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Expression of Recombinant Erythropoietin Gene in Transgenic Tobacco Plant

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형질전환 담배 식물체에서 재조합 erythropoietin 유전자의 발현

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Erythropoietin (EPO) is a glycoprotein that mediates the growth and differentiation of erythroid progenitors. In order to produce recombinant human erythropoietin in tobacco plant, the EPO genomic DNA (5.4 kb) was cloned into plant expression vectors, pBI△GUS121, pBD△GUS121 and pPEV-1, and introduced in *Nicotiana tabacum* (var. Xanthi) via *Agrobacterium tumefaciens*-mediated transformation. After selection on MS media containing kanamycin (Km), 10 Km-resistant plants were obtained per each construct. The correct integration of EPO genomic DNA in the genome of transgenic plant was confirmed by polymerase chain reaction (PCR). Northern blot showed that transcripts of 1.8 kb length were produced in leaves of the plants, but there was no difference of mRNA amount according to promoter number and 5'-untranslated sequence (UTS). The proteins obtained from leaves of transgenic plants were immunologically detected by Western blot using rabbit anti-human EPO polyclonal antibody. The expressed protein appeared as smaller band of apparent mass of 30 kDa as compared to the EPO protein from human urine (37 kDa), suggesting that the modification (glycosylation) system in tobacco plant might be different from that of mammalian cells.

Key words: kanamycin-resistant, integration, mRNA amount, glycosylation

INTRODUCTION

With the advent of plant genetic engineering, production of foreign proteins in transgenic plants is rapidly becoming general technology for a variety of plant species including many economically important crops (Gasser and Fraley, 1989). The availability of gene transfer system has already led to several important insights into the regulation of gene expression and protein function in plants. Combined with efficient expression system for the introduced genes, gene transfer could turn plants into a suitable alternative for the production of desirable products. Plants have been exploited either for the production of pharmaceutical protein such as human serum albumin (Sijmons et al., 1990), surface antigen of hepatitis B virus (Mason et al., 1992: Thanavala et al., 1995), immunogloblin (During et al., 1990: Fielder and Conrad, 1995), and industrial enzyme (Pen et al., 1992), or for the overproduction, derivatization, and bioconversion of compounds that naturally occur in the host plant.

Erythropoietin (EPO) is a haemopoietic hormone specific to cells of erythroid lineage and mediates the growth and differentiation of erythroid progenitors through its interaction with a specific receptor (Krantz, 1991). The hormone is produced primarily by the kidney in the adult and by the liver during fetal life (Fried, 1972: Zanjani et al., 1981). The nucleotide sequence of the human erythropoietin gene was reported (Kenneth et al., 1985) and the biologically active protein was expressed in chinese hamster ovary cell (CHO)

with the erythropoietin gene linked to an expression vector system (Lin et al., 1985). EPO is a glycoprotein of MW 37 kDa composed of mature protein of 166 amino acids and signal peptide of 27 amino acids, and is characterized by its large carbohydrate chains, which occupy close to 40% of its total mass. When the human erythropoietin gene is introduced into host cells such as insect cell (Quelle et al., 1989), bacteria (Lee-Huang, 1984), or yeast (Elliott et al., 1989), the recombinant products lack carbohydrate moieties present in the native human hormone. The erythropoietin molecule contains four carbohydrate chains, three N-linked saccharide (to asparagines at positions 24, 38, and 83) and one O-linked saccharide (to a serine at position 126) (Sasaki et al., 1987). The importance of the carbohydrate portion of EPO is suggested by the conservation of the glycosylation sites in humans, monkeys, and mice. Site directed mutagenesis of the carbohydrate attachment sites in position 38, 83, and 126 resulted in lowered secretion of EPO in transfected baby hamster kidney cell cultures (Dube et al., 1988). It was also reported that in vivo, the carbohydrate moiety is responsible for the metabolic fate of the hormone (Tsuda et al., 1990) and N-glycosylation is essential for full biological activity in vivo, but O-glycosylation does not appear to be required for in vivo biological activity (Delorme et al., 1992). Therefore, these sugar moieties were thought to be important for the biological activity of EPO protein.

