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Studies on secretory peroxidase and production of recombinant glycoprotein in cultured tobacco cells

Shinya Matsumoto

1995

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To my parents and brothers, Tadanori, Kyoko, Junji, and Katsumi
and my wife Kyoko

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Abbreviations

PBS	: 10 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl
CM	: carboxymethyl
SSC	: 15 mM sodium citrate buffer, pH 7.0, 0.15 M NaCl
BY2	: <i>Nicotiana tabacum</i> L. cv. Bright Yellow 2
PAGE	: polyacrylamide gel electrophoresis
SDS	: sodium dodecyl sulfate
ELISA	: enzyme-linked immunosorbent assay
kDa	: kilodalton
RZ values	: Reinheitszahl value
IEF	: isoelectric focusing
BSA	: bovine serum albumin
Epo	: erythropoietin
M-LS medium	: modified Linsmaier and Skoog medium
NPT-II	: neomycin phosphotransferase type II
kb	: kilobases
PCR	: polimerase chain reaction
MES	: 2-[<i>N</i> -morpholino] ethanesulfonic acid
NAD	: nicotinamido adenine dinucleotide
NADP	: nicotinamido adenine dinucleotide phosphate
EDTA	: ethylenediaminetetraacetic acid
h	: hour
min	: minute
EIA	: enzyme immunoassay
rHuEpo	: recombinant human erythropoietin
ECL	: enhanced chemiluminescence
PNGase F	: peptide-N ⁴ -(<i>N</i> -acetyl- β -glucosaminyl) asparagine amidase
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Con A	: Concanavalin A
WGA	: wheat germ agglutinin
cDNA	: complimentary DNA
mRNA	: messenger RNA
mAb	: monoclonal antibody
ASA	: 5-aminosalicylic acid
SSPE	: 0.15 M NaCl, 12 mM sodium phosphate, pH 7.4, 1 mM EDTA
Endo H	: endo-b-N-acetylglucosaminidase H

Introduction

The demands for recombinant proteins is increasing in therapeutic, agricultural, and food manufactural area. The special demand for the recombinant proteins in therapeutic area has aroused because some recombinant hormones and cytokines, their native forms being difficult to obtain, proved significantly effective for improving several disease; recombinant erythropoietin (Epo) in chronic renal failure [1], and insulin in diabetes [2]. Therefore the stable supply of the recombinant proteins is desired. Currently most of the recombinant proteins used in therapeutic area are produced in cultured animal cells. One of the problems, however, in this system is that it is costly to maintain the system, as cultured animal cells require serum. On the other hand, the establishment of foreign gene transfer techniques, such as *Agrobacterium*-mediated gene transfer [3, 4], electroporation [5, 6], and the particle gun [7] have opened the possible exploitation of plants as a novel host system of recombinant protein production. Plant cells are capable of producing recombinant proteins which require eukaryote-specific post-translational modifications in order to express their activities effectively; glycosylation, disulfide bond formation, processing of N-terminal signal peptide and specific proteolytic cleavage. Plant is also suitable as host as obtaining the large amount of biomass can be achieved easily and at lower cost. However, the attempt to use plant cell as host cell of the useful recombinant proteins has begun only recently.

The purpose of my study is to evaluate the usefulness of plant cell as a novel host cell of recombinant protein production. I have used the suspension cultured tobacco cells to perform my study. The suspension cultured tobacco cells are considered to be a suitable system as their growth is so rapid, producing large biomass in short culture time. The easy application of biological method to the suspension cultured tobacco cells is also one of the reasons I have chosen this system.

The one of the desirable forms of recombinant protein production in plant cells is that the produced recombinant protein is secreted into their surrounding environment of the cell, as it facilitates the purification procedure of the protein. In case of plant cells, however, this requires a produced protein to penetrate the cell wall. The average pore size of cell wall is estimated to be 5 nm [8]. Considering the pore size, the proteins

more than 20,000 dalton can not penetrate the cell wall. There have been known, however, that proteins, with their size being more than 20,000 dalton, exist in the intercellular spaces of plant tissues [9] and in the spent medium of suspension cultured tobacco cells, showing the proteins penetrated the cell wall [10]. Whether the penetration is due to non-specific leakage of the proteins through cell wall, or to an unknown mechanism which actively transports the proteins across cell wall, is currently unclear. The understanding of the protein secretion mechanism in plant cells will contribute to the enhancement of the secretion of produced recombinant protein. To begin with, I have focused on the proteins that are secreted into the spent medium of suspension tobacco cells. In Chapter 1, the properties and the secretion manner of the proteins were determined biochemically. Chapter 2 describes the structure of the proteins determined from their cDNAs, and the relation of their structure and secretion of the protein is discussed.

The protein synthesis system of plant is essentially identical to that of animals [11]. Indeed, several mammalian proteins have been produced in plants, including human serum albumin [12], enkephalin [13], and monoclonal antibodies [14, 15, 16]. These recombinant proteins synthesized in plant cells were almost indistinguishable from those produced in mammalian cells, confirming that the plant cells can produce functional mammalian proteins. As described above, plant cell possesses desirable features suitable for the production of mammalian protein. In fact, a proposal has been made to use plant-produced hepatic B virus major surface antigen as an oral vaccine [17]. Hiatt *et al.* [18] have also suggested the possibility of generating secretory IgA by crossing plants expressing the individual components of secretory IgA. Secretory monoclonal IgA antibodies are impossible to obtain by current hybridoma procedures.

Although plant cells are considered to possess suitable features for the production of mammalian proteins used in therapeutics, the *in vitro* and *in vivo* biological functions of such proteins produced in plants have not been analyzed. To evaluate the usefulness of mammalian proteins produced in plants, it is important to analyze their *in vitro* and *in vivo* properties compared with those produced in mammalian cells. Thus, I attempted the production of Epo in suspension cultured tobacco cells. Epo is a principal cytokine involved in the regulation and maintenance of a physiological level of circulating erythrocytes. Epo supports the

survival of erythroid precursor cells and stimulates their proliferation and differentiation through interaction with the Epo receptor integrated into the plasma membrane [1]. In Chapter 3, the production in tobacco cell of Epo, whose signal peptide was deleted, was described. In Chapter 4, Epo cDNA was introduced by *Agrobacterium*-mediated gene transfer technique, and production of Epo was attained. Characterization, biological activities and secretion of tobacco-produced Epo were described and the usefulness of plant-produced recombinant mammalian protein was discussed.

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Chapter 1

Cationic peroxidase isozymes released into the medium of cultured tobacco cells; isolation, characterization, and expression

INTRODUCTION

The presence of cell walls endows plant cells with the features distinct from other higher eukaryotes. The walls are responsible for resistance of the cells to the osmotically generated turgor pressures and for cellular adhesion, finally giving the plant its shape and stability. However, the composition of cell wall components and their spatial arrangement should be variable during proliferation, expansion, and maturation of the cells [1]. Moreover, the cell wall structure can be markedly altered in response to changes in environmental conditions such as mechanical wounding, infection, and occurrence of elicitors [2, 3]. Much information on the cell wall architecture is currently available [4-7] but the functions of the cell wall components and their intermolecular interaction in the dynamic alteration of the cell wall structure have not been fully elucidated.

