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ORIGINAL RESEARCH PAPER

Functional analysis of an *Aspergillus ficuum* phytase gene in *Saccharomyces cerevisiae* and its root-specific, secretory expression in transgenic soybean plants

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Abstract Phytases release inorganic phosphates from phytate in soil. A gene encoding phytase (*AfPhyA*) was isolated from *Aspergillus ficuum* and its ability to degrade phytase and release phosphate was demonstrated in *Saccharomyces cerevisiae*. A promoter from the *Arabidopsis Pky10* gene and the carrot extensin signal peptide were used to drive the root-specific and secretory expression of the *AfPhyA* gene in soybean plants. The phytase activity and inorganic phosphate levels in transgenic soybean root secretions were 4.7 U/mg protein and 439 μM, respectively, compared to 0.8 U/mg protein and 120 μM, respectively, in control soybeans. Our results demonstrated the potential usefulness of the root-specific promoter for the exudation of recombinant phytases and offered a new perspective on the mobilization of phytate in soil to inorganic phosphates for plant uptake.

Keywords *Aspergillus ficuum* · Phytase · Root-specific expression · Secretory protein · Transgenic soybean

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Introduction

The predominant form of organic phosphates in soil is phytate (Turner et al. 2002). Phytase catalyzes the dephosphorylation of phytate to inositol and orthophosphate (Wodzinski and Ullah 1996). The activity of phytase in plant roots is probably insufficient for effective utilization of phytate (Richardson et al. 2000) and plants may depend on soil microorganisms to obtain phosphates from phytate (Richardson et al. 2001b).

In tobacco, phosphate starvation triggered a significant increase in root-associated phytase activity and the elicited phytase might then be targeted to the secretion pathway (Lung and Lim 2006). Application of a fungal phytase to sterile cultures of *Trifolium subterraneum* seedlings enabled the plants to use phytate as the only phosphate source and the plants grew as well as inorganic phosphate-supplied plants (Hayes et al. 2000). An improved ability to grow on

phytate was also observed for sterile cultures of wheat inoculated with a phytase-secreting soil bacterium (Richardson et al. 2000). *Arabidopsis* lines engineered to secrete an *Aspergillus niger* phytase had a 20-fold increase in root phytase activity and grew better than the wild type on medium with phytate as the only phosphate source (Richardson et al. 2001a). All these studies point to the potential for improving inorganic phosphate acquisition and utilization efficiency of plants by biotechnology approaches.

However, altering the phytate content in the whole transgenic plant has the potential disadvantage of affecting many other cellular processes. Most recently, the *Aspergillus niger phyA2* gene was over-expressed in maize seeds using a construct driven by the maize embryo-specific globulin1 promoter and phytase activity in transgenic maize seeds reached a 50-fold increase compared to non-transgenic maize seeds (Chen et al. 2008). In the present study, we approached the reduction of phytate level in soybean by expressing a functional *Aspergillus ficuum* phytase gene in root tissue to modify its phytase composition and direct it for secretion into the surrounding soil. Different from previous supplementation studies using active enzyme, the phytase accumulation in transgenic soybean roots is altered and is supposed to stimulate the absorption of essential mineral elements due to the sufficient phytase activity in the rhizosphere.

Materials and methods

Cloning of *Aspergillus ficuum* phytase gene, *Arabidopsis pyk10* promoter sequence, carrot extensin signal sequence and vectors construction

