Expression of erythropoietin in Indian tetraploid potato variety [v1; ref status: not approved 2, <http://f1000r.es/USGpfK>]

Priti N. Desai, Harish Padh

B.V. Patel Pharmaceutical Education and Research Development (PERD) Centre, Ahmedabad, India.

Correspondence to Harish Padh: hpadh@yahoo.com

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Abstract With the advent of protein-based biotech drugs in the market, the quest for the “perfect” protein expression system, which is both economical and effective, has come into focus. Currently bacteria, yeast, insect cells, mammalian cells, transgenic animal and transgenic plants are widely used for the expression of therapeutic proteins. Among these, transgenic plants provide advantages in terms of low production cost, lower capital investment in infrastructure, and suitable post-translational modifications. The major limitation of plants as an expression host is the low level of transgene expression. To increase the expression of heterologous proteins in plants, a number of approaches have been used. One of the approaches is to increase the transgene expression by using tissue-specific promoter(s) which can concentrate the protein of interest in targeted tissues and, thus, prove advantageous in downstream purification. In the present report, a protocol for expression of heterologous protein erythropoietin in potato tuber using *patatin*, the tuber-tissue-specific promoter, was standardized. Expression vectors for production of the erythropoietin gene under tissue-specific promoter were successfully constructed. For production of a transgenic plant, tissue culture techniques for regeneration of the whole plant from single explants were standardized. Polymerase chain reaction (PCR) analysis was performed to confirm the stable integration of the erythropoietin gene in the potato plant by using sequence-specific primers.

Introduction

In the past decade, transgenic plant expression systems have emerged as a serious competitive force in the large-scale production of recombinant proteins. The first plant-derived heterologous proteins have already reached the market^{1,2} and detailed economic evaluations have demonstrated their competitiveness against established market sectors^{3,4}. Several plant-derived recombinant therapeutic proteins which are in the final stage of clinical trials include human blood products, vaccine, antibody and growth hormones⁵. As with a number of products coming to the market, molecular farming in plants is finally coming of age. There have been technological developments on many levels, including transformation methods, control of gene expression, protein targeting and accumulation, the use of different crops as production

platforms⁶ and modifications to alter the structural and functional properties of the product. One of the most driving factors has been yield improvements, as product yield has a significant impact on economic feasibility. To increase the final yield, a number of approaches can be used. One of them is to use a tissue-specific promoter, which can concentrate the protein of interest into targeted tissue and prove advantageous during downstream purification⁷.

In the present report, a protocol for expression of therapeutic proteins in potato tuber by using *patatin*, a tuber-specific promoter, was made. Indian tetraploid potato variety, *Kufri Bahar* was used for the expression of the therapeutic protein, as it is widely grown in Gujarat and other region of India. Potato is a vegetative propagated plant, which minimizes the spread of

transgene contamination through pollen, and high tuber biomass makes it suitable for bulk production⁸.

Erythropoietin (EPO) was chosen as a protein of interest for tuber-specific expression, and to evaluate the capability of performing complex glycosylation in the potato plant. Erythropoietin is a highly glycosylated protein and glycosylation is necessary for its *in vivo* activity⁹. Presently, recombinant erythropoietin available on the market is produced from mammalian cells. Mammalian systems have disadvantages in term of cost, scalability and safety^{10,11}. Recombinant human erythropoietin is widely used to treat anemia associated with chronic renal failure, rheumatoid arthritis, acquired immune deficiency syndrome (AIDS), and malignancies, as well as other types of anemia¹².

Recombinant EPO was expressed in tobacco BY2 cells. However, the expression level was very low (0.0026%) and expressed protein remained active only *in vitro*¹³. Thus, to increase the expression, researchers tried to express this protein in a whole plant using constitutive promoter (tobacco and Arabidopsis) where the side effect of over expression was observed¹⁴. Overexpression of EPO protein resulted in stunted vegetative growth, late flowering and male-sterility¹⁵.

