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# Molecular Cloning and Transgenic Expression of a Synthetic Human Erythropoietin Gene in Tobacco

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**Abstract** Erythropoietin (EPO) is a hormone belonging to a group of hematopoietic growth factors that control the proliferation and differentiation of bone marrow cells. It induces the production of erythrocytes, thereby increasing the amount of circulating hemoglobin and oxygen. Previous attempts to transgenically express human EPO in plants failed to succeed because the plants exhibited abnormal morphology and infertility. In the present work, we describe the generation of fertile transgenic tobacco plants able to express a synthetic version of human EPO. A 582-bp fragment of the human EPO gene was synthesized using a PCR-based method and ligated into pCR-Blunt. After sequencing, the human EPO fragment was transferred to pWUbi.tm1 and the expression cassette was then transferred to the binary vector pWBVec4a. After *Agrobacterium*-mediated transformation of *Nicotiana tabacum* SR1 plants, integration of the transgene into $T_0$ and $T_1$ plant genomes was confirmed by PCR. The human EPO gene was found to be expressed in tobacco leaves at the mRNA and protein levels. Self-crossing allowed us to obtain $T_1$ plants exhibiting Mendelian segregation of the transgene. None of the plants presented any kind of malformation or deformity.

**Keywords** *Agrobacterium* · Recombinant protein · Erythropoietin · Heterologous expression · Molecular breeding · Transgenic tobacco · Ubiquitin promoter

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## Introduction

All mammalian cells require oxygen for oxidative respiration and other biochemical reactions. Hemoglobin in red blood cells acquires oxygen from the lungs and makes it available to other tissues. The number of circulating red blood cells is the main determinant of tissue oxygenation. Each day, normal human beings replace 1% of their circulating red blood cells with an equal number of reticulocytes. An excess of oxygen, however, can lead to oxidative damage of multiple cellular components and, under extreme conditions, cellular death. Hence, daily production of reticulocytes must be strictly regulated, and the mediator of the homeostatic control of red blood cell number is erythropoietin [1].

Erythropoietin (EPO) is an endogenous cytokine essential for the development and maturation of red cell ancestors, and it is capable of regulating the levels of circulating red blood cells. EPO is produced in the kidneys and liver of embryos, and it is secreted into the circulation as a consequence of certain states of hypoxia and in response to cobalt chloride ($CoCl_2$). Hypoxia has been shown to be the main physiological stimulation that increases the expression of the EPO gene [1, 2].

The human EPO gene is located in chromosome 7q11–22 and is transcribed into a pre-mRNA composed of five exons. Post-transcriptionally, it generates one single mature transcript that is translated into a polypeptide chain containing 193 amino acids. During post-translational modifications, glycosylation occurs with the addition of *N*-linked oligosaccharides to Asn-24, Asn-38, and Asn-83; and an acidic *O*-linked oligosaccharide to Ser-126. Additionally, two disulfide bonds are formed between Cys-7 and Cys-161 and between Cys-29 and Cys-33, and this occurs concomitantly with the removal of an N-terminal hydrophobic secretory sequence of 27 amino acids. It is thought that Arg-166 in the C-terminal portion of the EPO protein is cleaved off before its release into circulation. Therefore, the primary structure of this mature protein contains only 165 amino acids. The molecular masses of the non-glycosylated EPO and its glycosylated form are estimated at 18 and 30 kDa, respectively [3].

Purified EPO is clinically used in the treatment of anemia caused by several conditions, including chronic renal disease; kidney, bone marrow, and stem cell transplants; frequent dialysis; malignancies such as tumors and cancers; and anemia associated with surgery or human immunodeficiency virus (HIV) infection. Additional therapeutic EPO applications include the treatment of autoimmune disorders, hemolysis, acute renal insufficiency, recovery after blood transfusion, ischemic brain damage, spinal marrow, neurological injuries, and congestive cardiac diseases [3–5].

The human gene encoding EPO was first cloned by Lee-Huang in 1984 [6] and its production was immediately and successfully achieved by transgenically expressing it *in vitro* in mammalian cells such CHO [7], COS-1 [8], COS-7, and BHK [9] cells. In trials to reduce production costs, the human EPO gene was also expressed in bacteria [6], yeasts [10], and insect cells [11], but these systems proved to be incapable of executing the correct post-transcriptional and post-translational processing necessary for *in vivo* protein activity. More recently, transgenic pigs were generated for the production of recombinant EPO in their milk. However, transgenic EPO expression resulted in high embryonic mortality, in addition to sterility [12].

A potentially lower-cost alternative method of producing active EPO in appreciable amounts is to express it in a plant system. Many pharmacologically important animal proteins like insulin, interferon, lactoferrin, and glucocerebrosidase are successfully being expressed in plants [13]. Indeed, human EPO has been produced in tobacco cells in culture, and the recombinant protein proved to be active *in vitro* [14]. Nevertheless, no transgenic

plants were regenerated from the transformed cells. Ten years later, both tobacco and *Arabidopsis thaliana* plants were regenerated from tissues transformed with the EPO gene [15]. Although both plants were capable of expressing the human EPO gene, the accumulation of human EPO in plant tissues was reported to cause sterility and morphological deformities.