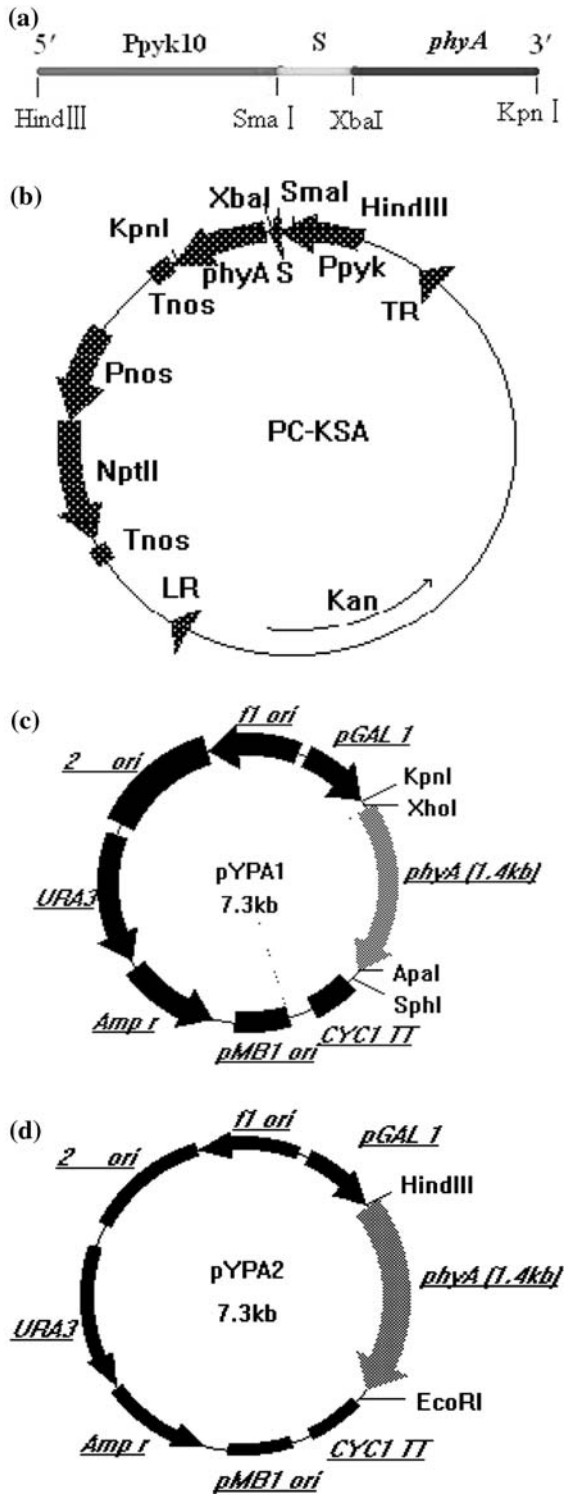
For phytase gene cloning, total RNA was extracted from *Asp. ficuum* AS3.324 by the method of guamidine thiocyanate (Chomczynski and Sacchi 2006). A phytase cDNA was generated by RT-PCR according to the published genomic DNA sequence of *Asp. ficuum*. The promoter sequence of *Arabidopsis Pyk10* gene and the carrot extensin signal sequence were cloned from genomic DNA extracted by the CTAB method from leaf and root tissue, respectively. Supplementary Table 1 lists the three pairs of cloning primers, the GenBank Accession numbers of the corresponding genes, the added restriction sites and individual PCR programs. The carrot extensin and fungal phytase fragments were then cloned into the pMD18-ProPyk10 under the control of the *Pyk10* promoter sequence, which was designated as a KSA expression cassette (Fig. 1a). After digestion by *Hin*dIII/*Kpn*I, the KSA expression cassette was cloned into the *Agrobacterium* expression vector, pPC-GENERAL and resulted in the final expression binary vector, pPC-KSA (Fig. 1b).

Functional analysis of *Aspergillus ficuum* phytase gene in *S. cerevisiae*

The *Asp. ficuum* phytase coding sequence with its native signal peptide was cloned by RT-PCR with added *Xho*I and *Apa*I sites, respectively, using the sense primer (5′-CTCGA**GATG**GGCGTCTCTGCT GTTCTACTTC-3′) and antisense primer (5′-GGG**CC CCTA**AGCAAAACACTCCGCCCAATCA-3′). The *AfPhyA* gene was then cloned into the shuttle plasmid pYES2 to form the pYPA1 expression vector (Fig. 1c). Meanwhile, the *AfPhyA* coding sequence without its native signal peptide was introduced into pYES2 to form the pYPA2 expression vector (Fig. 1d). The two plasmids were then introduced into *S. cerevisiae* INVSc1 and the transformed cells were screened on URA^- selective plates and analyzed by PCR for the presence of the inserted gene. After the culture has grown overnight, 20 g galactose/l was added to induce phytase expression and extracellular/intracellular phytase activity were assayed according to the method previously described (Li and Ljungdahl 1996).