In this study, to explore the possibility of therapeutically using recombinant mammalian proteins produced in plants, we constructed plant expression vector containing EPO genomic DNA under the control of CaMV 35S promoter and investigated the differences of expression level of the protein according to the change of promoter number and different 5'-UTS.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids

λhE1 clone (Lin et al., 1985) was obtained from ATCC and used as a source of EPO genomic DNA. λhE1 phage plaques were amplified in *E. coli* LE392 according to a laboratory manual of Molecular Cloning (Sambrook et al., 1989). The binary vectors, pBI121, pBD121, and pPEV-1 were purchased from Clontech (USA) and used to construct EPO expression vectors, pBIGEP, pBDGEP, and pEVGEP, which were screened in *E. coli* DH5α grown in Luria-Bertani (LB) medium (10 g of bactotrypton, 5 g of yeast extract, and 10 g of NaCl per 1 liter) supplemented with 50 μg/mL of kanamycin. The resulting plasmids were then mobilized to *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) by the freeze-thaw method (Holster et al., 1978) and selected in YEP medium (10 g of bactopeptone, 10 g of yeast extract, and 5 g of NaCl per 1 liter) containing 5 μg/mL of tetracycline and 10 μg/mL of kanamycin.

Materials

All restriction enzymes and DNA modifying enzymes were purchased from POSCOCHEM and Boeringer-Mannheim. [α-^{32}P]dCTP (3,000 Ci/mmole) was obtained from Amersham and Prime-a-Gene Labeling system from Promega. Rabbit anti-human EPO polyclonal antibody was obtained from GENZYME. Alkaline phosphatase- conjugated second antibody, BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitroblue tetrazolium) were purchased from PIERCE. Oligonucleotides were synthesized in Korea Biotech. Inc. All other materials were reagent grade and obtained from commercial source.

Plant transformation

From colonies of *A. tumefaciens*, plasmid DNAs were prepared and confirmed by restriction pattern in agarose gel electrophoresis after digesting with *Pst*I. Leaf discs of *Nicotiana tabacum* (var. Xanthi) were infected with *Agrobacterium tumefaciens* harboring co-integrated Ti vectors by the method of Horsch et al. (1985). Plant transformants were selected on MS (Murashige and Skoog, 1962) agar medium supplemented with 500 mg/L carbenicillin, 100 mg/L kanamycin, 0.5 mg/L NAA, and 2 mg/L BAP (McCormick et al., 1986). Regenerated plantlets obtained from hormone-free MS medium with 100 mg/L of kanamycin were checked for correct integration of the constructs in the tobacco genome by polymerase chain reaction.

DNA analysis by PCR

Genomic DNA was prepared according to the method of Murray and Thompson (1980). The presence of the recombinant gene in regenerated tobacco plants was confirmed using PCR with the primers 5'-GGGGAATTCAAGGAAGC CATC-3'and 5'-GGGGATCCCTGGACAGTTCCTCT-3' specific for the EPO gene. DNA fragments were amplified in

a total volume of 50 μl with 10 mM Tris-HCl, pH 8.3, 40 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.01% gelatin, 1.5 μM each specific primer, and 2.5 units of Taq polymerase (Korea Biotech.). PCR condition was : one cycle at 95°C 5 min/40°C 30 s/72°C 2 min, and following 29 cycles at 95°C 1 min/40°C 30 s/72°C 2 min, and finally 72°C 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized after staining with ethidium bromide.

