7 showing simple integration patterns

Transgenic line	No. of bands on Southern blots (T_0 generation)	No. of plants PCR^{+b}	No. of plants PCR^{-b}	Expected ratio	χ^{2c}
L6	2	78	2	15:1	0.75
L9	2	72	8	15:1	2.61
L12	1	54	16	3:1	0.08
L15	3	59	15	63:1	180.76

[a] *Aspergillus ficuum* phytase gene

[b] PCR$^+$, PCR-positive plants; PCR$^-$, PCR-negative plants

[c] Tabulated χ^2 value at 5% probability for 1 *df* is 3.84

Table 2 Segregation of *phyA* in the T_2 generation of transgenic lines of cotton

Transgenic line	No. of plants PCR$^+$	No. of plants PCR$^-$	Expected ratio	χ^2
L6	225	17	15:1	0.866
L9	240	12	15:1	1.24
L12	182	59	3:1	0.012
L15	198	3	63:1	0.042

See footnotes to Table 1 for explanation of terms

Extracellular phytase activity in transgenic roots

High levels of extracellular phytase activity were observed in the roots from transgenic lines L6, L9, L12, and L15, with no difference being found among them. However, compared with wildtype plants, the enzyme activities in the T_2 and T_3 transgenic lines were approximately 3.1- and 3.2-fold higher, respectively (Fig. 5).

Seedlings of T_3 transgenic lines grew better under phosphate-deficient conditions

Based on our extracellular phytase data, we chose transgenic Lines L9 and L12 for an in-depth analysis of their performance in the presence of exogenous phytate under phosphate-deficient conditions. Growth was compared between their representative T_3 progenies and the wildtype plants. Tissue dry mass of L9 and L12 progenies increased by 1.22- and 1.57-fold, respectively, over that of the wildtype control (Fig. 6). The higher shoot-to-root ratio of the transgenic plants (3.97–4.37) compared with that of the wild type (2.97) is believed to be an adaptation to P deficiency.

Phosphorus analysis of T_3 transgenic plants expressing the phytase gene

When grown in the presence of either phytate or KH_2PO_4 (Pi) as their sole P source, both wildtype and transgenic plants (T_3 generation) had similar amounts and concentrations of total P in their shoots (Table 3). However, when supplied with phytate, all transgenic plants accumulated higher amounts and concentrations of total P in their shoots, thereby demonstrating that cotton plants expressing *phyA* were better able to obtain P from phytate.

Discussion

Cotton plants are difficult to manipulate in tissue culture (Khan et al. 2010). Therefore, techniques that treat shoot tips are a possible alternative approach for recovering
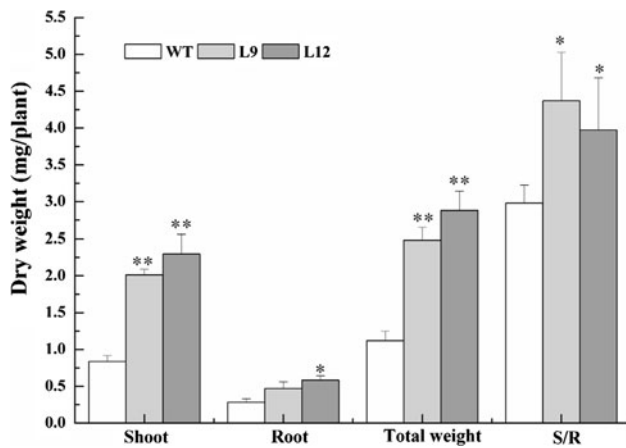
Fig. 6 Performance of plants grown on inorganic phosphate (Pi)-deficient media supplied with phytate. Seeds were germinated on MS agar for 15 days, and the seedlings then placed in pots containing sterilized vermiculite. Nutrient solution supplemented with 0.2 mM phytate was added for 30 days. Within each growth parameter, asterisks indicate significantly different values (*$P < 0.05$, **$P < 0.01$). S/R Shoot-to-root ratio

plants from callus tissue (Saeed et al. 1997). All methods described previously have utilized shoot apices for regeneration (Gu et al. 2008; Tsai et al. 2009; Parimalan et al. 2010; Zhang et al. 2010). As a result, the introduction of DNA into shoot meristem cells has led to the production of chimeric plants; that is, the transformed cells directly give rise to germ-line tissue and the introduced DNA is then passed on to the progeny. In our study, Line L15 appeared to be chimeric for the introduced gene because its segregation ratio (T$_1$ population) did not fit the theoretical Mendelian segregation ratio of 63:1. Interestingly, however, the T$_2$ population for that same line was inherited in a nearly Mendelian fashion, indicating that we had obtained homozygotes of those transgenic plants.