While in the present project we had chosen to express EPO protein in a tissue-specific manner, by using a tissue-specific approach, we aimed to decrease the metabolic burden to the whole plant and concentrate the protein in targeted tissue, where it can be easily purified. In the present study, potato tubers were selected as an organ of choice for the production of EPO since the same standard processes used in the starch industry may be adapted with little modifications to separate proteins. Also, the tubers, as most of the storage organs, offer a low hydrolytic profile, which facilitate protein stability.

Several genes have been expressed in potato tubers using different techniques with varying degrees of success^{15–17}. Considerable success has been achieved with the patatin promoter, which confers tuber specificity^{16,18}. Most of the reports which use potato tuber as an expresser of therapeutic proteins had generally used the diploid potato variety^{19–23}. However, we aim to use the Indian tetraploid potato variety *Kufri Bahar*, which is widely grown in Gujarat and other parts of India²⁴.

Materials and Methods

Plasmids, bacterial strains and plants

In the present report, plasmids, bacterial strains and plants were used, as described in [table 1](#).

Growth and maintenance of bacterial cultures

Escherichia coli DH5 α and *Agrobacterium* LBA4404 cultures were grown in luria broth (LB) medium. *E. coli* cultures were grown at 37°C while *Agrobacterium* cultures were grown at 28°C at 175 rpm. When necessary, antibiotics were added for *E. coli*

(chloramphenicol [Sigma] 35mg/ml) and for *Agrobacterium* (chloramphenicol 35 mg/ml and rifampicin [Hi-media] 50mg/ml).

Construction of plant rEPO expression vectors

All enzymes which were used for cloning were purchased from MBI fermentas. Tuber-specific promoter patatin (B33) was excised from the vector pBinB33 using *Eco* RI and *Bam* HI Restriction Endonuclease (RE) sites and cloned into binary vector pCambia1281Z using the same RE sites. pCambia1281Z contained GUS as a reporter protein. The patatin gene was cloned upstream to the GUS gene at *Eco* RI and *Bam* HI RE sites, resulting in a plasmid named pPERDB33 ([Figure 1a](#)). For the expression of the EPO gene, the cDNA sequence of EPO was excised from the pIRsepo and cloned in to pPERDB33 in place of the GUS gene by using *Nco* I and *Bst* EII RE sites ([Figure 1b](#)). The resulting plasmid was named pPERDB33cEPO, which has the EPO gene downstream to the patatin promoter ([Figure 1b](#)). pPERDB33cEPO was then introduced in to *E. coli* DH5 α for maintenance and further experimentation. pPERDB33cEPO was also introduced into *Agrobacterium tumefaciens* strain LBA 4404 by using triparental mating²⁵.

Establishment of *in vitro* potato plant culture

Shoot cultures of the variety *Kufri Bahar* were established from sprouted buds excised from the tubers. The buds were surface sterilized with 0.1% mercuric chloride for 3 min followed by a rinse with 70% ethanol. After repeated washing with sterile distilled water, the buds were cultured on Murashige and Skoog (MS) medium supplemented with 0.1 μ M GA₃. The pH of the medium was adjusted to 5.7 and all the cultures were solidified with 0.8% agarose. The cultures were exposed to 16 hours light, which provided 1600 lux intensity of light, and maintained at 24 $\pm$ 2 °C temperature with 50–60% relative humidity. The buds developed into plantlets in 4 weeks. Nodes from these plantlets were cultured on MS medium containing 3% sucrose for raising the fresh cultures. Shoots from the fresh cultures were cultured into the MS medium supplemented with 8% sucrose and 6 μ M BA to produce the microtuber. Inter-nodal segments, leaf discs and microtuber discs were routinely used for *Agrobacterium*-mediated transformation. To raise the shoots from inter-nodal segments, leaf discs and microtuber discs were used. Direct shoot regeneration was observed in all three explants in the two stage medium. In stage 1, all the explants were inoculated in PSRI 1 medium (MS medium + 1mg/l thiamine + 2% sucrose + 11 μ M zeatin + 1 μ M NAA + 0.05 μ M GA₃ + 0.8% agar) for 20–30 days and then shifted to the PSRI 2 medium (MS medium + 1 mg/l thiamine + 2% sucrose + 9 μ M zeatin + 0.1 μ M NAA + 0.05 μ M GA₃). This medium was standardized for direct shoot regeneration from internodes by Millam (2006)²⁶.