Northern blot analysis

Total RNA was extracted from leaves of tobacco by Phenol-SDS method (Draper et al., 1988). Isolated total RNA was denatured, electrophoresed in 1.0% agarose-formaldehyde gel and transferred to GeneScreen-plus membrane (DuPont, New England Nuclear) according to a laboratory manual of Molecular Cloning (Sambrook et al., 1989). The amounts of total RNA loaded were normalized with those of ribosomal RNA. The membrane was hybridized at 42°C in 2 × PIPES (0.8 M NaCl and 20 mM PIPES, pH 6.5), 50% deionized formamide, 0.5% SDS, and 100 μg/mL denatured salmon sperm DNA using ^{32}P-labeled EPO partial cDNA as a probe. The final washing was carried out in 0.1 × SSC (15 mM NaCl and 1.5 mM sodium citrate, pH 7.0) and 0.1% SDS at 60°C for 15 min. The filter was visualized by autoradiography.

Protein Gel Blot Analysis

The protein extraction procedure was done as described by Hurkman and Tanaka (1986). Leaves were cut from the plant, immediately frozen in liquid nitrogen, and stored at -70°C until use. Soluble proteins were extracted from 500 mg of leaf tissues. The tissues were homogenized on ice with a Polytron homogenizer in three volumes of 10 mM sodium phosphate buffer (pH 6.0) and 1% sodium bisulfite as a reducing agent. The homogenate was centrifuged at 14,000 × g for 10 min. The protein content of leaf tissue was measured as described by Bradford (1976). Prior to fractionation on SDS-PAGE, protein was denatured at 100°C for 5 min in a solution containing 100 mM dithiothreitol, 2% SDS, 50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and 0.1% bromphenol blue. Electrophoresis was performed at 20 mA in 12% polyacrylamide gel containing 0.1% SDS (Laemmli, 1970) and electroblotted to Immobilon-P membrane (Millipore, Bedford, MA). Blots were blocked for 1 hr in phosphate buffered saline (PBS) containing 3% skim milk and incubated with polyclonal anti-EPO antibody (1:1,000 dilution) for 1 hr. After washing unbound primary antibodies with washing buffer (PBS + 0.5% Triton X-100) three times for 10 min each, the blot was treated with alkaline phosphatase-conjugated goat anti rabbit antiserum and developed with BCIP/NBT substrate solution.

RESULTS AND DISCUSSION

Amplification and Subcloning of λHE1 clone containing EPO genomic DNA

To amplify EPO genomic DNA, *E. coli* LE392 cell was grown in LB broth containing 0.2% maltose and 10 mM $MgCl_2$. When the optical density of cells reached to 0.5 at A_{600}, cells were harvested, suspended in 10 mM $MgCl_2$, infected with λHE1 lysates, and induced to confluent lysis. The bacteriophage particles were pooled with SM buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin) and λDNA containing genomic EPO gene was prepared using Wizard Lambda purification system (Promega). To confirm whether this is a real clone, restriction pattern was analyzed by 0.8% agarose gel electrophoresis after digesting the λDNA with various enzymes (data not shown). To handle DNA manipulation easily, λHE1 DNA was digested with *Hin*dIII and *Bam*HI. The 5.4 kb fragment was eluted and subcloned in pBlue-SK vector digested with the same enzymes. The constructed pBlue-SK:gEPO plasmid was used as a source of genomic DNA.

Construction of Plant Expression Vector Containing the EPO Genomic DNA

To remove the β-glucuronidase (GUS) gene, pBI121 and pBD121 vectors were digested with *Sma*I and *Sst*I, treated with T4 DNA polymerase to remove overhanging ends and self-ligated to form pBIΔGUS121 and pBDΔGUS121 containing a unique *Bam*HI site between CaMV 35S promoter and nopaline (NOS) synthase termination site. Using pBIΔGUS121, pBDΔGUS121, and pPEV-1 vectors, we made three chimeric expression constructs containing the EPO genomic DNA. Vector preparation was done with *Bam*HI digestion and followed by filling-in with Klenow enzyme. The EPO genomic DNA was prepared from pBlue-SK:gEPO DNA as follows. The plasmid was digested with