Recent experiments indicate that plant peroxidase plays a role not only in biosynthesis of secondary cell wall including lignin formation [8] and suberization [9] but also in the dynamic alternation of cell wall structure during expansion of cells. For example, plant peroxidase has been implicated in the regulation of cell elongation [10], and cross-linkage of cell wall proteins such as extensin [11] and proline-rich protein [12]. During isolation of proteins from the spent medium of the suspension-cultured tobacco BY2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cells, I found three novel peroxidases that were released into the medium in the cell expansion phase, indicating that these peroxidases might be involved in the alteration of the cell wall structure. Plant cell lines that can be cultured in suspension are advantageous for investigation of the cell wall metabolism during cell expansion because their growth profile in suspension culture consists of the cell proliferation phase and the subsequent expansion phase. Therefore, molecular studies of these peroxidases would provide a good system to investigate the cellular phase-dependent alteration of cell walls.

Herein, I report the isolation and properties of three novel peroxidases released into the spent medium of suspension-cultured tobacco cells. The recognition specificity of the mAbs against these peroxidases is also described. The cellular phase-dependent expression of one of the peroxidases and its release into the spent medium were examined using Northern hybridization and a sandwich-type ELISA.

MATERIALS and METHODS

Materials

The following materials were obtained from the sources indicated: carboxymethyl (CM)-Toyopearl (TOSOH); hydroxyapatite and Affi-Gel Hz (Bio-Rad); Sephadex G-100 (Pharmacia); microtiter plate for ELISA (NUNC); monoclonal antibody isotyping kit, Hybond-N (+) and Multiprime DNA labeling system (Amersham); alkaline phosphatase-conjugated goat anti-mouse IgG (Cappel); biotinyl N-hydroxysuccinimide ester (E.Y. Laboratories); alkaline phosphatase-conjugated streptavidin (Oncogene Science); protein A column (KURABO); protein A bearing *Staphylococcus aureus* (ZYMED); soybean and horseradish peroxidases (Sigma). Commercially available 5-aminosalicylic acid was recrystallized in the presence of Na₂S₂O₅ [13]. All other chemicals used were of the highest purity available.

Enzyme activity

Peroxidase activity was assayed at 25 °C in a total volume of 0.2 ml of 5 mM 5-aminosalicylic acid, 17 mM H₂O₂ in phosphate buffer (10 mM sodium phosphate buffer, pH 7.4). The enzyme activity was expressed as the increase in absorbance at 450 nm/minute/ml of enzyme solution, which was monitored in a microplate reader (Bio-Rad model 3550).

Purification of peroxidases

Tobacco BY2 cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) were cultured as described previously [14]. All procedures for the purification were carried out at 4 °C. After removal of cells from the 7-day cultured medium by filtration, CM-Toyopearl (6 ml/l of the filtrate) was added and gently stirred for 3 h. The proteins bound to the gels were eluted with 0.5 M NaCl in phosphate buffer and the eluate was dialyzed against phosphate buffer. The dialysate was applied to a CM-Toyopearl column (1.7 x 7 cm) pre-equilibrated with phosphate buffer and the bound proteins were eluted with a gradient increase of NaCl (0-0.3M) in phosphate buffer. Peroxidase activity was distributed into three

fractions, Peaks I, II and III in the order of elution. The major protein in each peak migrated in SDS-PAGE with a size of 34K, 38K and 40K, respectively. The pooled peaks without dialysis were applied to hydroxyapatite columns (1.2 x 7.4 cm) pre-equilibrated with phosphate buffer containing 0.3 M NaCl. The peroxidase activity in Peak I did not bind to the column and those in Peaks II and III did and were eluted with a gradient increase of NaCl (0.3-0.6 M) in phosphate buffer. The resulting peroxidase-active fractions were concentrated and further purified on Sephadex G-100 columns (1.9 x 53 cm) pre-equilibrated with PBS (10 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl).

Preparation of monoclonal antibodies

Five female Balb/c mice (9 weeks-old) were immunized with the isolated 40K and hybridomas producing mAb against the antigen were established essentially as previously described [15] except that alkaline phosphatase-conjugated goat anti-mouse IgG was used as the second antibody. The 10 mAbs obtained were all IgG1 (κ) and designated as K3 and K6-14. Electrophoretically pure antibodies were obtained from the ascitic fluid of K6, 7, 10, 12 and 14 with protein A columns.

Protein electrophoresis and protein detection

SDS-PAGE was performed under non-reducing condition with a Mini protean II apparatus (Bio-Rad) [16]. For Western analysis, the proteins in a gel were transferred to a polyvinylidene difluoride membrane with a Mini Trans Blot apparatus (Bio-Rad). The culture supernatant of each mAb-producing cell was used as the primary antibody and the bound antibody was detected by using peroxidase-conjugated anti-mouse IgG. Acidic-disc PAGE was carried out to estimate the isoelectric points of the proteins [17].

Immunoprecipitation and neutralization of peroxidase

Serial dilutions of purified mAbs were incubated with 0.25 μ g of 40K in 55 μ l of PBS containing 0.1% BSA at 25 °C for 1 h. Then, excess amounts of anti-mouse IgG and protein A bearing *S. aureus* were added and incubated at 25 °C for 30 min. After the antibody-antigen complex was precipitated by centrifugation, the peroxidase activity in the supernatant was measured.

Isolation of 40K with an immunoaffinity column

The mAb K10 was coupled to Affi-Gel Hz according to the manufacturer's protocol. Forty two mg of the proteins recovered by a batch-wise purification with CM-Toyopearl was applied to 2 ml of

immunoaffinity column containing about 10 mg of mAb K10. The column was pre-equilibrated with phosphate buffer containing 0.5 M NaCl. After washing the column thoroughly with the buffer, 40K was eluted with 2 M MgCl_2 . The column was reusable after washing with phosphate buffer containing 0.5 M NaCl.

Quantification of 40K by the use of monoclonal antibody

To wells of the microtiter plates was added 50 μl of purified mAb (10 $\mu\text{g/ml}$ of mAb K12 or K14). After incubation of the plates at 37 °C for 1 h, the wells were washed with PBS three times, and the uncoated sites were blocked with 1% BSA. After washing the wells with PBS three times, samples containing 40K were added to each well and the plates were incubated at 37 °C for 1 h. The wells were washed five times with PBS containing 0.05% Tween 20 and once with PBS. For the direct peroxidase-binding assay, the 40K bound to a well was assayed directly by addition of the peroxidase substrate mixture. For a sandwich-type ELISA, mAb K10 biotinylated as described below was added to the wells in which the 40K had been bound and incubated at 37 °C for 1 h. After washing as described above with 20 mM Tris-buffered saline (pH7.4) instead of PBS, the 40K bound to a well was assayed indirectly by using alkaline phosphatase-conjugated streptavidin and *p*-nitrophenylphosphate.

Biotinylated mAb K10 was prepared by mixing 1 mg each of the mAb and biotinyl *N*-hydroxysuccinimide ester in PBS for 3 h at room temperature and successive dialysis against PBS . Standard curves were drawn using the isolated 40K in both direct and indirect assays. Data were mean values of triplicate experiments.