Transformation of potato plants

Inter-nodal segments, leaf discs and microtuber discs from *in vitro* grown plants were used for transformation and regeneration, essentially as described by Millam (2006)²⁶

Table 1 Plasmids, bacterial strains and plant variety used in this study.

Plasmids/Bacterial strains	Properties/Purpose	Source
pBinB33 pCAMBIA 1281Z pRisepo	Contain Patatin Promoter Used as backbone vector Contain cDNA of <i>EPO</i> gene	Gifted Dr. Frederik Bornke, Germany Procured from CAMBIA, Australia Gifted by Dr. Kirill Alexandrov, Germany
<i>E. coli</i> DH5 α	Use for cloning and amplification all plasmids	B.V. Patel PERD centre's Depository
<i>E. coli</i> HB101	Used as helper Plasmid in triparental conjugation	Procured from DSMZ, Germany
<i>Agrobacterium tumefaciens</i> LBA 4404	Disarmed strains	Procured from Netherland culture collection (NCCB), Netherland
Plant Variety <i>Solanum tuberosum</i> (Kufri Bahar)	Used as an expression host for <i>EPO</i> gene	Gifted by Dr. N.H. Patel (Potato Research station, DISA)

Figure 1 Schematic diagram of vector construction for tissue-specific expression of the EPO gene in potato plants. **a)** Construction of vector containing patatin, pPERDB33 and, **b)** construction of vector containing EPO gene under patatin promoter.

with some modifications. The procedure described by Milam (2006)²⁶ for internodes was also used for leaf discs as well as microtuber discs. All three explants were incubated in liquid shoot regeneration medium (MS medium containing 1 mg/l thiamine + 2% sucrose + 11 μ M zeatin + 1 μ M NAA + 0.05 μ M GA₃) for 2–3 hours in presence of 20 μ M acetosyringone. After preconditioning, the explants were infected with *Agrobacterium tumefaciens* LBA4404 harboring the pPERDB33cEPO for 15 minutes. The explants were then blotted dry on sterile whatman No. 1 filter paper and were co-cultured on shoot regeneration medium containing 0.8% agar for 2 days. Following co-cultivation, the explants were transferred to shoot regeneration medium containing

0.8% agar and 500 mg/ml cefotaxime for 7 days for inhibition of *Agrobacterium* growth. After 7 days, explants were transferred to shoot regeneration medium containing 0.8% agar, 500 mg/ml cefotaxime and 7.5 mg/ml hygromycin for selection of transgenic shoots.

Analysis of transgenic plants

After selection of transformed plant in antibiotic selection medium, genomic DNA was isolated by using fresh leaf from the regenerated shoots²⁷. Genomic DNA was used as a template DNA for the amplification of the cEPO gene. After transformation, T-DNA was integrated into the plant nuclear DNA. In order to confirm the presence of cDNA sequence of EPO

in plant nuclear DNA, gene specific PCR was performed by using EPO cDNA-specific primer, forward primer: 5' CCAC-CACGCCTCATCTGTGAC 3' and reverse primer 5' TCT-GTCCCCTGTCCTGCAGGC 3'. PCR amplification was performed in 50 µl reaction containing primers (50 ng each), Taq DNA polymerase (1 unit), 200 µM dNTP, 1x PCR buffer and 50 ng of genomic DNA as template amplifying a 408 bp fragment of the EPO gene. The PCR cycling conditions were set as initial melting at 95°C for 5 minutes, followed by 30 cycles of amplifications with each cycle consisting of the following steps: 95°C for 15 seconds, 68°C for 20 seconds and 72°C for 1 minute.

Results

To produce the EPO protein in a tuber-specific manner, patatin promoter was first excised and ligated into the backbone vector pCambia1281Z to produce pPERDB33. The confirmation of pPERDB33 was done by RE digestion and agarose gel electrophoresis (figure 2).