In the present work, we tested the expression of a synthetic cDNA corresponding to the mature mRNA of the human EPO in transgenic tobacco plants. Unlike in the previously mentioned works, we tested a different promoter sequence with known weaker expression in plant tissues. The resulting transgenic tobacco plants expressed the EPO gene as well as accumulated the recombinant protein in leaves and other tissues. Equally importantly, the transgenic tobacco plants were morphologically normal relative to non-transgenic control plants, and they generated descendants with the proven presence of the transgene.

## Materials and Methods

### Synthesis, Cloning, and Sequencing of a Human EPO cDNA

The full coding sequence of the human EPO gene was synthesized by an oligonucleotide "overlapping" technique [16]. Based on a mRNA sequence available at GenBank (NM 000799.2), terminal overlapping oligonucleotides [16] were designed and synthesized (Invitrogen). The PCR products were cloned into pCR-Blunt (Invitrogen) according to the manufacturer's instructions. After *Escherichia coli* transformation, recombinant plasmid clones were fully sequenced in an ABI3100 Automatic Sequencer (Applied Biosystems) platform (ACTGene, Centro de Biotecnologia, UFRGS).

### Construction of the EPO Expression Cassette for Plant Transformation

The corresponding EPO cDNA sequence was excised from pCR-Blunt using the endonuclease *Eco*RI and transferred to pWUbi.tm1 [17] cut with *Eco*RI. In doing so, the EPO cDNA was ligated between the promoter followed by the first intron of a maize ubiquitin gene and the terminator *tm1'*. The expression cassette was transferred to the pWBVec4a binary vector [17] using *Eco*RI and *Not*I. The resulting recombinant plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 by heat shock [18]. Transformed agrobacteria were selected in LB medium containing 50 μg/mL spectinomycin and 50 μg/mL rifampicin.

### Tobacco Leaf Transformation

Leaf discs of the *Nicotiana tabacum* cultivar SR1 'Little Havana' were infected in accordance with the protocol established by Horsch et al. [19] with minor modifications. About 100 leaf discs were placed in infection plates containing MS medium, 100 μM acetoseryngone, and 0.75 mL of a stationary-stage culture of *A. tumefaciens* LBA4404:: pWBVec4a-HsEPO for about 20 min. Leaf discs were then transferred to co-culture medium composed of MS, 0.1 μg/mL 1-naphthaleneacetic acid (NAA), and 1 μg/mL 6-benzyl aminopurine (BAP) and were kept under a photoperiod of 16 h at 26 °C. Two days later, the leaf discs were placed on selection and regeneration medium (MS medium, 100 mg/L cefotaxime, and 10 mg/L Basta) for a period of 40 days under the same temperature and light regime. Regenerated seedlings were transferred to isolated bottles

containing complete MS medium supplemented with 100 mg/L cefotaxime and 10 mg/L Basta. After they eliminated the residual agrobacteria and reached the expected size, the plants were transferred to vases containing vermiculite and black soil (1:1) in a greenhouse with a photoperiod of 16 h at 26 °C.

## Analysis of Transgenic Plants by PCR

Plant DNA extraction was carried out [20] and 50 ng of genomic DNA from each potentially transformed plant and from untransformed (control) plants were used in PCR analyses to assess their transgenic status. Reactions were carried in a total volume of 25 μL containing 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of corresponding flanking primers EPO-F (5′-ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCC-3′) and EPO-R (5′-TCATCTGTCCCCTGTCCTGCAGGCCTCCCCTGTGTACAGCT-3′), and 2.5 U Platinum Taq DNA Polymerase (Invitrogen). Reaction conditions included an initial denaturing step at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and a final step of 72 °C for 10 min. PCR products were horizontally electrophoresed in 1% agarose gels and visualized by ethidium bromide staining under UV light (254 nm). Optimum specific primer pairs were designed using GenScript Corporation's Real-Time PCR Primer Design Tool (http://www.genscript.com/ssl-bin/app/primer). The primers were synthesized by Integrated DNA Technologies, Inc.

## Reverse-Transcription (RT)–PCR

Total RNA was extracted using PureLink Plant RNA Reagent (Invitrogen) in accordance with the miniprep protocol in the manufacturer's instructions. Five micrograms of each RNA sample was treated with DNase I (Invitrogen) at 25 °C for 15 min. One microgram of DNase I-treated total RNA was used for cDNA first-strand synthesis using the Superscript III RT Pre-amplification System (Invitrogen) and the $VT_{25}$ primer. Complementary DNA amplification by PCR was performed using the primers EPO-F and EPO-R, which are specific to the whole EPO coding sequence (582 bp), as well as RT5.1 (5′-CATGTGGA-TAAAGCCGTCAG-3′) and RT3.1 (5′-AGCAGTGATTGTTCGGAGTG-3′), designed to amplify an internal 126-bp region of the EPO gene. Additionally, a cDNA control fragment of 125 bp specific to the tobacco tubulin gene was also amplified with the primers TUB-F (5′-GCAAGTACATGGCTTGCTGT-3′) and TUB-R (5′-CACACTTGAATCCTGTTGGG-3′).