Northern hybridization and densitometric analysis

Total RNA was prepared from suspension cultured tobacco cells using bentonite (montmorillonite) [18]. Total RNA (20 μg) was fractionated by formaline-agarose gel (1.2%) electrophoresis followed by blotting onto Hybond-N (+). As a probe, the full length cDNA of 40K (Chapter 2) was labeled with ^{32}P using Multiprime DNA labeling system (Amersham). The membrane was incubated in hybridization buffer (0.75 M NaCl, 50% formamide, 5 x Denhardt's solution, 5 mM EDTA, 50 mM phosphate buffer pH 7.4, 100 $\mu\text{g/ml}$ denatured salmon sperm DNA) at 42 °C for 12 h as prehybridization and hybridized with the labeled probe at 42 °C overnight in the hybridization buffer. The membrane was then washed once in 2 x SSC (15 mM sodium citrate buffer, pH 7.0 containing 0.15 M NaCl), 0.1% SDS at 60 °C for 15 min and twice in 0.2 x SSC,

0.1% SDS at 60 °C for 30 min, followed by autoradiography. The membrane was also analyzed by a FUJIX BioImaging Analyzer BASS2000 (Fuji Photo Film) for densitometric analysis.

Other methods

Proteins were determined according to the method of Lowry *et al.* [19]. The amino acid sequence was analyzed with a gas-phase protein sequencer (Applied Biosystems model 476A). The amount of DNA was quantified by absorbance at 280 nm.

RESULTS and DISCUSSION

Isolation of three proteins from the spent medium of cultured tobacco cells

Figure 1 shows the proteins released into the medium of suspension cultured tobacco cells. No protein bands were detected in the medium for 1 h to three days after inoculation (lanes 2-4) but faint bands were found in the medium on the 5th day of culture (lane 5). They became clearly visible in the medium on the 7th day of culture (lane 6) and the pattern was similar in the medium on the 9th day of culture (not shown). The medium on the 7th day of culture was mixed with CM-Toyopearl and the bound proteins were eluted with 0.5 M NaCl. The migration pattern (lane 7) of the eluted proteins on SDS-PAGE was similar to that of the untreated medium, indicating that most proteins in the spent medium are cationic. There were seven major proteins (four proteins with a molecular size of 60-80 kDa, and three proteins with a size of 34, 38, and 40 kDa).

Figure 2 (absorbance at 275 nm) shows the profile of the proteins eluted from a CM-Toyopearl column by a gradient increase of NaCl. During fractionation, I have noticed that several fractions were tinted brown, suggesting the presence of peroxidase possessing heme. I thus measured the peroxidase activity and absorbance at 406 nm for the heme protein of the fractions eluted from a CM-Toyopearl column (Fig. 2). There were three peaks with peroxidase activity, which were roughly consistent with those with an absorbance at 406 nm. Major proteins in Peaks I, II, and III (in the order of elution) migrated in SDS-PAGE with a size of 34, 38, and 40 kDa, respectively (data not shown), suggesting that the 34K, 38K, and 40K found as major components in the spent medium (see Fig. 1) were peroxidases. Contribution to the total peroxidase activity applied was about 15, 30, and 30% for the 34K, 38K,

and 40K, respectively. The pooled fractions of the three peaks were subjected to further purification with hydroxyapatite columns and then the resulting peroxidase-active fractions were purified with Sephadex G-100 columns (data not shown). In both purification steps, the fractions with absorbance at 406 nm were consistent with those with peroxidase activity. The final preparation of the individual peroxidase was homogeneous upon analysis with SDS-PAGE (Fig. 3A) and these peroxidases were glycoproteins (Fig. 3B).

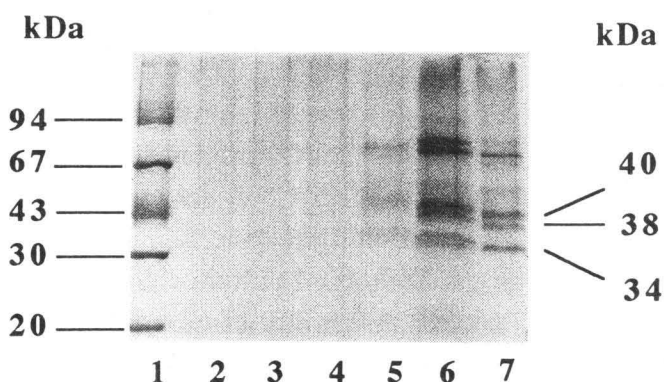


Fig. 1 Proteins in the spent media of tobacco cells cultured for different periods after inoculation.

The spent media of the cells cultured for different periods were evaporated to dryness by a centrifuge evaporator and the residual materials were dissolved in phosphate buffer with one-tenth volume of the individual spent medium. Five μ l of each sample was used for SDS-PAGE under the non-reducing condition. When the medium obtained on the 7th day of culture was treated with CM-Toyopearl gel as described in "Materials and Methods", the cultured medium was concentrated to one-tenth of its original volume. Lane 1, size markers; proteins in the spent media of the cells cultured for 1 h (lane 2), 1 day (lane 3), 3 days (lane 4), 5 days (lane 5), and 7 days (lane 6); lane 7, proteins in the medium obtained on the 7th day of culture were bound to CM-Toyopearl and eluted with 0.5 M NaCl. Numbers in both sides represent the molecular size in kDa.

Properties of the isolated 34K 38K, and 40K

Three peroxidases were sequenced in their amino terminal regions. The sequence for the 40K was AGNNVPRKNFYKSTRXPNAEQFRDIT, while that for the 38K was identical except that Ser¹³ of the 40K was replaced with Asn. This replacement was confirmed by the nucleotide sequences of cloned cDNAs (Chapter 2). Screening of these amino acid sequences

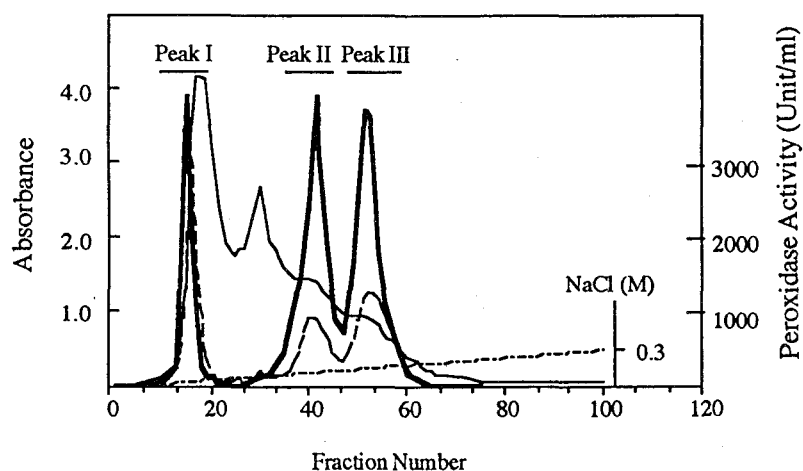


Fig. 2 Elution profile of proteins from CM-Toyopearl. The spent medium of tobacco cells cultured for 7 days was pre-treated with CM-Toyopearl gel as described in "Materials and Methods" and the resulting preparation containing 136 mg protein was applied to a CM-Toyopearl column. Proteins bound to the column were eluted with a gradient increase of NaCl. Fractions were 2 ml and the flow rate was 0.7 ml/min. (—) Absorbance at 275 nm; (.....) absorbance at 406 nm; (---) NaCl concentration; (———) peroxidase activity.