After confirmation of the right clone having patatin promoter upstream to the GUS gene, the clone was maintained in *E. coli*. The *Agrobacterium*-mediated method was used for expression of the EPO gene in the potato plant. Plasmid pPERDB33, which contains the GUS gene downstream from the

Figure 2 Confirmation of transformant by double RE digestion. Lane 1 contains Marker (lambda DNA digested with *Hind* III), lane 2 contains *Eco* RI and *Bam* HI digested plasmid from clone 23, lane 3 contains *Eco* RI digested plasmid from clone 23, lane 4 contains undigested plasmid from clone 23, lane 5 contains *Eco* RI and *Bam* HI digested plasmid from clone 26, lane 6 contains *Eco* RI digested plasmid from clone 26, lane 7 contains undigested plasmid from clone 26, Lane 8 contains undigested pCambia 1281Z, lane 9 contains *Eco* RI digested pCambia 1281Z, lane 10 contains *Eco* RI and *Bam* HI digested pCambia 1281Z, lane 11 contains undigested pBinB33, lane 12 contains *Eco* RI digested pBinB33, lane 13 contains *Eco* RI and *Bam* HI digested pBinB33 and lane 14 contains Marker (lambda DNA digested with *Bst* EII).

patatin promoter, was mobilized into *Agrobacterium* LBA4404 using tri-parental mating and confirmation of right clone, which was selected on the Luria agar plate containing rifampicin and chloramphenicol, and was done by the same method used for selection of the right clone in *E. coli*.

To clone the EPO protein into the vector pPERDB33 downstream to the patatin promoter the cDNA sequence of the human EPO gene was excised from plasmid pRISepo and ligated to the plasmid pPERDB33 in place of GUS. Confirmation of the right clone was done by RE digestion and agarose gel electrophoresis (Figure 3). The resultant plasmid which has the EPO gene downstream to the patatin promoter was named pPERDB33cEPO. pPERDB33cEPO was subsequently mobilized to the *Agrobacterium* LBA4404 using tri-parental mating and confirmed by RE digestion and agarose gel electrophoresis.

Figure 3 Confirmation of transformant (pPERDB33cEPO) by RE digestion. Lane 1 contains marker (lambda DNA digested with *Hind* III marker of MBI Fermentas), lane 2 contains pRISepo digested with *Nco* I and *Bst* EII, lane 3 contains pPERDB33 digested with *Nco* I and *Bst* EII and lanes 4 to 8 contain PCR positive clones.

The resultant plasmid pPERDB33cEPO was then introduced into potato plants by using *Agrobacterium*-mediated transformation. Transformed plants were selected on the PSRI medium containing 7.5 mg/L hygromycin and 500 mg/L cefotaxime. After 1 month, transgenic shoot formation was observed from the microtuber discs in the antibiotic selection medium (Figure 4). However, shoot formation was also observed from inter-nodal explants, but they did not survive for a longer time in the antibiotic stress medium.

Genomic DNA was isolated from the leaf of transformed plant. The relative quantity and quality of genomic DNA was analyzed using agarose gel electrophoresis. After transformation of

Figure 4 Shoot formations from transformed microtuber discs.

the plant by the *Agrobacterium* method, the T-DNA segment containing the EPO gene was integrated into the plant nuclear genome. To investigate the stable integration of gene of interest in to plant nuclear genome, gene-specific PCR was done. Isolated genomic DNA from the transformed plant was used as template DNA. For gene-specific PCR analysis, EPO specifically primed along with the plasmid DNA containing the EPO sequence (pPERDB33cEPO) as a positive control was used.

After PCR amplification, the PCR product was analyzed using 1.5% agarose gel (Figure 5). A 100 bp DNA ladder was used as marker. A 408 bp band corresponding to EPO amplicon was observed in the gel (Figure 5, lane 4), which was similar to the amplicon obtained from pPERDB33cEPO used as a positive control (Figure 5, lane 5). From this result it was confirmed that the EPO sequence was successfully integrated into the plant nuclear genome.