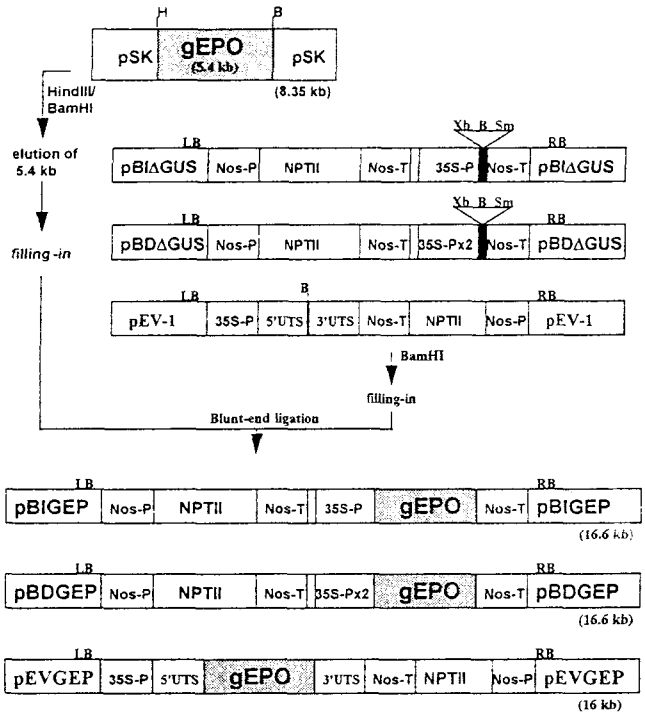

Figure 1. Diagrammatic representation of the construction of expression plasmids, pBIGEP, pBDGEP, and pEVGEP : Ap^r, ampicillin resistance gene: ori, DNA replication origin: gEPO, genomic EPO DNA: Nos-P, nopaline synthase promoter: Nos-T, nopaline synthase gene terminator: CaMV 35S-P, Cauliflower mosaic virus 35S promoter: NPTII, neomycin phosphotransferase II gene: LB, left border: RB, right border: 5'-UTS, 5'-untranslated sequence: 3'-UTS, 3'-untranslated sequence: B, *Bam*HI: H, *Hin*dIII: Sm, *Sma*I: Xb, *Xba*I.

*Bam*HI and *Hin*dIII, 5.4 kb fragment containing EPO genomic DNA was eluted, and the sticky ends were blunted with large fragment of DNA polymerase I. And then, the insert DNA was ligated with the vector described as above (Figure 1). After confirming the orientation by restriction pattern, real clones were obtained and named pBIGEP, pBDGEP, and pEVGEP expression plasmids, respectively.

Plant Transformation and Analysis of the EPO Genomic DNA

The constructed expression vectors have the EPO genomic DNA, cauliflower mosaic virus (CaMV) 35S promoter (one in pBIGEP or two in pBDGEP) including 5'-UTS and 3'-UTS (pEVGEP), the neomycin phosphotransferase II (NPT II) gene conferring kanamycin resistance upon the transformed tobacco cells, nopaline synthase (NOS) terminator, and the sequences of left and right border. The plasmids were introduced into *Agrobacterium tumefaciens*