with Swiss Plot and GenBank DNA Databank revealed that their homology to other plant peroxidases was very low, 20-30%, indicating that the 38K and 40K were novel peroxidases. So far I have been unsuccessful in either sequencing the amino terminal region of 34K or isolating its cDNA. Although the amount (1 nmole) of 34K used was sufficient for sequencing, few amino acids were detected even in the first cycle and the same was true in the following 25 cycles. Therefore, it is unlikely that the 34K is derived from random cleavage in the amino terminal region of 38K or 40K and therefore the amino terminal residue of the 34K is probably blocked. Table 1 summarizes the properties of these peroxidases. There was not much difference in the molecular size of individual peroxidases calculated from the migration in SDS-PAGE and that from the elution position of Sephadex G-100, indicating that they are present in monomeric form. These three peroxidases showed an absorption spectrum with the maximum at 406 nm, typical for the Soret

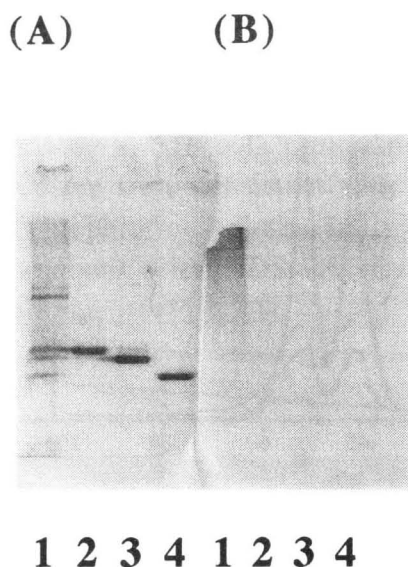


Fig. 3 SDS-PAGE of the purified peroxidases.

In (A), the gel was stained with Coomassie Brilliant Blue. In (B), the gel was stained with periodic acid-Schiff reagents for detection of glycoproteins. Lane 1, 10 μ g of protein in the medium obtained on the 7th day of culture, which were bound to CM-Toyopearl and then eluted with 0.5 M NaCl; lane 2-4, 1 μ g of the purified 40K, 38K and 34K, respectively.

band of heme proteins (not shown). The Reinheitszahl (RZ) value, the ratio of absorbance at 406 nm and 275 nm, is considered to be an index for purity of the peroxidase preparation; the RZ value for most of the pure enzymes was about 3. The IEF revealed that 34K was moderately cationic but the 38K and 40K were extremely cationic. The substrate (5-aminosalicylic acid) saturation kinetics of 38K and 40K was a typical Michaelis-Menten type, yielding the same K_m value of 1.0 mM and similar maximum velocity for both enzymes. By contrast, the initial reaction velocity of the 34K was nearly proportional to the substrate concentration up to 9 mM (the highest concentration tested), which suggests that the K_m value of this enzyme was far greater than 9 mM or the reaction kinetics of 34K was unusual for an unknown reason.

Table 1. Basic properties of three peroxidases isolated from the spent medium of the suspension-cultured tobacco cells.

The molecular size of the proteins was estimated by SDS-PAGE and gel filtration with Sephadex G-100. RZ represents the Reinheitszahl value (ratio of absorbance at 406 nm and 275 nm). The pI value was estimated by isoelectric focusing. To measure K_m for 5-aminosalicylic acid, the substrate concentration was varied from 0.2 to 9 mM at a fixed concentration (17mM) of H_2O_2 and the value was calculated from the Lineweaver-Burk plot. For the 34K, the substrate saturation was not observed even at the highest substrate concentration and therefore the K_m value for 5-aminosalicylic acid was not shown. To measure K_m for H_2O_2 , the substrate concentration was varied from 0.17 to 33 mM at a fixed concentration (5 mM) of 5-aminosalicylic acid. IC_{50} shows the NaN_3 concentration required for 50% reduction of the enzyme activity. Inhibition by NaN_3 was measured at 5 mM 5-aminosalicylic acid and 17 mM H_2O_2 varying NaN_3 concentrations from 0.01 to 1 M. ASA: 5-aminosalicylic acid.

Molecular size (kDa)				K_m		IC_{50}	
SDS-PAGE	Sephadex G-100	RZ	pI	ASA	H_2O_2	NaN_3	
				(mM)	(mM)		
40	46	3.47	>9.8	1.0	8.0	170	
38	44	3.23	>9.8	1.0	6.0	170	
34	33	2.67	9.0	—	0.67	35	

The 34K was also different from the other two enzymes in the affinity for H_2O_2 and in inhibition by NaN_3 , a typical inhibitor of heme enzymes; the concentration that caused 50% inhibition (IC_{50}) was lower than those of 38K and 40K.

Monoclonal antibodies and their properties

To further characterize these peroxidases, I prepared 10 hybridoma clones (K3, K6-K14) producing mAbs by the use of the 40K as an antigen. The subclass of all mAbs was IgG₁. To examine the recognition specificity of mAb, the peroxidase tested was fixed in a well of the microtiter plate and the mAb bound to the fixed peroxidase was detected. From the results shown in Fig. 4, they could be classified into three groups (Table 2). In group A to which only mAb K6 belongs, the mAb binds to tobacco 34K, 38K and 40K with similar efficiency and this antibody also cross-reacts with peroxidases from different sources such as

Table 2 Summary of the properties of monoclonal antibodies raised against the 40K. Details of the results on binding in an enzyme-immunoassay (Fig. 4), Western blotting (Fig. 5), immunoprecipitation of 40K and neutralization of 40K activity (Fig. 6) are described in the respective figures. +, positive; -, negative; nt, not tested.

Group		A		B				C			
Clones		K6	K3	K7	K8	K9	K11	K10	K12	K13	K14
Binding in enzyme- immunoassay	40K	+	+	+	+	+	+	+	+	+	+
	38K	+	+	+	+	+	+	-	-	-	-
	34K	+	-	-	-	-	-	-	-	-	-
Western blotting	40K	+	+	+	+	+	+	-	-	-	-
	38K	+	+	+	+	+	+	-	-	-	-
	34K	+	-	-	-	-	-	-	-	-	-
Immuno- precipitation of 40K		-	nt	-	nt	nt	nt	+	+	nt	+
Neutralization of 40K activity								+	-	nt	-

soybean and horseradish with less efficiency. The mAbs in group B (K3, K7-K9, and K11) recognize the 38K as well as the 40K but do not bind with the 34K. There was almost no interaction of the mAbs in group B with peroxidases from other plant sources. The mAbs in group C recognize the 40K almost exclusively.