## Quantitative RT–PCR

Quantitative reverse-transcription PCR (RT–qPCR) was carried out in an Applied Biosystems 7500 real-time cycler. The reaction settings consisted of an initial denaturation step of 5 min at 94 °C followed by 45 cycles of 10 s at 94 °C, 15 s at 60 °C, and 15 s at 72 °C. The samples were kept for 2 min at 40 °C for reannealing and then heated from 55 to 99 °C with a ramp of 0.1 °C/s to acquire data to produce the denaturing curve of the amplified products. RT–qPCRs were performed in final volumes of 20 μL composed of 10 μL of each RT sample diluted 50 times in 2 μL of 10× Platinum Taq PCR buffer (Invitrogen), 1.2 μL of 50 mM $MgCl_2$, 0.4 μL of 5 mM dNTPs, 0.4 μL of primer pairs at 10 μM each, 4.95 μL ultrapure water, 1.0 μL of SYBR-Green (1:100,000; Molecular Probes), and 0.05 μL of Platinum Taq DNA Polymerase (Invitrogen). The *N. tabacum* tubulin fragment was used as internal control to normalize the amount of mRNA present in each sample, using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [21].

### SDS–PAGE and Western Blotting

Leaf total proteins were prepared according to Jefferson and Wilson [22] with the following modifications: protein samples were extracted with 0.1 M sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% (w/v) Sarcosyl, 0.1% (v/v) Triton X-100, and 10 mM β-mercaptoethanol. After centrifugation (10 min at 13,000 rpm), the supernatant was collected and dialyzed against 100 mM sodium phosphate buffer, pH 7.0 exceeding 100 times the sample volume. Total protein was determined using the BCA Protein Assay Kit (Pierce). Twenty micrograms of protein was separated by denaturing (SDS) 12% polyacrylamide gel electrophoresis (PAGE). Another 20 μg of total protein and 2.5 ng of recombinant EPO were resolved in a second SDS–PAGE and transferred to a PVDF membrane (GE Healthcare) by gravity [23]. The membrane was blocked with 5% skim milk dissolved in 1× PBS. The membrane was incubated with an anti-EPO antibody (1:10,000) and a secondary antibody (1:5,000) in TPBS. Finally, the membrane was developed essentially according to the specifications of the ECL Western Blot Detection and Analysis System (GE Healthcare) and exposed to an X-ray film.

### Molecular and Genetic Analysis of $T_1$ Plants

Control and EPO-transformed tobacco plants were self-pollinated, and their seeds were surface-sterilized with 70% ethanol for 1 min and 1% hypochlorite for 20 min, followed by three washes with sterile distilled water. Seed germination and viability were evaluated in triplicate by incubation of 100 seeds in Petri dishes containing MS medium with or without 10 mg/L Basta for 1 month in a culture room with a photoperiod of 16 h at 25 °C. Viable $T_1$ (or $F_1$ control) plants were transferred to individual jars containing MS medium and 10 mg/L Basta. $T_1$ (and $F_1$) plant leaves, stems, and roots were analyzed by PCR and qPCR to determine the presence of the EPO transgene and mRNA.

## Results

### Synthesis and Cloning of a Human Erythropoietin cDNA

Based on the *Homo sapiens* EPO gene sequence available at GenBank (NM 000799 and NM 000799.2), a cDNA version was synthesized employing a nucleotide overlapping technique developed by our group [16]. One final PCR product of 582 bp was generated as designed and cloned into pCR-Blunt (Invitrogen; Fig. 1a). The resulting full coding sequence of the human EPO cDNA was submitted to automatic sequencing in order to confirm its integrity and fidelity. In this process, a single mutation at nucleotide 252 (G→A) was identified, but this modification (GGG→GGA) did not change the encoded amino acid (glycine), and so the identity of the sequence was confirmed.

The corresponding synthetic EPO cDNA was then transferred from pCR-Blunt to the vector pWUbi.tm1 [17] in order to adapt it to an expression cassette consisting of the promoter of a maize ubiquitin gene with its first intron and the terminator *tm1'*, which is part of the *Agrobacterium* tumor-morphology large gene. This expression cassette was transferred into the binary vector pWBVec4a [17] between the right and left borders of the T-DNA (Fig. 2). As shown in Fig. 1b, c, the cloning of the EPO cDNA into the expression cassette and the transfer of the complete cassette into the binary vector were shown be correct by endonuclease digestion followed by agarose gel electrophoresis. The binary