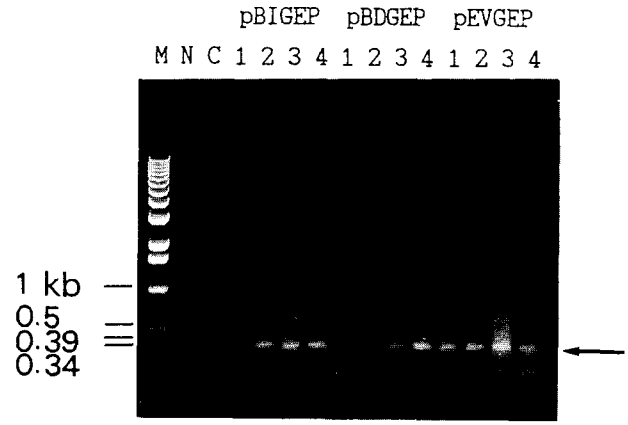

Figure 2. Identification of the integrated genomic EPO DNA by polymerase chain reaction. The genomic DNAs (0.5 μg) were amplified by PCR and the products were fractionated on 1% agarose gel. The arrow indicates a part of EPO DNA (330 bp length) integrated into plant genome. Numbers above lanes indicate the clone number of transformants with each construct: M, 1 kb DNA size marker: N, negative control (no template): C, nontransformed plant.

strain LBA4404 that contains the vir-helper plasmid. Leaf discs of *Nicotiana tabacum* (var. Xanthi) treated with the *Agrobacterium* strain were maintained on selective medium to promote the growth of kanamycin resistant transgenic callus tissue. We obtained about 10 kanamycin resistant calli per each plasmid construct. The transformed transgenic calli had a normal appearance and similar growth characteristics as the control calli, and kanamycin resistant plants were grown to maturity along with nontransformed plants obtained by leaf disc culture. No morphological differences were observed between the transformed and nontransformed plant. Four of the 10 transgenic plants per each construct were studied. The cell extracts from these leaves were prepared to determine the introduction of EPO genomic DNA into tobacco genome by PCR and the expression of EPO protein by Western blot analysis.

To determine the correct integration of the constructs in the tobacco genome, we checked the tobacco genomic DNAs by PCR using specific primers span from amino acid 116 to 166. As shown in Figure 2, the DNA fragments of 330 bp size were identified in transgenic plants containing EPO genomic DNA. However, another bands appeared in the range of 600-700 bp size in all samples including nontransformed plant might be considered as a nonspecifically amplified DNA fragment. All of the analyzed clones have the EPO genomic DNA, whereas there was no detectable DNA fragment in nontransformed plant.

Northern Blot Analysis

The steady-state level of EPO mRNA in wild type and transgenic plants of *N. tabacum* was analyzed by RNA gel blot. Thirty microgram of total RNA was subjected to agarose-formaldehyde gel electrophoresis and transferred to a GeneScreen-plus filter. The amounts of total RNA loaded were normalized with those of ribosomal RNA. The filter was hybridized with the EPO partial cDNA clone. A typical blot from such an experiment is shown in Figure 3. Transcripts of 1.8 kb length were detected in all transgenic plants integrated with each construct, indicating that mRNAs of the proper size were transcribed from the chimeric constructs and accumulated in the transformed plants. However, there was no difference in the amount of EPO transcript according to promoter number and 5'-UTS, but different levels of the EPO transcript were detected in transgenic plants containing same construct. In #3 transgenic plant of pBIGEP and #1 transgenic plant of pBDGEP construct, a two-fold to three-fold increase in the EPO transcript was observed, whereas the rest of plants showed a similar pattern. The same phenomena of different levels of mRNA accumulation were observed in transgenic plant integrated with other construct derived from the same cassette vector, suggesting that the effects of integrated copy number and insertion position in the genome