Next, I examined whether these mAbs could detect tobacco peroxidases by Western blotting; the preparation containing 34K, 38K and 40K was fractionated with SDS-PAGE, the fractionated proteins were transferred to a membrane, and the proteins were detected with the individual mAb (Fig. 5). The results of the mAbs in group A and B were in good agreement with those described above; the mAb K6 detected three peroxidases, and K3, K7-K9 and K11, which belonged to group B, detected both 38K and 40K but not the 34K. However, none of the mAbs

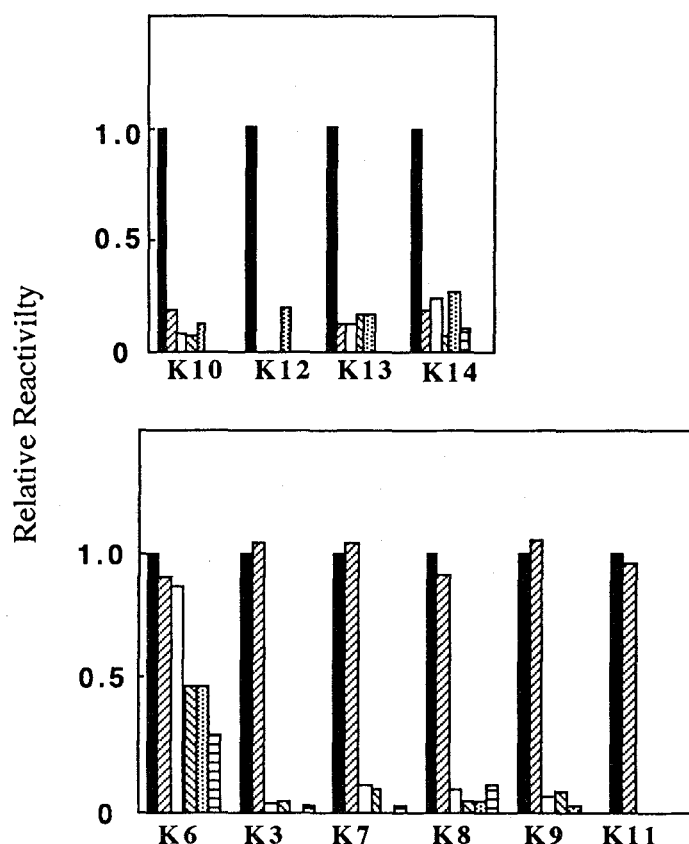


Fig. 4 Specificity of monoclonal antibodies.

Plant peroxidases (2 $\mu\text{g/ml}$) were fixed in wells of microtiter plates and the culture supernatant of the cells producing respective mAbs was added. Binding of mAbs to the fixed peroxidase was detected by using alkaline phosphatase-conjugated anti-mouse IgG. Results are expressed as relative reactivity when the reactivity of each mAb to 40K was defined as 1. (■), 40K; (▨), 38K; (□), 34K; (▩), soybean peroxidase; (▤), horseradish acidic peroxidase; (▥), horseradish cationic peroxidase.

in group C could react with the 40K transferred to a membrane, although they all bound to this antigen fixed in the well. The mAbs in group C seem to recognize the conformational epitope(s) that has been destroyed in the SDS-treatment. In fact, the 40K transferred to a membrane after SDS-PAGE did not exhibit any peroxidase activity (data not shown).

To determine whether the mAbs bind with the native 40K, the immunoprecipitation experiments were done; the 40K activity remaining in the

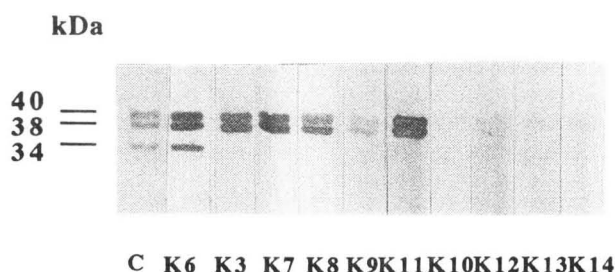


Fig. 5 Western analysis of monoclonal antibodies.

The mixture containing 0.5 μ g each of the purified 34K, 38K, and 40K were subjected to SDS-PAGE and transferred to a membrane. Lane C was stained with Coomassie Brilliant Blue. Other lanes were immunostained with mAbs. The lane numbers expressed as K3, K6 and others correspond to mAbs used for detection (see Table 2). Numbers in the left margin express molecular size in kDa.

supernatants after immunoprecipitation was assayed (Fig. 6). The mAbs K10, K12 and K14 in group C (K13, not tested) decreased peroxidase activity. Concomitant disappearance of 40K from the reaction mixture was confirmed by SDS-PAGE (data not shown). Unexpectedly, K6 in group A and K7 used as a representative in group B failed to precipitate the antigen. From the fact that these two mAbs interact with the 40K fixed in the well, I infer that fixation of the protein on the well induces a conformational change so that the epitopes are exposed to be accessible to the antibody molecules. Examples showing that the binding of an antigen to the solid phase causes a conformational change of the antigen, resulting in the appearance or disappearance of antigenic sites have been reported [20-22].

Of the mAbs in group C, which interacted with the native 40K, similar inhibition was also seen only in the case of K10 without addition of anti-mouse IgG and protein A bearing *S. aureus* and centrifugation, suggesting that the epitope of K10 were close to the active site of the 40K (data not shown). The mAb K10 did not inhibit the 34K and 38K activity and the other two mAbs (K12 and K14) showed no effect on any peroxidases (data not shown).

Finally, most, if not all, of the mAbs in groups A and B appear to recognize antigenic determinants that are exposed by a conformational changes of the antigen and the mAbs in group C recognize only those in the native conformation of 40K (Table 2). MAbs directed to

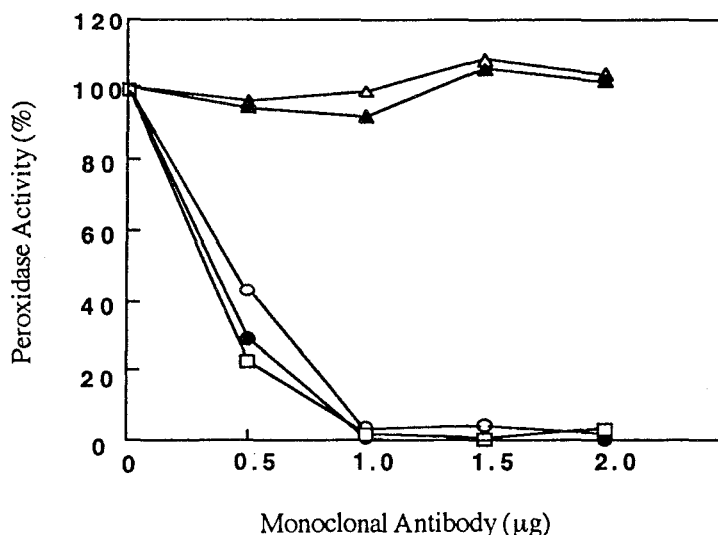


Fig. 6 Immunoprecipitation of 40K by monoclonal antibodies.