Discussion

The aim of the present study was to develop a protocol for production of heterologous proteins in potato tuber. Vectors

Figure 5 Analysis of PCR-positive transformed plants on 1.5 % agarose gel. Lane 1 contains Genomic DNA isolated from transformed plant 1, lane 2 contains DNA ladder as a marker (100 bp MBI fermentas), lane 3 and lane 4 contain PCR product of transformed plants, lane 5 contains positive control (PCR product of pPERDB33cEPO), lane 6 contains negative control (PCR product of pBSSK which does not contain EPO gene), lane 7 contains negative control of PCR reaction (master mix) and lane 8 contains genomic DNA isolate from transform plants.

for the production of the erythropoietin gene under tissue-specific promoter and tissue culture techniques for regeneration of a whole transgenic potato plant from single explants were standardized successfully. Direct shoot regeneration from a number of explants like leaf discs, *in vivo* tuber discs, *in vitro* tuber discs and internodes were successfully achieved. A very low plant transformation efficiency and a high percentage of necrosis were observed using *Agrobacterium*-mediated plant transformation. To reduce the necrosis of explants and increase the transformation efficiency time of co-cultivation, inoculum of the bacteria as well as the time for pre-selection were optimized. Hygromycin which was used in selection media caused necrosis of explants even at lower concentrations. Transgenic potato plants were confirmed with the gene-specific PCR, which proved that our gene of interest successfully integrated into the desired plant nuclear genome. However the PCR-positive plant was not able to form rooting when transformed to the rooting medium containing hygromycin. This may be because the hygromycin concentration was lethal when it directly came into contact with the shoot.

Overall, the study demonstrates the feasibility of designing vectors to be used in creating transgenic potato plants for tissue-specific heterologous protein production. However, a lot remains to be done in optimizing the transformation and selection processes before the process can be used for large-scale production of heterologous proteins using tissue-specific expression in tuber of *Solanum tuberosum*.

Authors' contributions

PND and HP reviewed the manuscript. PND was involved in drafting the manuscript and reviewing the literature. HP was responsible for supervising the production of the manuscript.

Competing interests

No competing interests were disclosed.

Grant information

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Laura Privalle

BASF Plant Science, Research Triangle Park, NC, USA

The idea presented by the authors is a good one and I was very interested in reading more about their success or their outcome. Unfortunately, I was quite disappointed as all that was presented in the paper was about a third of the story.

They demonstrated that they could transform potato; however potato transformation is not new and of itself is an insufficient single result not deserving of publication. They did not demonstrate expression of erythropoietin or that using the patatin promoter would give sufficient expression to be of interest. Patatin is a major protein in potato but protein levels in potato are still quite low (~2%). The idea of using the starch extraction method to enrich the protein fraction is a good idea but again they did not present any follow-up that showed it would work for this particular protein of interest. When additional information is available it should be considered for publication but not before. In addition, I would recommend the authors seek a person more conversant in English to assist them in writing the next version.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 17 October 2012

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Argelia Lorence

Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

As expressed by the authors, the aim of this study was to develop a protocol for the production of erythropoietin (EPO), a pleiotropic cytokine with remarkable tissue-protective activities in addition to its well establish role in red blood cell production, in the Indian tetraploid potato variety Kufri Bahar.

The recent FDA approval of Elelyso™, the first plant-made pharmaceutical for treatment of Type I Gaucher disease in humans, has renewed worldwide interest of using plants as viable production platforms for human and veterinary therapeutics.

Although improving the production of EPO in plants is a topic of relevance, unfortunately the data described in this work is too preliminary and falls short from accomplishing the goal set by the authors. The key information lacking here is the level of expression of EPO achieved in this system, the demonstration that the expression level is stable over generations of these transgenics, and more importantly the proof that the expressed EPO is functional. The authors also failed to include in the introduction and to compare their strategy with the one of other groups who have successfully produced EPO in other plant expression systems Conley et al (2009)¹ 2 Conley et al (2010)³, Sperb et al. (2011)⁴, Conley et al (2011)⁵, Kitter et al. (2012)⁶; Nagels et al. (2012)⁷ and Castilho et al. (2012)⁸.

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Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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