ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

An Improved Gene Expression System to Generate Transgenic Arabidopsis Thaliana Plants Harboring a Bacillus Ginsengihumi Phytase Gene.

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ABSTRACT

We constructed a new vector system for heterologous gene expression in Arabidopsis thaliana. The construct contains a codon-optimized sequence encoding Bacillus ginsengihumi phytase behind an inducible plant-specific promoter for expression in root epithelial cells. The new vector introduced into the plant A. thaliana by Agrobacterium mediated transformation. We obtained several generations of transgenic A. thaliana plants with integrated Bacillus ginsengihumi phytase gene, as well as with an empty vector as a negative control. We tested several transgenic plants harboring the phyCg construct under the control of phosphate-starvation inducible Pht1;2 promoter and show that the phyCg gene is expressed at the mRNA level. Further characterization of these lines of plants will help us to design an improved transgenic strategy for the development of a root-specific heterologous system for the expression of bacterial phytases in plants.

Keywords: *Bacillus* phytase, *Pht1;2* promoter, *Arabidopsis thaliana.*

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ISSN: 0975-8585

INTRODUCTION

One of the major limiting factors of crop yield is the lack of sufficient quantities of inorganic phosphorus in the soil. Much of the inorganic phosphate fertilizer applied to the soil often accumulates in the environment in the form of insoluble organic compounds, such as phytate (myo-inositol hexakisphosphate). Phytate is also considered the main form of phosphorus storage molecule in plant seeds [1]. Phytate content in natural soils can reach over 50% of the total phosphorus content [2]. Plants are unable to metabolize phosphorus from phytate, which also often becomes insoluble and forms complexes with metal cations and proteins. In contrast to plants, microorganisms and many bacteria and fungi are capable of cleaving phosphates off the phytate backbone, as they secrete a number of specific phosphatases (phytases) that hydrolyze phytate complexes to release inorganic phosphates and less phosphorylated phytate deriviatives. Plants can also synthesize phytases, but only as intracellular enzymes that are unable to scavenge phosphorus from the soil [3, 4].

As a promising alternative to applying rock phosphate fertilizer, plants can be engineered to secrete phytases of microbial origin into the soil, in particular, phytases from abundant soil bacteria, such as *Bacillus* [5]. Phytases isolated from *Bacillus* have higher thermal stability and optimum activity at neutral pH values, exhibit a unique Ca^{2+} - dependant mode of catalytic activity and very high substrate specificity for calcium-phytate complexes. All of these properties highlight the value of bacillar phytases as the enzymes of choice for plant biotechnology.

The aim of the work was to develop an improved gene expression system in *Arabidopsis thaliana* that is more suitable for expressing *Bacillus ginsengihumi* phytase in root epithelial cells. The development of such system will facilitate future studies to evaluate growth efficiency of such transgenic plants when grown in phosphorus-depleted soils.