Plant material and *Agrobacterium* transformation

Soybean [*Glycine max* (L.) Merrill, Chinese soybean cultivar genotypes Jilin35] seeds were used for *Agrobacterium* transformation. After excision of the apical and lateral meristem explant embryonic tips, the intact cotyledon was wounded by scalpel and ready for *A. tumefaciens* (LBA4404) transformation. Soybean hypocotyls were infected in MS liquid medium with 30 g sucrose/l, 100 μM acetosyringone, 1.5 mg N_6-benzylaminopurine (BAP)/l at pH 5.8. The explants were then transferred to inoculation media (MS with 30 g sucrose/l, 1.5 mg BAP/l) and incubated in the dark for 3 days before being transferred to selection medium (MS with 250 mg



◀ **Fig. 1** The physical map of *AfPhyA* gene expression vectors used in this study. **a** The expression cassette of SKA composed of the *AtPky10* promoter fused with the carrot extensin signal sequence and *AfPhyA* coding sequence. **b** The construct for *AfPhyA* gene (with native signal sequence) expression in *S. cerevisiae*. **c** The construct for *AfPhyA* gene (without a signal sequence) expression in *S. cerevisiae*. **d** The construct for *AfPhyA* gene (with carrot extensin signal sequence) expression in soybean plants

Cefotaxime/l, 100 mg kanamycin/l, 1.5 mg BAP/l, and 30 g sucrose/l). Resistant shoots were excised and transferred to shoot elongation medium (MS with 30 g sucrose/l, 1 mg gibberellic acid$_3$/l, 250 mg Cefotaxime/l, 100 mg kanamycin/l) for 2 weeks. When the shoot reached 2 cm long, they are transferred to rooting medium (MS with 30 g sucrose/l, 0.5 mg IBA/l) and rooted seedlings were transplanted to greenhouse for maturity.

Molecular characterizations of *AfPhyA* transformed soybean plants

Genomic DNA was isolated from soybean plants and PCR analyses were carried out using *AfPhyA* specific primers. Southern dot blot was carried out using genomic DNA and the promoter sequences of *AtPyk10* were labeled using DIG DNA Labeling Mix to produce probe. Detection was performed using the Luminescent Image Analyzer LAS-3000 (Fuji Film, Tokyo, Japan). Total RNA from root tissue of putative transformants was extracted (RNeasy mini kit, QIAGEN) and used as templates for the cDNA synthesis (iScript cDNA Synthesis kit, BioRad) to detect the transcription of *AfPhyA* gene.

Phytase determination in yeast culture supernatant and whole-root extract of transgenic soybean

The yeast cells were induced by 20 g galactose/l and continually growing for 36 h. To detect the phytase activity, 30 µl culture supernatant was mixed with 100 µl 10 mM sodium phytate and 500 µl 0.1 M sodium acetate (pH 5.0) and incubated at 37°C for 1 h before 300 µl 10% (v/v) trichloroacetic acid was added to terminate the reaction. Next, 500 µl color-developing solution [50% (v/v) acetone, 2.5 mM ammonium molybdate, and 0.63 M sulfuric acid] was added to the solution mixture and incubated at 37°C for another 10 min before the absorbance was measured at 820 nm (Heinonen and Lahti 1981). One unit of the phytase activity was expressed as the

amount of enzyme required to liberate 1 nmol inorganic orthophosphate (Pi) per min.