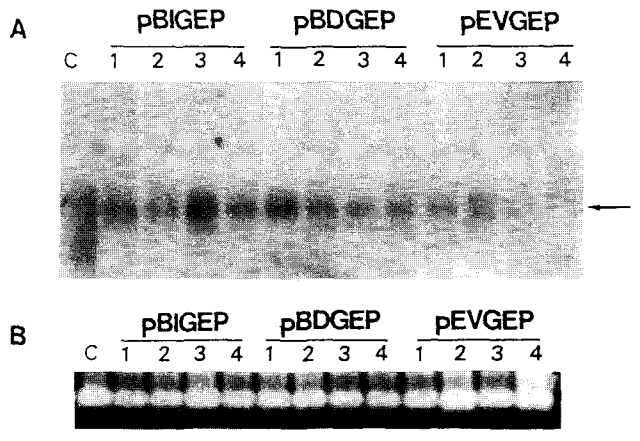

Figure 3. Northern blot hybridization of total RNA from leaves of nontransformed and transgenic plants. Total leaf RNA (30 μg) was denatured and separated on 1% agarose-formaldehyde gel, transferred to a GeneScreen-plus filter and then hybridized with the ^{32}P-labeled EPO partial cDNA (A). The amounts of total RNA loaded were normalized with those of ribosomal RNA (B). The arrow indicates EPO transcripts of 1.8 kb length expressed in transgenic plants. Numbers above lanes indicate the clone number of transformants with each construct: C, nontransformed plant.

may be responsible for the different levels of the EPO transcript. However, in #3 transgenic plant of pBIGEP, #1 of pBDGEP, and #2 and #3 of pEVGEP, the upper bands larger than 1.8 kb of EPO transcript were appeared, indicating that this might be caused by incomplete RNA processing.

Western Blot Analysis

To investigate whether EPO protein is expressed in the transgenic plant, soluble proteins were extracted from leaf tissue and extracts corresponded to 10 mg of leaf were fractionated on 12% SDS-PAGE. After transferred to PVDF membrane, Western analysis was carried out using anti-EPO polyclonal antibody. Although the EPO proteins were not detected on SDS-polyacrylamide gel, the immunoreactive bands were recognized in all transgenic plants tested by the anti-EPO antibody, but the extract of the control plant did not react with the antibody as shown in Figure 4. The difference of intensity among transgenic plants showed the EPO expression at different levels, which was roughly proportional to the levels of specific EPO mRNA for a given construct. The EPO protein produced in transgenic tobacco migrated in the SDS-PAGE with a molecular mass of 30 kDa which was smaller than 37 kDa of human urine EPO. This indicate that the modification system in plant is different from mammalian cells. When produced in mammalian cells, the mass of the recombinant human EPO varies depending upon the type of the host cells due to a difference in the N-linked oligosaccharides (Goto et al., 1988), suggesting that tobacco-produced EPO might has N-linked oligosaccharides that are smaller than those attached to recombinant human EPO.

To study further, we are going to purify EPO protein from the cell extracts of transgenic plant and analyze the difference of glycosylation between plant and animal system and the biological activity.

적 요

Erythropoietin (EPO)은 적혈구 모세포의 분화와 성장을 중재하는 당단백질이며 담배 식물체에서 재조합 사람 EPO를 생산하기 위해 CaMV 35S promoter를 갖는 발현 vector인 pBI△GUS121, pBD△GUS121, pPEV-1을 이용하여 5.4 kb의 EPO genomic DNA를 cloning 하였고 *Agrobacterium tumefaciens*에 의한 형질전환에 의해 *Nicotiana tabacum*