MATERIALS AND METHODS

Construction of expression vectors

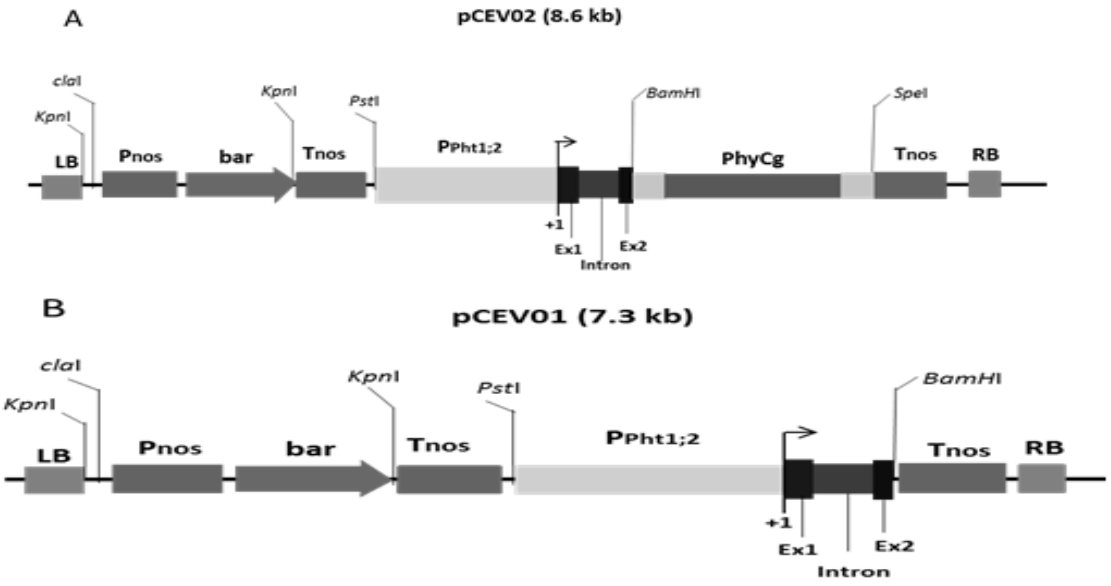

Figure 1: Map of binary vectors created for the purpose of phytase gene expression in *A. thaliana*. A- Binary vector pCEV02 for phyCg expression under the control of *Pht1;2*promoter. B - Binary vector pCEV01 without *PhyCg* gene, used as a negative control. Abbreviations: Pnos –nos promoter; Bar – BASTA resistance gene; Tnos – nos terminator; LB and RB –left and right T-DNA borders; $P_{Pht1;2}$ – Pht1;2 gene promoter; +1 – transcription initiation site; Ex1 and Ex2 – first and second exons present in the *Pht1;2* promoter sequence; Intron –intron present in the *Pht1;2* promoter sequence; SP- leader sequence of carrot extensin gene; *phyGg* – *B. gensengihumi* phytase gene. Restriction sites are marked by curved lines.

As an attempt to improve heterologous gene expression in *A. thaliana*, the nucleotide sequence of the coding region of *Bacillus ginsengihumi* phytase *phyCg* (GenBank NC_000964) gene was optimized using the CodonAdaptationTool software (http://www.jcat.de). Only codons most often preferred by *A. thaliana* were chosen. The optimized sequence also included in-frame nucleotides coding for the signal peptide of carrot extensin from the 5'-terminus, and His- and Strep-sequence at the 3'-end. The entire construct was chemically synthesized by GenScript USA Inc (http://www.genscript.com). The phosphatestarvation-inducible *A. thaliana* promoter Pht1;2 [6] was PCR-amplified from *A. thaliana* genomic DNA. The Pht1;2 promoter was then cloned into the pCBK05 vector [7] using restriction sites *PstI* and *BamHI*. The chemically synthesized construct ex-phyCg-6xHis-Strep was then inserted into the plasmid using *SpeI* and *BamHI* sites. The resulting plasmid was named the pCEV02 vector (Figure 1A), while the control vector without the phytase gene *PhyCg* was named pCEV01 (Figure 1B).

Plant materials and transformation

Arabidopsis thaliana wild-type (WT) ecotype Columbia seeds were planted in sterile moist soil and plants were grown in 12/12 h (day/night) photoperiod at 25°C.

pCEV01 and pCEV02 vectors were transformed into *A. tumefaciens* strain GV3101 by electroporation [8]. Transformed *A. tumefaciens* cells were grown in Luria-Bertoni with antibiotics kanamycin and gentamicin (100 ug / ml) at 26 °C. 3-4 week old *A. thaliana* plants were transformed by dipping the flowers into suspension of *A. tumefaciens* cells in 5% sucrose solution containing Silwet L-77 detergent [9]. The plants were then placed in conditions of high humidity and after two days returned to standard growth conditions. Transgenic plants were selected by sterilizing seeds in 2.5% sodium hypochlorite [10] and subsequently sowing them on MS medium plates (Murashige-Skoog) supplemented with 25 ug/ml of herbicide BASTA to select against untransformed plants [11]. Antibiotic vancomycin (100 ug /ml) was also added to the medium to prevent bacterial contamination.