For transgenic soybean plants, in vitro assays for root-released phytase activities were conducted after collection of secretory proteins in root-bath solution. In brief, the roots of 20-day-old soybean plants were put into a dialysis bag with 1 mM $CaCl_2$ and dialyzed against same solution. After 24 h, the roots were taken out and the collected root exudates were filtered, lyophilized and resuspended in 1 ml 50 mM sodium acetate buffer (pH 5.0). The phytase activity in 500 µl volume was assayed as described above and the overall volume of the root system was recorded. Inorganic phosphate concentrations were calculated from a standard curve determined using known concentrations of potassium phosphate. The amounts of protein were quantitated by Bradford method.

Results and discussion

Cloning of *Aspergillus ficuum* phytase gene, *Arabidopsis* pyk10 promoter sequence, and carrot extensin signal sequence

The cloned *Asp. ficuum* phytase cDNA (GenBank accession number AF537344) was 1,347 bp long and encoded a mature peptide composed of 448 amino acids with a molecular weight of 49 kDa and pI value of 4.96. Its protein sequence contained the reserved active-site sequence CQVTFAQVLSRHGARYPTDSKGK for histidine acid phosphatases (HAP) at position +52 to +74 and the underlined RHGARYPT part was the most reserved signature sequence for microbial phytase (Kostrewa et al. 1997), which was believed to be the phosphate receptor region.

The protein sequence also contained 10 typical potential *N*-glycosylation sites (Asn-X-Ser/Thr). The glycosylation has little effect on the substrate specificity of phytase but has significant effect on the molecular weight, protein structure, thermal stability and biological synthesis (Wyss et al. 1999). Database search of the deduced amino-acid sequence against other published microbial phytase revealed >90% similarity with phyA proteins from *A. niger* SK-57 (AB0227007), *A. ficuum* AS3.324 (AY013315), *A. ficuum* (AAB26466), and *A. niger (ficuum)* var. *awamori* (M94550).

Functional analysis of *Asp. ficuum* phytase gene in *S. cerevisiae*

Since that many histidine acid phosphatases have little or no activity against InsP6, we first tested the functionality and activity of the genetically engineered phytase protein in yeast system with or without its native signal peptide. Both the pYPA1 and the pYPA2 transformants showed moderate levels of extracellular (1.9 U/mg protein) and intracellular (2.1 U/mg protein) phytase activity, respectively, at 36 h after induction in YEPD medium, while the pYES2 (vector control) transformants had no detectable activity (Table 1). Six pYPA1 transformants produced phytase activities as 1.4–2.4 U/mg protein in the culture supernatant and nearly all the phytase produced was secreted into the medium because no intracellular phytase activity was detected. Without a signal sequence, the pYPA2 transformants exhibited similar intracellular phytase activities, which was 2.1 U/mg protein on average (Table 1). These data demonstrated that the specific product of the *AfphyA* gene is a functional phytase enzyme and indicated that higher activity did not correlate with protein secretion in *S. cerevisiae* in the present study. We also observed similar levels of cell growth (OD_{600}) of two kinds of transformed *S. cerevisiae* and due to its relatively low cost, YEPD medium would potentially be a good choice for phytase production in *S. cerevisiae*.

Molecular characterizations of *AfPhyA* transformed T_0 soybean plants

Genomic PCR amplification of the putative transformants using *AfPhyA* primers gave ~1.4 kb amplification products of transgene (Fig. 2a), which was also

Table 1 Phytase activity from *S. cerevisiae* INVSc1 strain transformed by pYPA1 (harboring *AfPhyA* gene with signal peptide) and pYPA2 (harboring *AfPhyA* gene without signal peptide)

Plasmids	Location	Phytase activity (U/mg protein)
pYPA1	Extracellular	1.9 ± 0.3
pYPA2	Intracellular	2.1 ± 0.2
pYES2	ND	ND

Each observation is the mean of 3 replicates ± standard deviation

ND Not detectable

observed for the positive control pMD18-*PhyA* vector, but not for the untransformed controls. Seventeen out of 62 transformed plants turn out to be positive in genomic DNA PCR analysis (Fig. 2a). Southern dot blot analysis of genomic DNA of PCR-positive plants using labeled *Atpyk10* promoter sequence disclosed clear hybridization dots in fourteen of seventeen PCR positive plants, which indicated the integration of intact *PhyA* expression cassette into their genomes (Fig. 2b).

