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Expression Of *Pantoea Agglomerans* Phytase From A Strong Constitutive Promoter In *Arabidopsis Thaliana* Plants.

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ABSTRACT

In this study we report construction of an efficient gene expression system in plants and subsequent characterization of transgenic *Arabidopsis thaliana* expressing a bacterial *paPhyC* phytase gene from *Pantoea sp*. Phytase gene expression is controlled by a strong 35S constitutive promoter from cauliflower mosaic virus. All identified transgenic plants had multiple T-DNA insertions in the genome. Expression of paPhyC phytase mRNA in plant tissue was confirmed by RT-PCR in the second generation of transgenic plants, and phytase protein expression was confirmed by Western blotting. Our data indicate that bacterial phytase expression in plants can be an efficient way to potentially increase crop performance in conditions of inorganic phosphorus deficiency in the soil.

Keywords*:* phytase *Pantoea sp.*, recombinant protein expression in plants, CaMV35S promoter, transgenic plants, phosphorus deficiency, *Arabidopsis thaliana*.

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INTRODUCTION

Phosphorus is one of the most important mineral elements for both eukaryotes and prokaryotes. Phosphorus is required for many enzymatic reactions, structural organization of nucleic acids and lipids and for many other aspects of cellular metabolism [1]. The level of bioavailable phosphorus in many natural ecosystems is declining, and some recent reports indicate that with the current consumption trajectories most reserves of rock phosphate fertilizer worldwide will be depleted by the end of this century [2].

Inorganic phosphorus in the form of phosphate is the major source of phosphorus for cellular metabolism, and many eukaryotes are unable to utilize organic phosphorus compounds. However, the main pool of phosphorus in the soil is actually present in the organic form, with up to 80% of it being in the form of phytate, a derivative of inositol hexakisphosphates [3]. The main forms of phytate in the soil are highly stable and insoluble. In addition, phytate is often considered an anti-nutritional factor for many mammals, as it has a tendency to bind and immobilize ions and proteins in gastrointestinal tracts. Furthermore, field run-offs carrying with phytates can lead to eutrophication of water reservoirs and further damage the environment [4; 5; 1].

Overall, the current dilemma of inorganic phosphorus depletion and pollution caused by excess phytate levels in the soil poses a long-term risk for sustainable agriculture. One of the proposed potential solution to this problem is the use of biotechnology to engineer plants capable of utilizing soil phytate for growth and development. Phytases are enzymes that hydrolyze phytate and release phosphates [6]. Plants have endogenous phytases in seeds but their activity in other plant tissues is very low. In contrast to many higher eukaryotes, microorganisms (bacteria and fungi) can scavenge phosphorus from phytate because they produce a variety of phytases with very different modes of action and specificity [7].

These important properties make microbial phytases particularly suitable for plant biotechnology, as genes coding for microbial phytases can be introduced into plant genomes to help alleviate the phosphorus deficiency problems. While some microbial phytases can be used to engineer that are able to grow on phytate-rich phosphate-depleted medium, others can be used to engineer low-phytate plants better suitable for animal feed and less likely to contribute to increased environmental pollution.

Here we report generation and subsequent characterization of transgenic *Arabidopsis thaliana* plants expressing a bacterial *paPhyC* phytase gene from *Pantoea sp.* The transgenic plants express paPhyC phytase at both the transcription and translation levels and can serve as a model for better understanding of the effects of bacterial phytase expression in plant tissues on plant metabolism, growth and development.

MATERIALS AND METHODS

35S::ext-phytase-His-Strep construct

The nucleotide sequence of *Pantoea sp. paPhyC* phytase (KJ783401) gene was codon-optimized to improve protein expression in *Arabidopsis thaliana* using the CodonAdaptationTool software (http://www.jcat.de).

The optimized sequence also included 5'-terminal in-frame nucleotides coding for the signal peptide of carrot extensin from the, and 3'-terminal 6xHis- and Strep-sequence. The entire construct was chemically synthesized by GenScript USA Inc (http://www.genscript.com). The ext-paPhyC-His-Strep sequence was subcloned into the pCBK05 vector using restriction sites *PstI* and *BamHI* under the control of 35S cauliflower mosaic virus promoter [8]. The resulting plasmid was dubbed pCEV04 and used to transform *Agrobacterium tumefaciens* GV3101 cells (Figure 1). As a negative control, a deriviative of this plasmid was also generated that did not contain the ext-paPhyC-His-Strep sequence .