Plant DNA extraction

Plant DNA extraction was performed by standard methods in CTAB buffer: 2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 0,2% β-Mercaptoethanol. The presence of T-DNA insertions in transformed plants was confirmed by PCR using primers to the phytase gene, to the *bar* gene on the vector and to the *A. thaliana* promoter Pht1;2. Wild-type *A. thaliana* genomic DNA was used as a negative control. PCR products were separated by electrophoresis in 1% agarose gel. Each individual transformant was selfed and used to established a separate transgenic line. Transgene copy number was established by following each transgenic line for three consecutive generations and analyzing the segregation pattern of BASTA resistance by counting the number of dead and surviving plants in the selective medium. The presence of a single-copy transgene in the genome of transformed *A. thaliana* plants was inferred in a case of 3:1 (surviving : dead) plant segregation in generation 2 (T2).

RT-PCR Analysis

RNA was isolated from roots of transgenic plants which were grown in liquid medium under the conditions of phosphorus starvation for 28 days. DNA-free RNA was extracted as described previously [12]. For RT-PCR analysis, 1 µg of RNA was used as a template with the first strand cDNA synthesis kit (Thermo Scientific). Potentially co-purifying genomic DNA was removed by DNase 1 digestion (Thermo Scientific). One µl of the first strand cDNA reaction was then used for PCR with gene specific primers and the products were separated on 1% agarose gel.

RESULTS AND DISCUSSION

Codon optimization and cloning of *B. ginsengihumi* phytase gene *(phyCg)*.

To take advantage of abundant sources of phytate in the soil, plants need to be able to secrete extracellular phytases into the rhizosphere. To generate transgenic plants harboring the bacillary phytase gene *phyCg*, we developed an improved system for gene expression in the roots. We took advantage of the previously characterized *Pht1;2* promoter from *A. thaliana* [13], which is specifically activated in the conditions

of inorganic phosphorus deficiency. Furthermore, the leader sequence of carrot extension was introduced into the construct to ensure robust protein secretion. The overall construct pCEV02 was designed to maximize control of gene expression (Figure 1A). As a negative control in expression analysis, we also generated vector pCEV01, which contains just the promoter *Pht1;2* without the bacterial *phyCg* gene (Fig. 1B).

Due to the degenerate nature of the genetic code, many amino acids are encoded by multiple codons, with only a subset of these being preferentially utilized by different organisms. To increase the efficiency of bacterial gene expression in plants and to avoid using rare *A. thaliana* codons, codon-optimization was conducted using the CodonAdaptationTool [14]. This allowed us to optimize nucleotide sequence of *B. ginsengihumi phyCg* gene for efficient expression in *A. thaliana*.

A. thaliana transformation and selection.

Both vectors were introduced into the *A. thaliana* genome by *A. tumefaciens*-mediated transformation. Seeds of plants exposed to *A. tumefaciens* were plated on Petri dishes with MS medium supplemented with selective herbicide BASTA and selection of transformed plants was performed on the basis of resistance to this herbicide. 13 individual first generation (T1) transgenic plants (transgenic group K1 harboring the full *Pht1;2::ex-phyCg* construct) were identified (Table 1). In addition, 5 transgenic plants harboring the negative control construct (containing only the *Pht1;2* promoter) were identified (transgenic group A1). After 2 weeks of growth on selective medium all transformants were transplanted from Petri dishes to sterile soil.

Genotyping and analysis of the second (T2) and third (T3) generations of transgenic plants.

The presence of transgene T-DNA insertion in the plant genome was additionally verified by PCR. The presence of the *Pht1;2*::*phyCg* construct was confirmed in all 7 individual T1 plants analyzed (Fig. 2), three of which were selected for further analysis. Furthermore, the presence of T-DNA with the negative control construct, containing only the *Pht1;2* promoter, was verified in the first 4 individual T1 plants analyzed (Fig. 3).