Purified 40K (0.25 μg) was incubated with the indicated amount of purified mAbs: (Δ) K6, (▲) K7, (○) K10, (●) K12 and (□) K14. After removal of the antigen-antibody complex, the remaining peroxidase activity was measured. Peroxidase activity in the absence of mAb was defined as 100%.

carbohydrate chains of glycoproteins must be able to react with the antigens irrespective of their conformation. Therefore, it seems unlikely that our mAbs were directed to a carbohydrate chain of the peroxidases. It is clear that the mAb K6 recognizes the epitope common in the 34K, 38K and 40K and this epitope is different from those recognized by the mAbs in group B. At present the epitope structures for the mAbs remain unknown.

Isolation and quantification of the 40K by the use of monoclonal antibodies

The features of the mAbs in group C have made it possible to develop an efficient isolation procedure and a sandwich-type ELISA for the 40K. To isolate 40K using mAb K10, the proteins recovered by a batch-wise purification of the spent medium with CM-Toyopearl was applied on a column containing the mAb K10-fixed gel. As shown in Fig. 7, most proteins appeared in the wash-through fractions and the 40K was

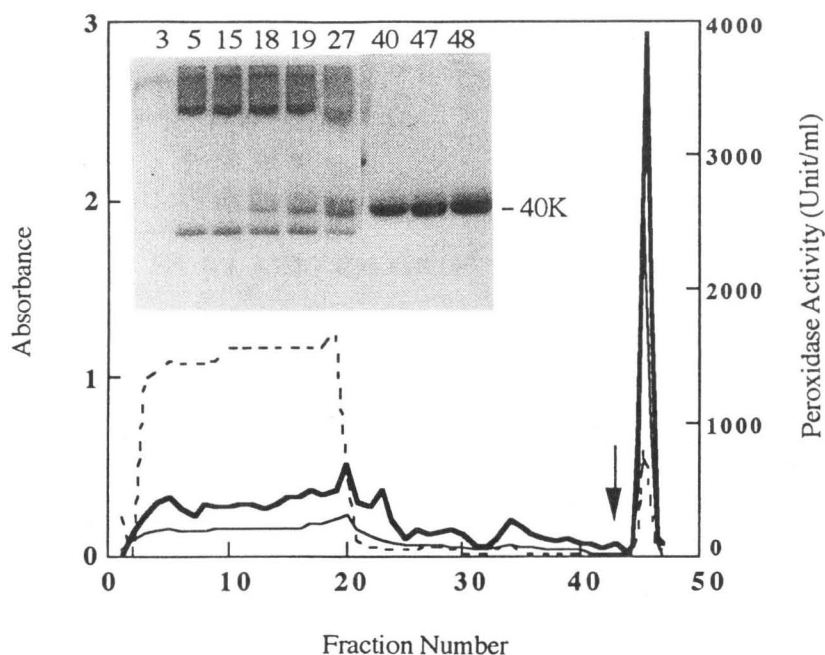


Fig. 7. Immunoaffinity purification of 40K.

The protein preparation obtained by the batch-wise purification of the culture medium with CM-Toyopearl was applied to a column of mAb K10-immobilized gels as described in "Materials and Methods". The column was washed and the 40K was eluted with 2 M $MgCl_2$ (a vertical line with arrowhead). Each fractions was 2 ml.

(- - - -) Absorbance at 275 nm; (—) absorbance at 406 nm; (———) peroxidase activity. The inset shows the results of SDS-PAGE analysis of the fractions obtained. Numbers in the inset indicate the fraction number.

eluted sharply with 2 M $MgCl_2$. The eluted 40K preparation was homogeneous on SDS-PAGE (inset). About 30% of the total peroxidase activity in the spent medium was recovered in the 40K preparation and this percentage was consistent with contribution of the 40K to the total activity recovered from a CM-Toyopearl column (see Fig. 2). The specific activity of the 40K eluted with $MgCl_2$ was similar to that isolated by conventional procedures. The 40K could be eluted from the immunosorbent gel of pH 2.5 but the specific activity was low compared with the 40K eluted with $MgCl_2$. Storage of the spent medium and also the enzyme preparations after fractionation with CM-Toyopearl caused marked inactivation of peroxidase activity and made the enzyme purification a difficult task. Isolation of the 40K using mAb was rapid with a high recovery.

The mAbs in group C were also used for ELISA of the 40K in the spent medium. The mAb K14 was fixed in a well and the sample containing the 40K was added to the well. To the well, the biotinylated-mAb K10 was added to bind with the 40K. The bound mAb K10 was detected by the use of alkaline phosphatase-labeled streptavidin. When various amounts (0.5-500 ng) of the isolated 40K were added to the spent medium obtained on the 3rd day of culture and was assayed by this sandwich-type ELISA, the 40K was almost fully recovered (data not shown). This method measures as little as 5 ng of the 40K. In an alternative method, the mAb K14 was fixed in a well and the sample containing the 40K was bound to the fixed mAb. The bound 40K could be determined by the measurement of its peroxidase activity. This direct

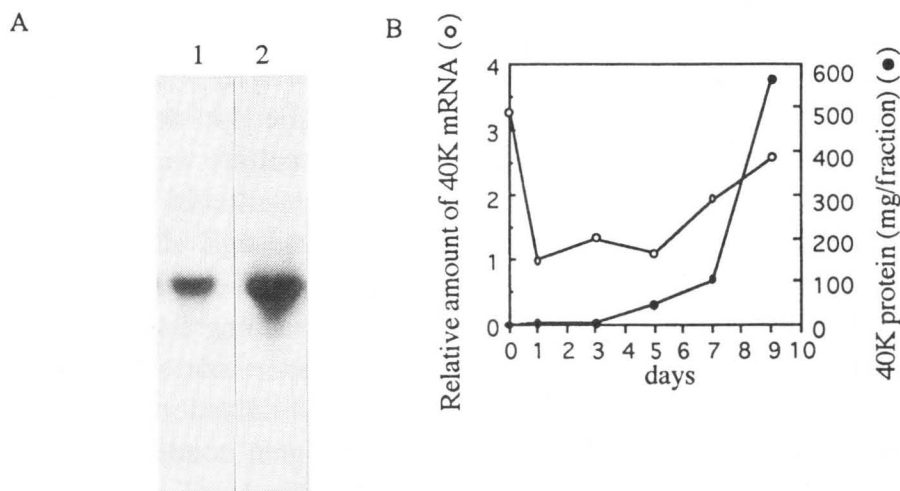


Fig. 8 Changes in the levels of 40K and its mRNA during cell culture. BY2 cells (0.5 g) was inoculated into 75 ml of the medium and cultured for the indicated periods. After filtration by suction, cells were used for the determination of mRNA content of the 40K and the filtrate was used for the determination of the 40K by the sandwich-type ELISA. (A) represents Northern blot analysis of mRNA for 40K. Lane 1; 1st day of culture; lane 2, 7th day of culture. (B) represents mRNA of 40K (○) and 40K in the culture medium (●). The relative amount of mRNA was determined by the densitometric analysis of Northern hybridization, defining the density of the 1st day of culture as 1. For determination of the 40K, the filtered medium (50 μ l each) was applied to a well of a microtiter plate coated with mAb K14 and the sandwich-type ELISA was carried out as described in "Materials and Methods". Results are expressed as ng of the 40K in the total medium.

peroxidase-binding assay is simple and rapid but not sensitive as compared with the sandwich-type ELISA. The mAb K12 could be substituted for the mAb K14.