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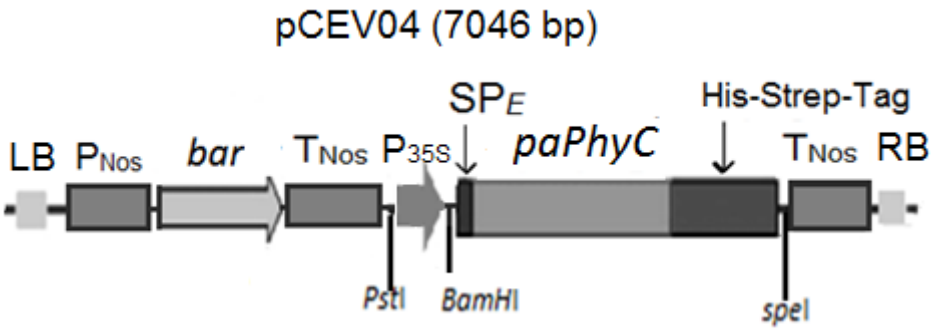

Figure 1: Map of T-DNA region in pCEV02 binary vector developed for constitutive expression of 35S::ex-paPhyC-His-Strep phytase construct in *A. thaliana*.

Abbreviations: Pnos –nos promoter; Bar – BASTA resistance gene; Tnos – nos terminator; LB and RB –left and right T-DNA borders; 35S – constitutive CaMV promoter; SP- leader sequence of carrot extensin gene; *paPhyC* – *Pantoea sp.* phytase gene fused to 6xHis-Strep sequence. Restriction sites are marked by curved lines.

Growth conditions and transformation of *Arabidopsis thaliana* plants

Arabidopsis thaliana (Columbia ecotype, Col-0) plants were used for Agrobacterium-mediated transformation by the floral dip procedure [9]. Seeds from these plants were sterilized with 70% Ethanol for 1 min, 50% bleach with TritonX-100 for 10 min, washed 5 times with distilled sterile water and planted on sterile plates with MS medium containing 25 mg/L BASTA to select for transgenic plants. Transgenic seedlings T1 that survived on BASTA were transferred into pots with soil and used to establish individual transgenic lines for further analysis. From each T1 plant, 50-100 individual seeds for the next (T2) generation of transgenic plants were again subjected to BASTA treatment on MS medium in Petri dishes to establish segregation ratio of survivor/dead plants. The obtained survival ratios were used to judge the number of T-DNA inserts per plant genome.

All wild-type (WT) and transgenic plants were grown in controlled-environment conditions (16 h light period/ 8 h dark period) at 22º C and 60% humidity. Before planting, soil was sterilized by autoclaving and all plants were watered every 2-3 days with distilled water.

Genotyping of transgenic plants and RNA expression analysis *DNA purification.*

Total DNA from plants was extracted using cetyltrimethyl ammonium bromide (CTAB) method (CTAB-buffer: 2% CTAB, 100 мM Tris-HCl, 20 мM EDTA, 1,4 M NaCl, 0,2% β-merkaptoethanol). 100 mg of plant tissue was homogenized in CTAB-buffer and incubated for 1 h at 65 C. Upon the addition of the chloroform samples were mixed by vortexing and centrifuged at 15000 g for 20 min. Supernatant was then mixed with 500 µl of isopropanol in a separate tube and the mixture was centrifuged for 15 min at 15000 g. The DNA samples were rinsed with 100 µl of 70% ethanol, air dried and resuspended in 50 µl HPLC-grade water containing 1 µg/ml RNAse A.

To verify the presence of the transgene sequence in genomes of plants from transgenic group E, containing the full length 35S::ext-paPhyC-His-Strep construct, PCR genotyping was performed using forward primer Pa-synDNA-F (5'-caagaaactcctgaagga-3') and reverse primer PaPhyC-mid-Rev (5'-tccatagccttcaaagcttg-3'). For plants from the transgenic group O (negative control plants), containing only the 35S promoter sequence, the PCR primers were 35S-Prom-F1 (5'-atcccactatccttcgcaa-3') and Ter-Nos-R (5'-catcgcaagaccggcaac-3'). The expected length of the PCR products is 400 bp for both E- and O- groups of plants.

RNA purification and RT-PCR analysis of paPhyC expression

Total RNA was extracted with the TRI reagent. First strand cDNA synthesis was carried out using 1 µg of total RNA, OligodT primer and RevertAid (H-) First Strand cDNA synthesis kit (Fermentas). The resulting cDNA was PCR-amplified with primers to the codon-optimized paPhyC gene.