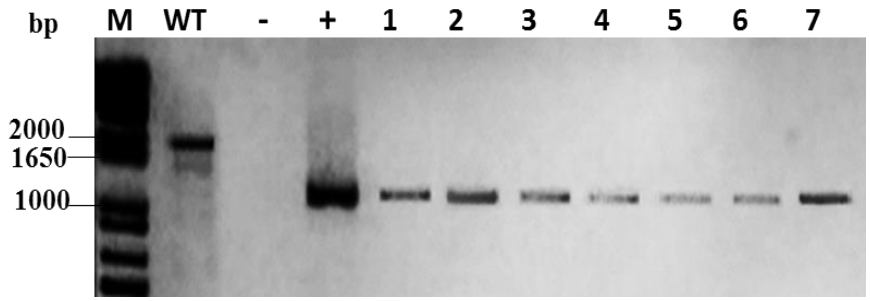

Figure 2:. PCR genotyping of transgenic T1 plants of K1 line. WT- PCR amplification of endogenous *A. thaliana Pht1;2* promoter in wild-type plants, used as a positive control; "-" – negative control: PCR using phyCg-specific primers on genomic DNA from wild type plants; "+" – positive control: PCR using phyCg-specific primers on pCEV02 plasmid; 1-7 – PCR on genomic DNA from transgenic plants of K1 line using phyCg-specific primers; M –DNA ladder.

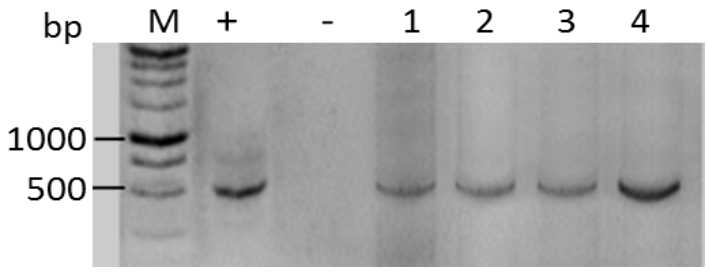

Figure 3:. PCR genotyping of transgenic T1 plants of A1 line. "+" – positive control: PCR using phyCg-specific primers on pCEV02 plasmid; "-" – negative control: PCR using phyCg-specific primers on genomic DNA from wild type plants; 1-4 – PCR on genomic DNA from transgenic plants of A1 line using primers to *bar* gene; M –DNA ladder

ISSN: 0975-8585

We then tested segregation ratio for BASTA resistance in the second and third generations of transgenic plants. Seeds from all 3 individual T1 lines of K1 transgenic group showed approximately 3:1 (alive:dead) segregation ratio on BASTA plates, suggesting that they all have a single T-DNA integration site per genome (Table 1). Similarly, all 3 individual T1 lines of the A1 transgenic group, harboring the negative control construct without the phytase gene, showed 3:1 (alive:dead) segregation ratio on BASTA plates. Seeds from all these T2 plants were then taken to establish clean transgenic lines homozygous for the corresponding transgene, as judged by 100% survival of T3 seedlings on selective medium (Table 1).

Table 1: Analysis of segregation ratios for BASTA resistance trait in the second and third generations of transgenic plants.

Transgenic plant ID in the first generation (T1)	Segregation ratio for BASTA resistance trait in the second generation (T2) **(alive/ dead)**	Deduced transgene copy number in transgenic plants	Transgenic plant ID in the second generation (T2)	Segregation ratio for BASTA resistance trait in the third generation (T3) **(alive/ dead)**
K1-1	82/22 **(3,7:1)**	1	K1-1-15	100/00 **(4:0)**
K1-7	96/26 **(3,7:1)**	1	K1-7-25	120/0 **(4:0)**
K1-15	109/27 **(4:1)**	1	K1-15-1	110/0 **(4:0)**
A1-1	76/24 (3:1)	1	A1-1-5	95/0 (4:0)
A1-2	213/72 **(3:1)**	1	A1-2-5	85/0 **(4:0)**
A1-4	79:25 **(3:1)**	1	A1-4-1	90/0 **(4:0)**

Expression of the *Pht1;2*::*ex-phyCg* transgene in plants.