The *AfPhyA* transgene was under the control of the promoter of *AtPyk10* gene. Pyk10 is a root and hypocotyl specific β-glucosidase/myrosinase from *Arabidopsis thaliana*, which is implicated to be involved in plant defense against herbivores and pathogens (Nitz et al. 2001). Transcript levels of *AfPhyA* in transgenic plants were determined by RT-PCR and the results showed a strong and de novo expression of transgene on the transcriptional level (Fig. 2c). This transcriptional expression was only found in the root tissues of transformed soybean plants, but not in other tissue and organ types (data not shown), which indicated the expression specificity of the transgene driven by the *AtPyk10* promoter.

Phytase activity was elevated in T_1 soybean root tissue as a secretory protein

The inclusion of a plant signal peptide sequence is required for the extracellular secretion of active fungal phytase when the gene was overexpressed in plants (Richardson et al. 2001a). We used the carrot

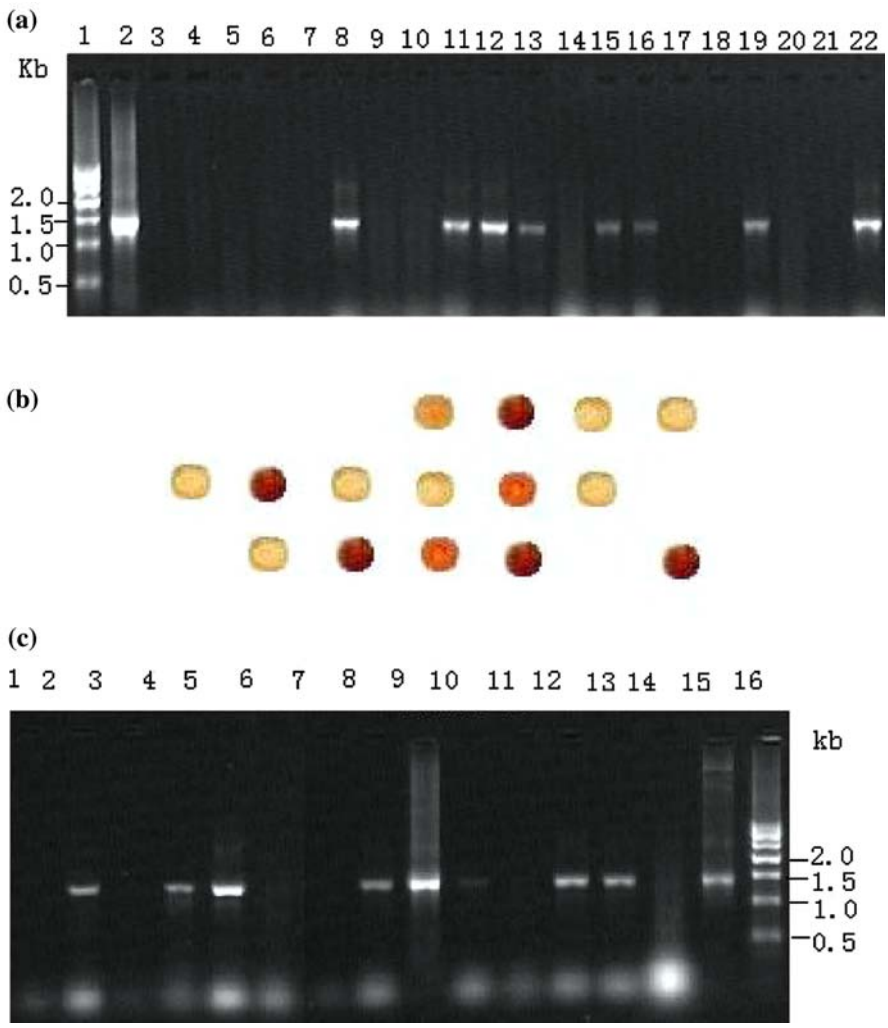


Fig. 2 Molecular characterization of putative *AfPhyA* transformed T_0 soybean plants. **a** Genomic PCR of the kanamycin-resistant soybean plants. **b** Southern dot blot of putative transgenic soybean plants. **c** RT-PCR results of transgenic soybean plants using *AfPhyA* gene-specific primers on root tissues. Lane 1-14: independent transgenic plants; Lane 15: Positive control; Lane 16: DNA molecular weight marker