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Western-blot analysis

Expression of PaPhyC phytase in transgenic plant tissues was confirmed using Western blot analysis. Plants were grown sterilely in liquid MS medium for 3-4 weeks, grinded in liquid N_2 with mortar and pestel and transferred to the Protein Extraction Buffer (15 mM MES/Ca buffer containing 1 mM cysteine and 1 mM EDTA). Homogenized tissue was spun at 12000 g for 10 min at 4 C. Supernatant was then collected an stored in -20 C. 30 µl of protein sample was mixed with Protein Sample Ladder, incubated for 5 min at 85º C and subjected to SDS-PAGE (12.5% Acryl-amide gel). Separated proteins were transferred onto a PVDF membrane for 50 min at 90 V. The membrane was blocked with 5% Skim Milk (Sigma Aldrich, Germany) in PBS-T buffer (Phosphate buffer, 0.1 % Tween-20) for 1 h at RT with shaking. PVDF membrane was then incubated with primary antibodies (6x-His Epitope Tag Monoclonal Antibody (HIS.H8), Thermo Scientific) at a dilution of 1:3,000 for 1 h at RT with shaking. The membrane was washed for 10 min three times in PBS-T buffer and incubated with secondary antibodies (Pierce Goat Anti-Mouse IgG, (H+L), Peroxidase conjugated, Thermo Scientific) at a dilution 1:10,000 for 30 min. After washing in PBS-T and PBS three and two times, respectively, the membrane was visualized using a chromogenic substrate SuperSignal West Pico Stable Peroxidase Solution and SuperSignal West Pico Luminol/ Enhancer Solution (Thermo Scientific).

RESULTS

Generation of transgenic *A. thaliana* plants harboring T-DNA insertions with the ex-paPhyC-His-Strep construct

The sequence of *Pantoea sp.* paPhyC gene was codon-optimized for expression in *A. thaliana* and cloned as a fusion with 5'-terminal carrot extensin leader sequence and 3'-terminal 6xHis-Strep peptide tags (the ex-paPhyC-His-Strep construct) into pCEV04*A* vector under the control of a strong constitutive viral promoter CaMV35S (Figure 1). As a negative control, an empty vector construct containing only the 35S promoter was also prepared. Both vectors were used to transform *A. thaliana* Col-0 plants by *A. tumefaciens*-mediated transformation.

Seeds of plants exposed to *A. tumefaciens* were plated on Petri dishes with MS medium and seedlings were selected based on their ability to grow in the presence of the selective herbicide BASTA. Three primary transgenic plants harboring the ex-paPhyC-His-Strep construct were obtained in T1 generation and each was used to establish an independent transgenic line (transgenic group E). In addition, three primary transgenic plants harboring just the empty vector with 35S promoter alone (negative control) were obtained in the T1 generation and each was also used to establish an independent transgenic line (transgenic group O).

To further verify the presence of the corresponding transgenes, DNA from each primary T1 transformant was used in PCR with either paPhyC- or 35S-specific primers (Figure 2). The presence of the expected size of PCR product in the E-line plants and not in the negative control O-line plants (Figure 2A) indicates that the T-DNA harboring the modified paPhyC transgene was stably integrated into the *A. thaliana* genome. As expected, both the E-line and the O-line plants harbor 35S promoter sequences (Figure 2B).

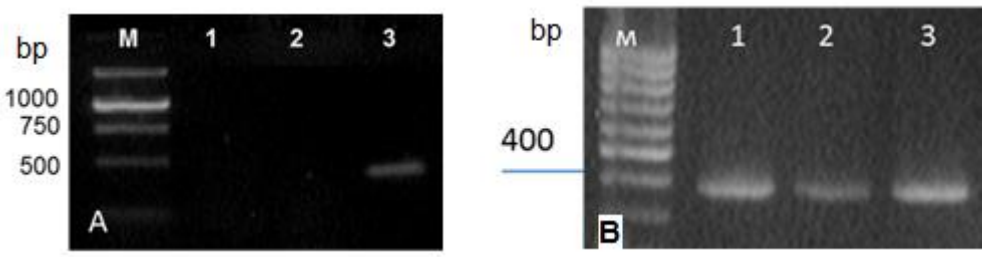

Figure 2: PCR genotyping of transgenic T1 plants of E- and O- lines. A, PCR amplification of paPhyC gene in transgenic T1 plants of E- and O- lines. Lanes 1, 2 – PCR with genomic DNA from negative control lines O1-1 and O1-2; lane 3, amplification of paPhyC sequence in E-1 line. The expected PCR product size is ~ 500 bp. M, molecular size markers. B, PCR amplification of the *bar* gene in transgenic T1 plants of negative control O line (lanes 1,2) and E-1 line (lane 3). M, molecular size markers.