To analyze the expression of phytase gene *B. gensengihumi* under phosphate starvation conditions, transgenic plants were grown in liquid minimal medium without any source of phosphorus for 28 days. Total RNA was then extracted from roots and by RT-PCR was performed to detect phytase gene expression at the mRNA level. To differentiate between PCR products produced from mRNA and potentially contaminating genomic DNA templates, specific primers were chosen to detect PCR products from the spliced mRNA template, as the *Pht1;2* promoter sequence includes a 256-bp intron (Figure1).

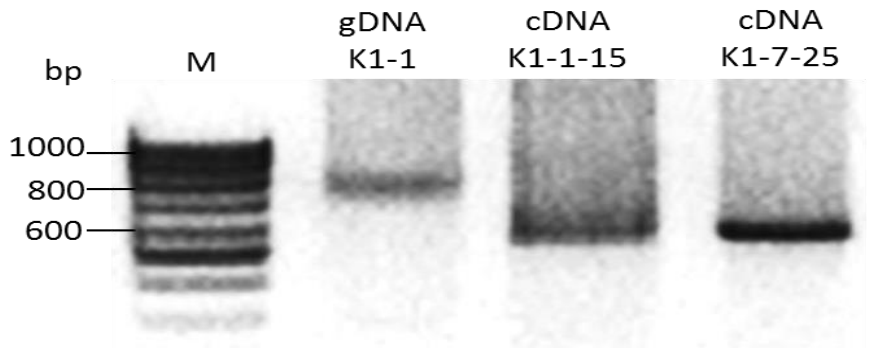

Figure 4: RT – PCR analysis of transgenic plants of K1 line. Expression of phyCg mRNA is detected in two transgenic plant of K1 line. As expected, the size of the RT-PCR product is smaller than that of a PCR product amplified from genomic DNA with the same primers.

The results of RT-PCR assay confirmed that the *Pht1;2::ex-PhyCg* construct is indeed expressed at the mRNA level. The size of the RT-PCR product in two K1 lines is ~610 bp (Figure 4, lanes 2,3), consistent with amplification from the spliced mRNA template. As expected, negative control PCR with the same primer pair

amplifies a ~860 bp product from genomic DNA (Figure 4, lane 1). Sequencing of RT-PCR products amplified from K1 plants also confirmed that the intron is efficiently spliced out to generate a mature form of phyCg mRNA. Taken together, these data strongly support the conclusion that the codon-optimized *phyCg* gene is efficiently expressed in transgenic *A. thaliana*.

CONCLUSION

The rapid improvements in plant genomics tools, as well as genetic engineering techniques, create ample conditions for the development of new plant biotechnology. Generation of transgenic plants expressing microbial phytases for the purpose of utilizing soil phytate is a promising direction in addressing the current shortage of phosphorus for plant growth and crop yield. Of particular practical interest are bacillar phytases with unique physicochemical properties adapted to the conditions in the soil, such as narrow specificity and a relatively small molecular weight [15, 16, 17]. To further explore this direction, we have constructed a heterologous gene expression system in *A. thaliana* plants using a codon-optimized bacillar phytase *phyCg* from *Bacillus ginsengihumi*. We generated several transgenic plants harboring the *phyCg* construct under the control of phosphate-starvation inducible *Pht1;2* promoter and show that the *phyCg* gene is expressed at the mRNA level. Further characterization of these transformants will help design an improved transgenic strategy for the development of a root-specific heterologous system for the expression of bacterial phytases in plants, thus facilitating the transition to a more sustainable agriculture.

ACKNOWLEDGEMENT

This work was supported by the Russian Foundation for Basic Research (project no. 12-08- 22 00942a) and by the subsidy allocated to Kazan Federal University for the state assignment in the sphere of scientific activities (project №14-83 0211/02.11.10083.001). This work was performed in accordance with the Russian Government Program of Competitive Growth of Kazan Federal University.

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