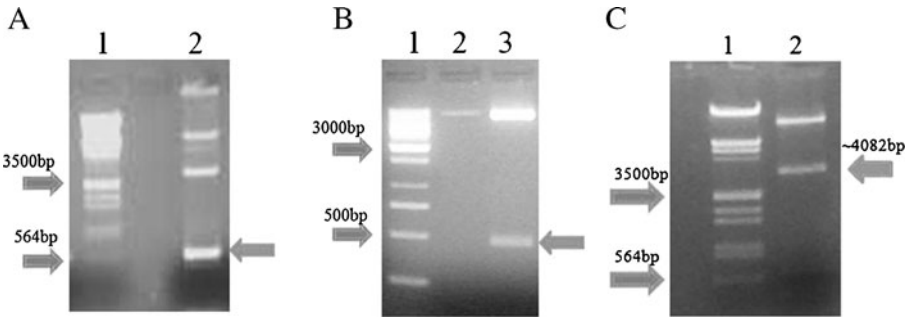


**Fig. 1** Cloning of a PCR-based synthetic human EPO cDNA. **a** *Lane 1* λ DNA cleaved with *Eco*RI/*Hin*dIII as a molecular size marker, *lane 2* pCR-Blunt::HsEPO exhibited the expected 582-bp fragment corresponding to the HsEPO cDNA. **b** *Lane 1* GeneRuler 1-kb DNA Ladder (Fermentas) as a molecular size marker, *lane 2* pWUbi-tm1′, *lane 3* pWUbi-tm1′::HsEPO exhibited the expected fragment corresponding to the HsEPO cDNA. **c** *Lane 1* λ DNA cleaved with *Eco*RI/*Hin*dIII as a molecular size marker, *lane 2* pWBVec4a-HsEPO exhibited the expected 4,082-bp fragment corresponding to the $P_{ubi}$-HsEPO-$T_{tm1'}$ expression cassette

vector pWBVec4a contains the phosphinothricin (PPT) acetyltransferase gene (*pat*) from *Actinomyces coelicolor* under control of the 35S CaMV promoter and the *tm1′* terminator as a selectable marker. This selectable marker cassette is capable of conferring tolerance to PPT and herbicides derived from it like Basta (Fig. 2). Finally, the resulting recombinant binary plasmid was introduced into *A. tumefaciens* LBA4404 in order to transform tobacco (*N. tabacum*) plants.

## Generation of Transgenic Tobacco Plants Containing EPO and DNA Analysis

Using the traditional tobacco leaf disc transformation method [19] with minor modifications, only two transgenic tobacco plants were recovered after transformation and selection under 10 mg/L Basta. Considering that 100 leaf discs were initially treated with *A. tumefaciens*, this efficiency of 2% was considered very low when compared to our previous transformation efficiencies of up to 45%. In those cases, we employed binary vectors containing the kanamycin resistance gene (*npt*II) like pMOG402 [24] and pCAMBIA2300 [25].

Primary PCR analysis of the two regenerated tobacco plants confirmed their transgenic status because a band of approximately 600 bp was generated with EPO-specific 5′- and 3′-flanking primers (EPO-F and EPO-R), and no band was obtained when DNA from wild-type untransformed tobacco plants was assayed (Fig. 3).

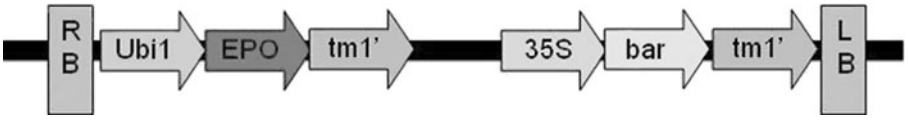


**Fig. 2** Schematic representation of the T-DNA of the pWBVec4a-HsEPO binary vector. The HsEPO (EPO) cDNA was cloned under the control of the ubiquitin promoter (Ubi1) and the *tm1′* terminator (tm1′). The phosphinothricin acetyltransferase gene (*bar*) under the control of the 35S CaMV promoter (35S) and the *tm1′* terminator (tm1′) is the selectable marker cassette. Both constructions are between the right (RB) and left (LB) borders of the T-DNA. *Boxes* and *arrows* are not drawn to scale

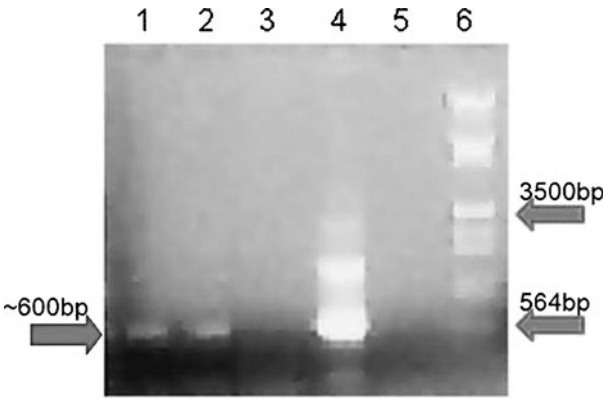


**Fig. 3** PCR analysis of EPO-transgenic and wild-type tobacco plants. The image presents the result of agarose gel electrophoresis where products of PCR employing the EPO-F and EPO-R flanking primers were resolved. *Lanes 1* and *2* EPO-transgenic plants 1 and 2, *lane 3* wild-type tobacco (negative control), *lane 4* pCR-Blunt::EPO (positive control), *lane 5* PCR mix without template DNA (negative control), *lane 6* λ DNA cleaved with *Eco*RI/*Hin*dIII as a molecular size marker. DNA samples derived from transgenic plants and pCR-Blunt::EPO exhibited the expected 582-bp fragment corresponding to the synthetic EPO cDNA