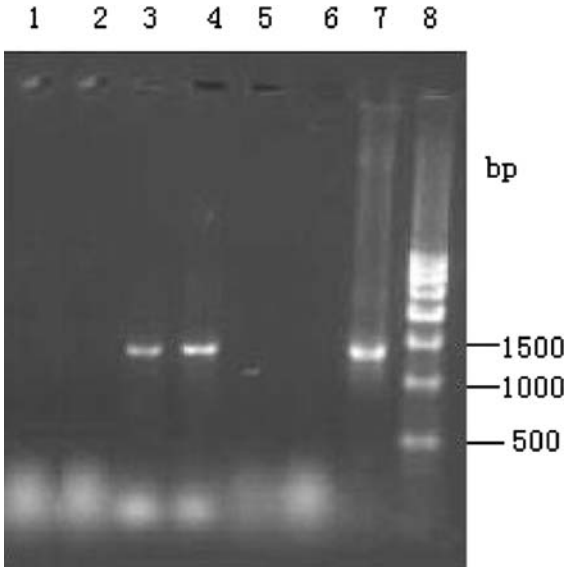


Fig. 3 Genomic DNA PCR results of T_1 generation transgenic soybean. Lane 1-7: independent T_1 transgenic lines; Lane 8: DNA molecular weight marker

Table 2 Phytase activities and Pi content of crude root exudates in incubation media from 20-day-old *AfPhyA* transgenic soybean T_1 plants and control plants

Plants	Pi (μM)	Phytase activity (U/mg protein)	Phytase activity (U/cm^3 root volume)
Control	120 ± 16	0.8 ± 0.1	8.6 ± 1.2
T1-1	268 ± 23	10.2 ± 0.4	112.9 ± 3.9
T1-2	491 ± 30	1.5 ± 0.2	17.7 ± 2.5
T1-3	557 ± 38	2.4 ± 0.3	26.8 ± 3.7

Each observation is the mean of 3 replicates ± standard deviation

extensin signal sequence for secretion of AfPhyA in soybean roots. Soybean plants of T_1 generation containing a phytase transgene were generated from seven T_0 different transgenic lines and only three lines survived and regarded as positive transformants due to their genomic DNA PCR results (Fig. 3). The phytase activity in root excretions of three independent T_1 soybean transgenic plants was 10.2, 1.5, 2.4 U/mg protein, respectively, compared to the 0.8 U/mg protein in control root excretions. When phytase activity was compared based on the volume of root system, the control plant had an average activity of 8.6 U/cm^3 root, while the transgenic root showed activity as high as 113, 18 and 27 U/cm^3 root, respectively (Table 2). Measurement on substrate controls without growing plants revealed that the natural decomposition of phytate was negligible. Hence, the presence of inorganic phosphates in the collected root secretions implied the extracellular phytase activity of the cultured plants. When Pi in the secretions of control plants was at 120 μM, its concentration in the secretions of transgenic plants was 268, 491, and 557 μM, respectively (Table 2). Therefore, expressing phytase during root development as a secreting protein may offer an effective strategy for improving Pi availability in the root surrounding soil.

In summary, the expression of fungal phytase could be localized in plant root tissue specifically by using a heterologous promoter sequence and directed the soluble proteins into the surrounding soil through the guide of a carrot extensin signal peptide. The altered phytase expressing localization and distribution resulted in an increased phytase secretion by root and inorganic phosphate content in the root surroundings without significant deleterious effects on plant growth. This strategy could enable the cultivars to utilize phosphate already present in soil more efficiently and it could also be a more environment-friendly and economically feasible phytoremediation way for improving crop production in low Pi soils.

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