Cell culture and secretion of the 40K in the spent medium

The 34K, 38K, and 40K isozymes were undetectable in the spent medium of cell culture at an early period after inoculation and became abundant at a later period (see Fig. 1). To further analyze this temporary appearance of these enzymes in the culture medium, I focused on the 40K because this protein could be measured quantitatively with our ELISA. Figure 8B shows an increase in the 40K in the spent medium during the cell culture. The 40K was undetectable for three days after inoculation, but it became detectable in the 5th day and thereafter increased.

I next examined the change of mRNA for the 40K during cell culture. As Fig. 8A shows, Northern hybridization with the cDNA revealed 1.3 kb band in the RNA preparations obtained not only on the 7th day of culture but also on the 1st day of culture, although the 40K was undetectable in the spent medium on the 1st day of culture. Furthermore, the band signal on the 7th day of culture was more intense than that on the 1st day of culture. The results of densitometric quantification of the mRNA hybridizable with the 40K cDNA (Fig. 8B) indicated that the mRNA content was unchanged at a basal level for 5 days of culture and then continued to increase, reaching a level three-fold greater than the basal level. This increasing pattern correlates well with that of the 40K in the spent medium. The hybridization and washing-steps of the filter were performed under stringent conditions to detect only highly homologous mRNAs but the 1.3 kb band may include mRNAs of both 38K and 40K because they are highly homologous in base sequence and similar in length (Chapter 2).

To know the cell stage when the 40K is secreted in the spent medium, I measured the cell weight during cell culture and DNA per cell weight (data not shown). The wet weight of cells in a culture flask increased very rapidly after a lag period of three days but the increase became sluggish after the 7th day of culture. The DNA content per wet weight of cells increased for five days after inoculation and then abruptly decreased, indicating that the cells proliferate during the initial five days of culture and thereafter they expand. It has been shown with the tobacco BY2 cell line that the cells proliferate actively for 4-5 days after inoculation and thereafter the increase in cell number levels off [23].

Taken together, the BY2 cells actively divide for 4-5 days after inoculation and after this proliferation stage the cell expansion is the prime characteristic of the cells. These findings indicate that the massive secretion of the 40K into the spent medium occurs in the cell expansion phase and this secretion is accompanied with an increase of the mRNA.

In this study, I isolated three cationic peroxidases with a size of 34, 38, and 40 kDa from the spent medium of suspension-cultured tobacco BY2 cells. Plants have a number of peroxidase isozymes and in the tobacco plant there are at least 12 isozymes that are classified into three groups by their charges; the anionic, the moderately anionic, and the cationic [8]. A tobacco anionic peroxidase was cloned and suggested to play a key role in lignin formation [8]. Analysis of the tissue specificity of tobacco peroxidase isozymes with IEF has indicated that the root tissue expressed all of the peroxidase isozymes, and wounding of the plant induced the expression of cationic isozymes in the leaf [24]. The presence of a cationic peroxidase in tobacco vacuoles was reported [25]. To my knowledge, the release of peroxidase isozymes into the culture medium of tobacco cells has not been documented. The sequence of the amino terminal region (this chapter) and cDNA of the 40K (Chapter 2) revealed the 26-amino acid signal peptide. The 40K and two other peroxidases are released into the medium during the cell expansion phase but not during the cell proliferation phase. The mRNA for the 40K was expressed in the cell proliferation phase and increased in the cell expansion phase. These findings suggest that the 40K exists in the cell wall compartment during the cell proliferation phase and is released into the medium in the cell growth phase. This cellular phase-dependent release may reflect the alteration in the interaction of the 40K with the cell wall components and also the increase in its mRNA.

In potato, expression of a suberization-associated highly anionic peroxidase was shown to be induced by abscisic acid [9]. Wounding and fungal attack triggered the expression of tomato anionic peroxidase and its expression was developmentally regulated [26]. Monoclonal antibodies to wall-localized peroxidases of corn seedlings identified two anionic isozymes [27]. Release of cationic peroxidases into the spent medium of cultured cells has been shown in peanut and *Lupinus polyphyllus*, and their cDNAs have been cloned [28, 29]. Monoclonal antibodies against the peanut cationic peroxidase were subdivided into four groups according to their epitope [30]. All of these antibodies cross-reacted

weakly with the anionic peanut peroxidase. My molecular tools including cDNA and monoclonal antibodies should prove useful for studies on the function of the tobacco cationic peroxidase in the cell wall metabolism.

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Chapter 2

Nucleotide sequence of cationic peroxidases that are abundantly secreted by cultured tobacco cells

INTRODUCTION

Plant cell wall is a structural compartment which metabolizes actively during proliferation, differentiation, and expansion of the plant cell [1]. One of the key enzymes that are considered to be involved in the cell wall metabolism is plant peroxidase. There are evidences that indicate the involvement of plant peroxidases in cell wall metabolism such as lignin formation [2], suberization [3], polymerization of extensin monomer [4, 5] and proline-rich protein [6] and the elongation of the cell [7]. I have found two cationic peroxidase isozymes, designated 40K and 38K, that are abundantly secreted by suspension cultured BY2 tobacco cell during cell expansion phase (Chapter 1). The increase of their mRNA was also observed during the phase [8]. As the cell wall structure is considered to be rearranged actively during the cell expansion phase [1], these peroxidase isozymes may be involve in the regulation of the cell wall metabolism when plant cell increases its size. Therefore the isolation and characterization of their cDNA may contribute to the understanding of the cell wall metabolism.

40K and 38K were purified from the spent medium of suspension cultured BY2 cells as described [Chapter 1 and ref. 8] and their N-terminal amino acid sequences were determined. As shown in Fig.1, the N-terminal amino acid sequence of the isozymes coincided perfectly except that Ser¹³ in 40K was replaced with Asn in 38K. The N-terminal amino acid sequence of the isozymes did not show significant homology with known amino acid sequences in GenBank and EMBL DNA Data Bank, showing 40K and 38K were the novel peroxidases. Two oligonucleotides that corresponded to the anti-sense nucleotide sequence of the amino acid sequence were synthesized and used as probes for screening 40K and 38K cDNA. Inosine was used to reduce the degeneracy of the probes (Fig. 1). The oligonucleotide probes were ³²P-labeled using [γ -³²P] ATP and T4 polynucleotide kinase.

BY2 tobacco cell was maintained as described previously [9]. The cells were harvested 4 days after inoculation and total RNA was prepared as described by Murray *et al.* [10]. PolyA⁺ RNA was isolated with

	1	
40K	A	G N N V P R K N F Y K S T R
38K	A	G N N V P R K N F Y K N T R
oligonucleotide	3'	CGICCITTRTTACAIGGIWCITT5'
	16	
40K	X	P N A E Q F V R D I T W S K
38K	X	P N A E Q F V R D I T W S K
oligonucleotide	3'	GGITTTCGICTMGTMAAACAIWCICTITAITGIACC5'
	31	
40K	A	K
38K	A	K

Fig. 1 N-terminal amino acid sequence of 40K and 38K and nucleotide sequence of synthesized oligonucleotide probes.