## RNA and Protein Expression of Transgenic Tobacco Plants Containing the Synthetic EPO cDNA

The transcriptional expression of the EPO transgene in tobacco plants was confirmed by RT–PCR and RT–qPCR. The amplified cDNA yielded an EPO PCR product of the expected size (approximately 600 bp) when assayed using the two 5′- and 3′-flanking primers (Fig. 4a) and a product of 125 bp when using internal primers. Electrophoretic analysis of the RT–PCR products revealed that, by visual observation, transgenic tobacco plant "2" accumulated about twice as much EPO mRNA as did plant "1" (Fig. 4a). In order to confirm this observation, quantitative (real-time) RT–PCR was carried out using the pair of internal primers. The results in Fig. 4b show that plant "2" presented 1.85 times more EPO mRNA than plant "1".

The transgenic tobacco plants were also tested for the presence of the EPO protein by SDS–PAGE and western blot. After numerous SDS–PAGE assays employing different amounts of total protein extract resulting from different extraction procedures, it was not possible to clearly visualize distinct bands of around 20–30 kDa in the transformed or wild-type plants (results not shown). These results indicate that the EPO protein, if present, was likely at very low concentrations in the leaf, stem, and root tissues of the transgenic tobacco plants. Nevertheless, the use of an anti-EPO antibody in western blot assays allowed us to visualize a 30-kDa band that corresponded to that of a commercially available positive control EPO protein (Fig. 5). Interestingly, the tobacco-derived EPO seemed to be heavier than the control sample, with a band at approximately 40 kDa.

## Morphology and Viability of HsEPO-Transgenic Tobacco Plants

Earlier attempts to express the human erythropoietin (HsEPO) gene in plants resulted in either abnormal phenotypes or infertility [15]. The transgenic tobacco plants generated in the present work and kept *in vitro*, as well as plants transferred and maintained in soil in a greenhouse, presented no morphological differences from control plants of the same age

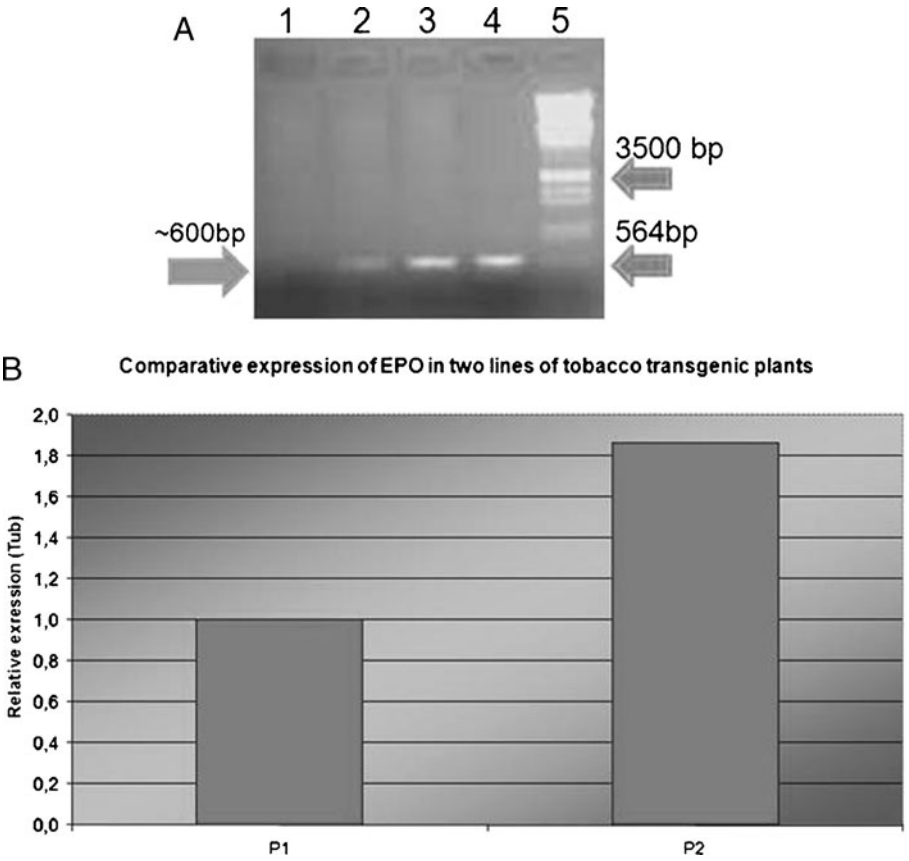


**Fig. 4** Evaluation of recombinant EPO gene expression in plants by PCR. **a** Analysis of EPO-transgenic and control tobacco plants by conventional RT–PCR. Transgenic-derived mRNA samples allowed the amplification of the ~600-bp fragment corresponding to the synthetic EPO cDNA. *Lane 1* wild-type tobacco (negative control), *lanes 2* and *3* transgenic plants 1 and 2, respectively, *lane 4* pWBVec4a-HsEPO (positive control), *lane 5* λ/*Eco*RI/*Hin*dIII molecular marker. **b** Quantitative (real-time) RT–PCR analysis of relative EPO expression with total RNA isolated from transgenic and control tobacco plants. EPO expression values were normalized with values obtained for an internal control gene (tubulin) and values obtained for control untransformed tobacco plants. To compare the levels of expression of the two plants, the expression value obtained for plant "1" was set to 1. All data are presented as the mean of three technical replicates