paPhyC phytase expression in transgenic plants

Expression of recombinant paPhyC phytase in transgenic plants was confirmed at the levels of both transcription and translation. RT-PCR with paPhyC gene-specific primers confirmed that the paPhyC mRNA is expressed in all three transgenic E-line plants (Figure 3, lanes 2,4,6). The absence of contaminating genomic DNA in extracted RNA samples was confirmed using the negative control PCR primers to the 35S sequence (Figure 3, lanes 1,3,5). These RT-PCR data indicate that paPhyC mRNA is stably expressed in E-line plants.

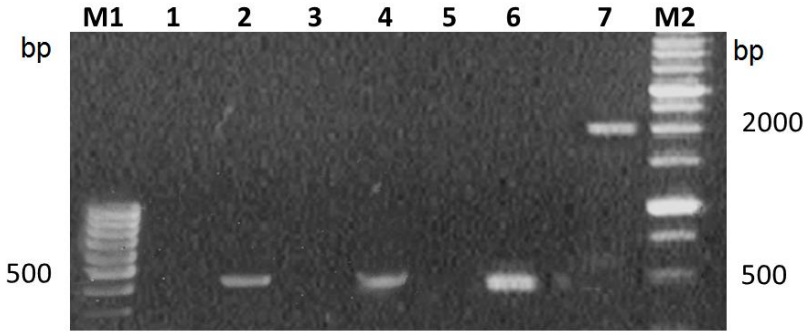

Figure 3: RT-PCR analysis of paPhyC phytase mRNA expression in transgenic plants of E line. Expression of paPhyC mRNA is detected in all three transgenic E lines (E1-1, lane 2; E1-2, lane 4; E1-3, lane 6). No PCR product is detected with primers specific to the 35S promoter sequence in the same E lines (lanes 1, 3, 5), indicating that RT-PCR products present in lanes 2, 4, 6 are amplified from *paPhyC* mRNA and not from potentially co-purifying plant genomic DNA. A positive control for 35S-specific PCR (from pCEV02 plasmid template, lane 7) with the same primers confirms that PCR primers work properly. M1, M2 - DNA molecular size markers.

To further verify paPhyC expression at the protein level, Western blot analysis was performed on plant protein extracts from transgenic E and O lines. As expected, a single protein band was detected in protein extracts from E-lines, but not from O-lines (Figure 4). The molecular weight of the detected protein is ~50 kDa, which correlates well with the predicted molecular weight of the recombinant ex-paPhyC-His-Strep protein. We conclude that the 35S::ex-paPhyC-His-Strep construct represents an effective gene expression system in *A. thaliana* and that the transgenic plants we generated efficiently express bacterial *paPhyC* phytase at both the transcriptional and translational levels.

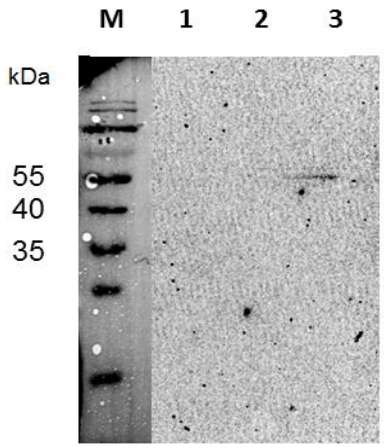

Figure 4: Western blot detection of recombinant PaphyC phytase protein in transgenic plants of E line. Whole-plant protein extracts were prepared from wild-type (lane 1, negative control), T3 trangenic plants harboring only the CaMV35S promotor (lanes 2, negative control) or T3 trangenic E line plants harboring the full 35S::ex- PaphyC-6xHis-Strep expression construct (lane 3). The ~50 kDa phytase-specific band is only detectable in E-line plants.