Phosphorus deficiency in soil is a major problem of agricultural production worldwide. However, most soils actually do contain significant amounts of total P, which occurs in both inorganic and organic fractions and is also accumulated with P fertilization. An important component of soil organic P occurs in the form of phytate. Although plants have numerous mechanisms for increasing the availability of soil P, their utilization of phytate-P is very limited due to the low activity of phytase within the rhizosphere. For example, such activity around *Arabidopsis* roots contributes only 0.5% of the total phosphomonoesterase activity. To the best of our knowledge, no extracellular plant phytase has even been reported. The restricted bioavailability of phytate-P has also been observed with several agricultural crops, including wheat and various forage species (Hayes et al. 2000; Richardson et al. 2000). Although two phytase genes have been cloned from maize, the phytases encoded by these genes are not secreted (Maugenest et al. 1999). Because of this lack of extracellular activity, plants utilize soil phytate rather poorly. In contrast, transgenic *Arabidopsis* plants expressing extracellular *Aspergillus* phytase can obtain P directly from phytate (Richardson et al. 2001). The addition of purified phytase to the rooting medium has the same effect (Findenegg and Nelemans 1993), underlining the importance of P availability for plant nutrition. Hence, direct enzyme secretion from the roots could be one of the most efficient and economically feasible strategies for improving phytate-P utilization by the plant.

To test this hypothesis specifically, we generated transgenic cotton plants that expressed the *Aspergillus* phytase gene. We found that the levels of phytase activity were more pronounced in the transgenics than in the wild type. When supplied with phytate, seedlings of the former clearly out-performed the wildtype control in terms of biomass accumulations by 1.22- to 1.57-fold. This result demonstrated that the extracellular secretion of *Aspergillus* phytase enabled our transformed plants to obtain their P requirements directly from phytate. Our finding supports previous observations that the P nutrition of plants supplied with phytate can be improved significantly when either the medium is inoculated with soil microorganisms that possess phytase activity (Richardson et al. 2000, 2001) or a purified

Table 3 Content of phosphorus (P) in shoots of wildtype and transgenic lines of cotton when grown with different sources of P

Parameter	Treatment	Wild type	Transgenic lines			
			L6	L9	L12	L15
Total shoot P content (mg)	No P	1.12 ± 0.04	0.96 ± 0.06	1.64 ± 0.26	1.34 ± 0.36	1.24 ± 0.31
	Phytate	4.57 ± 2.43	23.01 ± 4.01	24.01 ± 2.01	25.01 ± 3.01	22.31 ± 3.02
	Pi	21.2 ± 1.07	24.02 ± 4.07	23.12 ± 2.02	25.42 ± 1.12	22.42 ± 1.02
Shoot P concentration (% dry weight)	No P	0.12 ± 0.04	0.14 ± 0.03	0.33 ± 0.02	0.42 ± 0.06	0.23 ± 0.01
	Phytate	0.39 ± 0.09	0.99 ± 0.08	1.34 ± 0.08	1.52 ± 0.04	1.02 ± 0.09
	Pi	0.86 ± 0.01	0.85 ± 0.02	1.06 ± 0.04	1.44 ± 0.06	1.26 ± 0.06

Pi, Inorganic phosphate

Values shown as the mean of three replicates each ± standard deviation

phytase is added to the rooting medium (Findenegg and Nelemans 1993; Hayes et al. 2000). Taken together, these observations confirm that a lack of phytase activity around the plant roots is a significant factor that limits the utilization of P from phytate.

The root-specific *pyk10* cloned from *Arabidopsis thaliana* and its pyk10 promoter drive the expression of the β-glucuronidase (*gus*) reporter gene in root tissues (Nitz et al. 2001). To hydrolyze phosphorus from organic P substrates, phytase must be extracellularly secreted from the roots into the rhizosphere. In our experiments, we used not only the pyk10 promoter but also a extensin leader signaling peptide from carrot to drive transgene expression. The results showed that this promoter successfully led to the overexpression of *phyA* in cotton plants. Assays of enzyme activity revealed that recombinant phytase was accumulated in the solution. This finding is very valuable in terms of improving plant-P acquisitions because although phytate makes up 60–80% of soil organic P, it is usually poorly utilized by plants (Turner et al. 2002; George and Richardson 2008).

Being able to improve the capacity for plant-P uptake will reduce the need for agronomic fertilizers and the negative environmental impacts associated with their heavy use. In this investigation, we demonstrated that P uptake was enhanced in our transgenic cotton lines. Therefore, we suggest that these plants could be important for agricultural production in soils with a low P content. Moreover, our results clearly indicate that when roots secrete extracellular phytase, their ability to utilize P from phytate is markedly improved. This is a positive step toward finding an alternative to the use of fertilizer(s). It also helps address the poor growth of crop plants in regions where the content of inorganic P in the soil is low or not available in forms that can be directly absorbed by plants. Using cotton as our example, we have shown that it is possible to improve the performance of plants grown in an artificial medium containing only phytate as the P source. This was achieved by over-expressing the gene for a secretable form of phytase in the roots. Our laboratory is currently focusing on testing these transgenic cotton plants under field conditions.

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