grown under identical conditions (Fig. 6a). The HsEPO transgenic plants blossomed at the same time as wild-type tobacco with normally appearing visible floral structures (Fig. 6b, c). They were allowed to self-fertilize and set seeds like wild-type plants. A sample of 12 individual $T_1$ progeny from each transgenic plant was grown on selective MS medium containing Basta. Only two plants from each transgenic line were recessive for the *ppt* transgene. This result was also confirmed by detection of the HsEPO cDNA by PCR and the mRNA by qRT–PCR (Fig. 7).

Plants 1 and 2 were self-fertilized, and the seeds were germinated in MS medium to test the efficiency of germination and to analyze the F1 generation (Table 1). For each line, 90 seeds were allowed to germinate. A, B, and C represent different experiments. Ninety-seven percent of the seeds from the control plants germinated, while 86% from plant 1 germinated and 62% from plant 2 germinated. When Basta was added to the

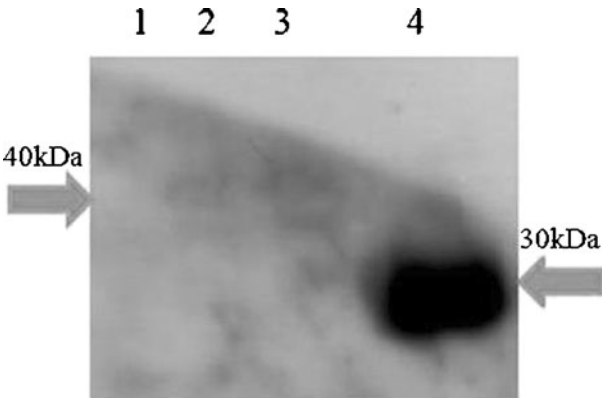


**Fig. 5** Detection of the EPO protein in transgenic and control tobacco plants. Western blot analysis of transgenic and control tobacco plants. Twenty micrograms of total protein extracted from leaves of wild-type tobacco (*lane 1*) and from transgenic lines 1 (*lane 2*) and 2 (*lane 3*); and commercial EPO (*lane 4*, positive control). Faint bands corresponding to the synthetic EPO protein are visible in *lanes 2* and *3* with sizes of ~40 kDa. Positive control EPO showed the expected size of ~30 kDa (*lane 4*)

medium, the efficiency of germination declined for plants 1 and 2 by 76% and 32%, respectively.

Only three plants did not show segregation of the gene for erythropoietin, and all plants positive for the presence of the gene showed some level of expression.

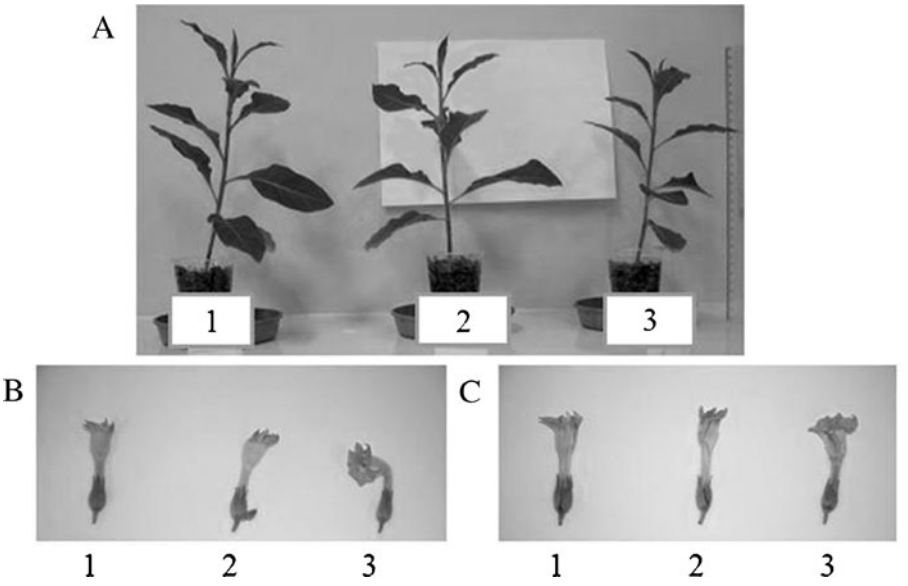


**Fig. 6** Plants and flowers from transgenic tobacco lines expressing HsEPO and a wild-type line. *1* and *2* EPO-transgenic plants 1 and 2, *3* wild-type tobacco (negative control). **a** Two-month-old plants kept in soil in a greenhouse. The overall phenotypes of the transgenic and untransformed plants were indistinguishable. **b** Entire and **c** longitudinally cut flowers showed no clear anatomical or morphological modifications