DISCUSSION

While phytase genes have been identified in a number of prokaryotes and eukaryotes, fungal and bacterial phytases appear to have the highest phytate-degrading activity. HAPs (histidine-acid phytases) is a

group of phytases that shows pH optimum below 7 [10]. In a few recent studies HAPs from *A. niger* and *E. coli* have been used to generate transgenic plants expressing bacterial and fungal phytases [11, 12]. In this study, we used a modified paPhyC phytase from *Pantoea sp.* to construct an efficient bacterial phytase gene expression system in *A. thaliana* and assayed several transgenic lines for phytase expression.

Three individual transgenic lines of *A. thaliana* harboring the bacterial phytase gene (E lines) were identified. Previous studies of transgenic *Trifolium subterraneum* plants expressing *phyA* phytase gene from *A. niger* have shown that transgenic lines with multiple T-DNA insertions had high phytase activity. Similar results were obtained in studies with transgenic *B. napus* and maize plants expressing phytase genes from *E.coli* and *A. niger,* respectively [George et al., 2004; Chen et al., 2008]. These reports indicate that plants with multiple T-DNA insertions appear to show higher phytase activity. As all 3 E lines identified in our study appear to also harbor multiple T-DNA insertion (data not shown), these lines may also harbor increased levels of phytase acticity.

The availability of strong constitutive promoters, such as CaMV35S, provides an efficient way to increase transgene expression. While several studies used a number of tissue-specific or inducible promotors to direct microbial phytase expression in plants [13; 14; 15; 16], the strong CaMV35S promoter may provide better expression levels and overall higher enzyme activities. Our data indicate that both the paPhyC mRNA and protein are stably expressed in transgenic *A. thaliana* plants over several generations. Thus, generation of transgenic *A. thaliana* expressing the 35S::ex-paPhyC-His-Strep construct represents a unique avenue to analyze the effects of recombinant bacterial phytase expression on plant physiology, growth and development. Further explorations of bacterial phytase expression systems in plants can be an efficient way to potentially increase crop performance in conditions of inorganic phosphorus deficiency in the soil.

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REFERENCES

[1] Gaxiola R.A. Edwards M., Elser J.J. Chemosphere 2011; 84: 840–845.
[2] Hurley B. A., Tran H. T., Marty N. J., Park J., Snedden W. A., Mullen R.T., Plaxton W. C. Plant Physiology 2010; 153: 1112–1122.
[3] Wang Y., Ye X., Ding G., Xu F. PLoS ONE 2013; 8(4): 1-9.
[4] Yip W., Wang L., Cheng C., Wu W., Lung S., Lim B.-L. Biochemical and Biophysical Research Communications 2003; 310: 1148–1154.
[5] Raboy V. Inositol Phosphates. Linking Agriculture and the Environment. Seed Phosphorus and the Development of Low-phytate Crops, CABI, 2007, pp. 111-133.
[6] Yao M.-Z., Zhang Y.-H., Lu W.-L., Hu M.-Q. W., Wang A.-H. Liang Journal of Applied Microbiology 2011; 112: 1–14.
[7] Singh B., Satyanarayana T. Physiol. Mol. Biol. Plants 2011; 17: 93–103.
[8] Riha K., Watson J.M., Parkey J., Shippen D. E. EMBO J. 2002; 21(11): 2819–2826.
[9] Clough S.J. Bent A. F. The Plant Journal 1998; 16(6): 735-743.
[10] Mullaney E. J. Ullah H.J. Inositol Phosphates. Linking Agriculture and the Environment. Phytases: Attributes, Catalytic Mechanisms and Applications, CABI, 2007, pp. 97-111.
[11] George T. S., Richardson A. E., Hadobas P. A., Simpson R. J. Plant, Cell and Environment 2004; 27: 1351–1361.
[12] Chen R. Xue G., Chen P., Yao B., Yang W., Ma Q., Fan Y., Zhao Z.,. Tarczynski M.C, Shi J. Transgenic Res 2008; 17: 633-643.
[13] Lei Z.Y., Zhao P., Cao M.J., Cui R., Chen X., Xiong L.Z., Zhang Q.F., Oliver D.J., Xiang C.B. J. Integr. Plant Biol. 2007; 49 (4): 556−567.
[14] Drakakaki G., Marcel S., Glahn R.P., Lund E.K., Pariagh S., Fischer R., Christou P., Stoger E. Plant Molecular Biology 2005; 59: 869–880.
[15] Mudge S.R., Frank W.S., Richardson A.E. Plant Sci. 2003; 165(4): 871-878.
[16] Binka A., Orczyk W., Nadolska-Orczyk A. J. Appl. Genetics 2012; 53: 1–8.