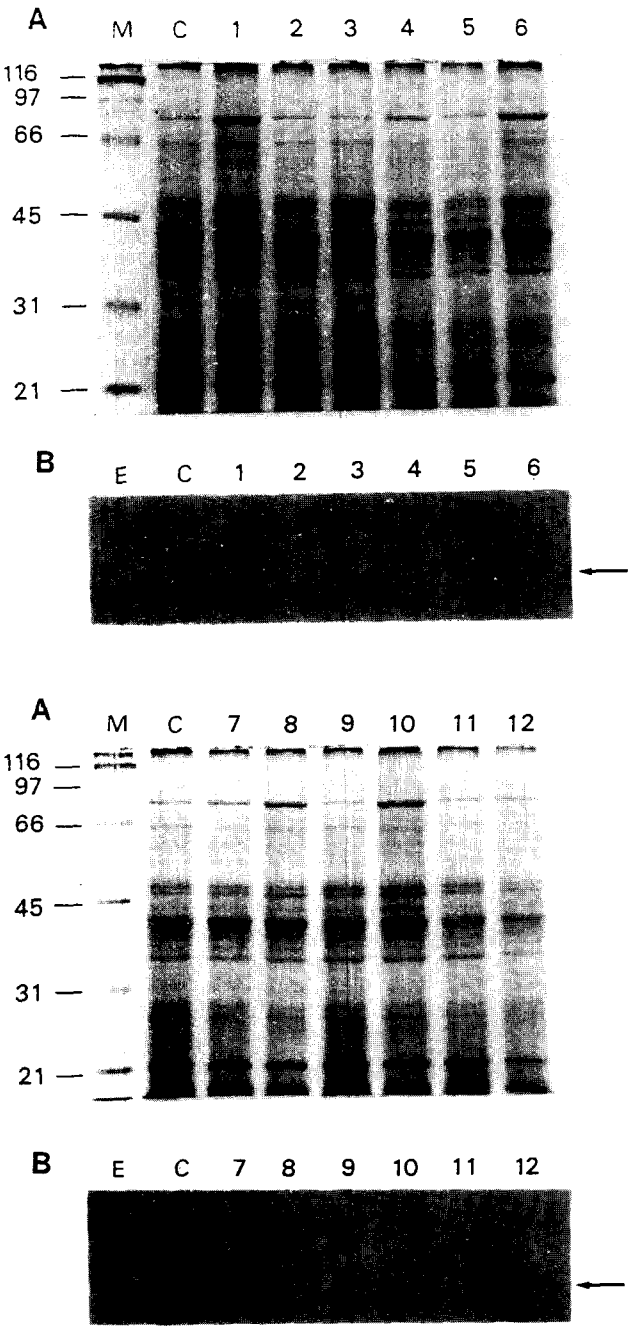

Figure 4. Immunodetection of erythropoietin protein in transgenic plants. Total protein extract corresponded to 10 mg of leaf tissue was fractionated by SDS-PAGE and stained with Coomassie blue (A) or blotted onto a PVDF membrane, probed with anti-EPO polyclonal antibody, and developed with BCIP/NBT reagents (B). Numbers at the left are protein size marker in kDa. The arrow indicate immunoreactive EPO protein from transgenic tobacco plants : M, protein size marker: C, nontransformed plant: E, EPO protein (37 kDa) from human urine: 1, #1 plant of pBIGEP: 2, #2 plant of pBIGEP: 3, #3 plant of pBIGEP: 4, #4 plant of pBIGEP: 5, #1 plant of pBDGEP: 6, #2 plant of pBDGEP: 7, #3 plant of pBDGEP: 8, #4 plant of pBDGEP: 9, #1 plant of pEVGEP: 10, #2 plant of pEVGEP: 11, #3 plant of pEVGEP: 12, #4 plant of pEVGEP.

(var. Xanthi)으로 도입되었다. Kanamycin을 포함하는 MS 배지에서 각각의 construct에 대하여 10 Km 저항성 식물체들이 얻어졌다. 형질전환된 식물체의 게놈에 EPO genomic DNA의 정확한 결합은 polymerase chain reaction에 의해 332 bp의 DNA 조각에 의해 확인되었으며 Northern blot 결과 1.8 kb의 전사체들이 식물체 잎에서 발현 축적되는 것이 확인되었다. Promoter의 수나 5'-UTS 서열에 의한 mRNA 양에는 변화가 없었지만 식물체 게놈에 결합된 위치 및 copy number에 의해 mRNA 수준에 영향을 주는 것으로 밝혀졌다. EPO 항체를 이용한 Western blot 결과 식물체에서 발현된 EPO 단백질의 크기는 동물세포에서 발현된 37 kDa 보다 작은 30 kDa 이었다. 이는 식물체에서 modification (glycosylation) system은 동물세포에서와는 다르다는 것을 보여준다.

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