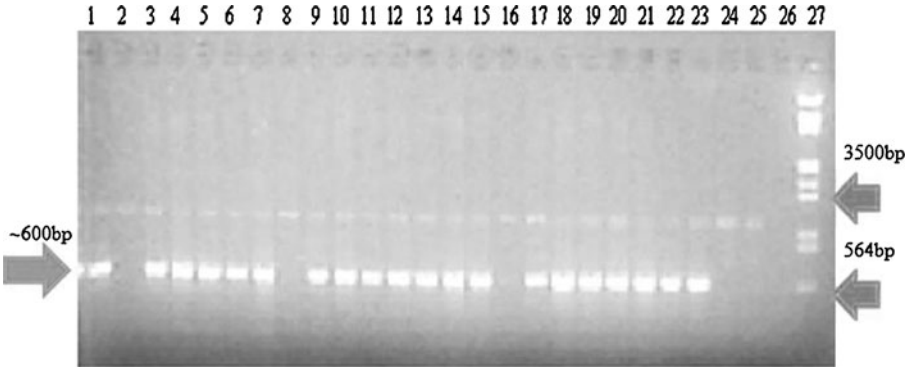


**Fig. 7** PCR analysis of $T_1$ and $F_1$ tobacco plants. Agarose gel electrophoresis of PCR products employing the HsEPO 5′- and 3′-flanking primers. *Lanes 1–12* $T_1$ samples derived from the self-pollination of transgenic plant "1", *lanes 13–23* $T_1$ samples derived from the self-pollination of transgenic plant "2", *lanes 24–26* $F_1$ samples of wild-type tobacco, *lane 27* λ *Eco*RI/*Hin*dIII molecular size marker. Transgenic samples exhibited the 582-bp fragment corresponding to the HsEPO cDNA

In preliminary results, the descendants of plant 1 designated plants 1/1 and 1/10 showed high levels of expression of the erythropoietin gene by real-time PCR, as did the descendants of plant 2 designated plants 2/10 and 2/11 (results not shown).

## Discussion

The production of recombinant proteins in plants presents several advantages over other methods. It is estimated that the cost of recombinant protein production in plants may be 10 to 50 times lower than producing the same protein in *E. coli* [26]. Classic methods of protein expression require significant investments in the purification steps required for large amounts of recombinant protein and may require expensive cell or tissue culture media [27]. The expression of genes in animal cell culture allows the correct synthesis of human and other mammalian products. However, in addition to being an expensive mode of production, the products and cells in animal cell culture are very sensitive to ambient changes, especially when cultivated on an industrial scale [28]. By contrast, transgenic plants can produce high levels of functional and safe recombinant protein, and their cultivation on an agricultural scale requires only the transgenic plant, water, minerals, and sunlight [27], offering advantages over all other previously established expression systems in terms of capacity, flexibility, scale, and cost of production. Furthermore, the area where

**Table 1** Efficiency of germination

| Medium | Descendants of | No. of seeds | A (30 seeds) | B (30 seeds) | C (30 seeds) | Media | Standard deviation | Total germination | Efficiency % |
|---|---|---|---|---|---|---|---|---|---|
| Without Basta | Control | 90 | 28 | 30 | 30 | 29.33 | 1.15 | 88 | 97 |
| | 1 | 90 | 26 | 28 | 24 | 26 | 2 | 78 | 86 |
| | 2 | 90 | 17 | 21 | 18 | 22 | 2.08 | 56 | 62 |
| With 40 mg/L Basta | Control | 90 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1 | 90 | 23 | 25 | 21 | 23 | 2 | 69 | 76 |
| | 2 | 90 | 12 | 10 | 7 | 9.66 | 2.51 | 29 | 32 |

transgenic plants are planted can be adapted and altered yearly according to the demand for product without additional costs [29].

Since the first experiments by Jackson et al. [30] and by Cohen et al. [31] proving the feasibility of heterologous protein expression, scientists have struggled with the dream of producing high-value medicines in the most straightforward way possible. Human genes encoding pharmacologically active peptides are among these valuable medicines, and plants, essentially because they depend on sun and water, are the lowest-cost vectors for genetic engineering reviewed by [26, 32, 33]. After 36 years of continuous research, it is surprising that only a few recombinant human proteins produced by transgenic plants are commercially available (see 29, 34]. The reasons for this poor success rate range from basic difficulties concerning reliable expression systems to biosafety and regulatory policies regarding the cultivation, sale, and consumption of genetically modified organisms and their derivatives. Nevertheless, confident that genetic engineering will also prove itself in the area of molecular farming, we tested the abilities of transgenic *N. tabacum* (tobacco) and *Oryza sativa* (rice) plants to express a cDNA sequence encoding the human erythropoietin (HsEPO) peptide.

Although HsEPO-transgenic rice plants could not be unambiguously regenerated and kept in culture, we successfully generated two transgenic lines of tobacco containing the HsEPO cDNA integrated into their genomes. The expression of HsEPO in a transgenic plant system has been previously published. In 1995, Matsumoto et al. [14] generated BY2-tobacco cells in suspension producing the EPO peptide at up to 0.0026% of total protein. That recombinant EPO was found to have activity only *in vitro*. According to the authors, no plants were regenerated from those cells, which prevents us from drawing further comparisons with our work. To our knowledge, after 1995 only one single paper, published by Cheon et al. in 2004 [15], described EPO gene expression in transgenic plants. In that work, Cheon et al. [15] were able to transform and regenerate *Arabidopsis* and tobacco plants containing a HsEPO cDNA. Unfortunately, all $T_0$ plants showed malformations and male sterility. It was shown in that study that overexpression of EPO causes pleiotropic effects in plants including vegetative growth retardation, arrangement of leaves in rosettes, bloom slowing, deformed flower buds, and sterility. Details of the causes of these abnormal phenotypes were not specified.

Unlike those in the previously mentioned work, the plants we obtained did not show any malformations when compared to wild-type plants, neither in the two $T_0$ tobacco lines nor in the two $T_1$ progeny. Vegetative growth and overall leaf, stem, leaf, root, and flower organ anatomy were identical to those of control plants. Additionally, our transgenic and control plants flourished, set seeds after self-fertilization, and generated viable descendants, showing that they were perfectly fertile. Seed germination rates were similar for all the plants, and the fertilization rate of the transgenic plants was even higher than that of control plants when the number of fruits and seeds per fruit were counted (results not shown).

Our failure to obtain HsEPO-transgenic rice plants and the low efficiency of our tobacco plant transformation (two plants out of 100 leaf discs) might be due to some negative effect of the HsEPO gene product on plant cells. In addition to the results presented here, two other transformation attempts were performed in our laboratory with 200 tobacco leaf discs in total, and not a single plant was regenerated under Basta selection (results not shown). As mentioned before, such low efficiency is not normal in our experience. When selecting transgenic events, only those plantlets with clear healthy phenotypes were collected from Petri dishes, and therefore, we believe that the HsEPO protein has a negative effect on plant cells, as previously described by Cheon et al. [15]. This was later corroborated by the

observation that HsEPO was at such a low level in the leaves of the two transgenic lines obtained that it was only faintly detectable even with anti-HsEPO antibodies.

Some differences between the work done by Cheon et al. [15] and ours are worth mentioning. First, we used a different version of the HsEPO cDNA that contained a silent point mutation. Both sequences are available at GenBank under the accession numbers NM_000799 and NM_000799.2 [gi:62240996]. Second, we worked with different varieties of tobacco. While we used *N. tabacum* SR1 Little Havana, Cheon et al. [15] used the Xanthi variety. However, the most important difference was the gene constructs used. Our construct contained the ubiquitin (*ubi*) promoter and the *tm1′* terminator from maize controlling the expression of the HsEPO cDNA. The maize *ubi* 5′-sequence is known to be a very strong promoter in monocotyledonous (monocot) cells, but less effective in dicotyledonous (dicot) cells [35–42]. We used this promoter to control the expression of the HsEPO cDNA in rice (a monocot) and tobacco (a dicot). Again, this may explain (at least in part) why we obtained two normal tobacco plants expressing HsEPO mRNA and its protein but no such rice plants. The strong expression of EPO in rice tissues might have resulted in toxic effects and prevented the regeneration of viable plants. Cheon et al. [15] used the cauliflower mosaic virus (CaMV) 35S promoter to drive HsEPO cDNA expression, which is a promoter for high-level transcription in dicot plants [26]. In both dicots tested (*Arabidopsis* and tobacco) with this high level of expression, they obtained plants with morphologically affected phenotypes that were sterile.

Although not always comparable due to differences in many other variables involved, the scientific literature is rich in examples of works employing both the *ubi* and 35S promoters. For instance, Upadhyaya et al. [40] demonstrated that the Ubi-1 promoter produces 30-fold higher β-glucuronidase (GUS) activity in transgenic rice plants than the 35S promoter. In the optimization of sorghum transformation parameters using genes encoding green fluorescent protein (GFP) and the GUS protein as visual markers, Jeoung et al. [41] also tested the *ubi* and 35S promoters, showing that for both reporter genes, the expression driven by the *ubi* promoter was higher than the expression driven by the 35S 5′-end sequence. These works show the important of the choice of promoters. According to Ma et al. [26], the advantages of strong promoters for driving gene expression in transgenic plants include increased stability of the encoded protein and protein accumulation in vegetative organs. This is true for non-toxic proteins, but it seems not to be the case for the HsEPO peptide.

One of the future prospects for this work will be to introduce into the construct containing the EPO gene a seed-specific promoter such as the zein promoter of maize, which demonstrably leads to gene expression only in the grain [43, 44]. We also want to find ways to increase the percentage of recombinant protein produced by testing new constructs or even by trying to determine whether some type of post-transcriptional or post-translational silencing can be reversed.

The objectives of this study were achieved by proving that it is possible to obtain plants expressing EPO with normal morphology and reproductive capability. We believe that the level of recombinant protein production can be improved not only for EPO but also for various other human proteins expressed in plants, opening new prospects for the large-scale production of human proteins of pharmacological interest.

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