N-terminal amino acid sequences of 40K and 38K determined by protein sequencer are shown in upper two lines. Sequences of synthesized oligonucleotide probes are shown in the third line. The oligonucleotide sequence correspond to the anti-sense strand of the underlined amino acid sequence. X, unidentified amino acid residue; I, inosine residues; W, G/T mixture; M, T/C mixture; R, A/G mixture.

Oligotex-dT30 (Takara). Oligo-dT primed cDNA was synthesized with 5 µg polyA⁺ RNA using cDNA Synthesis Kit (Pharmacia) and subsequently inserted into the phage vector λZAP-II (Stratagene) at *EcoRI* site. 6x10⁵ pfu of the library were screened with the ³²P-labeled oligonucleotide probes. Hybridization was carried out overnight at 37°C with a final wash at 37°C in 0.1 x SSC and 0.1% SDS for 1 h. Thirteen phage clones that hybridized to both oligonucleotide probes were isolated. As their inserted cDNAs showed the identical restriction map profiles, the three phage clones (pC, pD, pQ) which contained the longest cDNAs were selected and the nucleotide sequences of the cDNAs were determined by the dideoxy chain-terminal method [11] with an ABI 373A DNA sequencer (Applied Biosystems). As pD and pQ encoded polypeptides which included the defined N-terminal amino acid sequence of 40K and 38K respectively, I concluded that pD and pQ encoded cDNA of 40K and 38K respectively. The phage clone pC contained short cDNA which encoded the partial amino acid sequence of 40K.

Figure 2 shows the nucleotide sequence of 40K and 38K cDNA. 40K and 38K cDNA consisted of 1244 and 1234 nucleotides respectively. Both cDNA contained 5'-noncoding region (61 nucleotide residues for 40K and 74 residues for 38K) and 3'-noncoding region (187 nucleotide

40K 1 ----- --AGGTTTA AGTCCGTCGA CATTATTGTC TTGTTTTCAT TTCCTAGCAA
 ***** * * * *
 38K 1 CTTAATTCTA ATTAGGTTTA AGTCCTTTGA CATTATTGTC TTGTTTTCAT TTCCTAGCAA
 48 GGAATAAAAA GAAATGCGA ACTGCTCAAT TACTGCTACT AAGTAATATT TTTCTTGTT
 ***** * * * *
 61 GGAATAAAAA GAAATGAGA ACTGCTCAAT TACTGCTACT AAG-----T TTTCTTGTT
 108 TTCTTTCTAT TGTGTTTGT GGAGTTTCGG GTGCAGGAAA TAATGTGCCA CGCAAGAATT
 ***** * * * *
 115 TTCTTTCTAT TGTGTTTGT GGAGTTTCGG GTGCAGGAAA TAATGTGCCA CGCAAGAATT
 168 TCTACAAGAG CACTCGTTGT CCAATGCTG AACAATTGT TAGAGATATC ACTTGGAGCA
 ***** * * * *
 175 TCTACAAGAA CACTCGTTGT CCAATGCTG AACAATTGT TAGAGATATC ACTTGGAGCA
 228 AAGCTAAGAA TGACTCCAGC TTGGGTGCTA AATGCTTCG ACTCCACTAC CATGATTGTT
 ***** * * * *
 235 AAGCTAAGAA TGACGCTACT TTGAGTGCTA AATGCTTCG ACTCCACTAC CATGATTGTT
 288 TCGTTAGGGG ATGTGATGCA TCGATATTGC TCGATAAAGT TGGAAACAGAT CAATTGAGA
 ***** * * * *
 295 TCGTTAGGGG ATGTGATGCA TCGATATTGC TCGATAAAGT TGGAAACAGAT CAATTGAGA
 348 AGGAGGCCAG GCCAAACCTC TCCTTGGGTG GTTTCGATGT AATTGATGAT ATCAAGCGAC
 ***** * * * *
 355 AGGAGGCCAG GCCAAACCTC TCGTTGGGTG GTTTCGATGT AATTGATGAT ATCAAGCGAC
 408 AAGTTGAGGA AAAATGTCCT GGGATTGTTT CTGTGTCAGA CATTTTAGCA TTAGCTACTC
 ***** * * * *
 415 AAGTTGAGGA AAAATGTCCT GAGATTGTTT CTGTGTCGA CATTTTAGCA TTAGCTGCTC
 468 GTGACGCTGT TTCTTTTCGA TTAAAGAAAT CACTATGGGA CGTGGCGACC GGAAGAAAAG
 ***** * * * *
 475 GTGATGCTGT TTCTTTCTCT TTAAAGAAAT CGCTATGGGA TGTGGCGACC GGAAGAAAAG
 528 ACGGGAATGT TTCCTTGCA AGTGAAGTGA ATGGAAACTT ACCTTCACCA TTCTCAGATT
 * * * *
 535 ATGGGAATGT TTCCTTGGG AGTGAAGTGA ACGGAAACTT ACCTTCACCA TTCTCAGATT
 588 TTGCCACCCT TCAGCAACTG TTGCAAAGA AAGGCCTAAA TGTCAATGAT CTTGTTGCAT
 ***** * * * *
 595 TTGCCACCCT TCAGCAACTC TTGCAAAGA AAGGACTAAA TGTCAATGAT CTTGTTGCAT
 648 TATCAGGTGC TCACACCATT GGTGTTGCTC ATTGTGGAGC TTTCTCTAGA AGACTCTTCA
 ***** * * * *
 655 TATCAGGTGC TCACACAATT GGTGTTGCTC ATTGTGGAGC TTTCTCTAGA AGACTCTTCA
 708 ATTTCACAGG CAAAGGTGAC GTGGATCCAT CTTGAGTTC CACTTATGCT GAATCTTTGA
 ***** * * * *
 715 ATTTCACAGG CAAAGGTGAC ATGGATCCAT CTTTAAATCC TACTTATGTT GAATCTTTGA
 768 AACCAATTGT CCCTAATCCA GCCAACCCAG CCACTACAGT GGAATGGAC CCTCAGAGCT
 ***** * * * *
 775 AACCAATTGT CCCTAATCCA GCAAACCCAG CCACTACTGT GGAATGGAC CCTCAGAGCT
 828 CAACTTCATT TGACAGCAAT TATTCAACA TCCTTACTCA GAACAAAGGG TTGTTCCAAT
 ***** * * * *
 835 CAACTTCATT TGACAGCAAT TATTCAACA TCCTTACTCA GAACAAAGGG TTGTTCCAAT
 888 CTGATGCAGC CCTCCTCACA GACAAAAAAT CAGCTAAAGT TGTAAAAACA CTGCAAAAAA
 ***** * * * *
 895 CTGATGCAGT CCTCCTCACA GACAAAAAAT CAGCTAAAGT TGTGAACAG CTGCAAAAAA
 948 CTAATGCCTT TTTTCTGAG TTGCTAAAT CCATGCAGAA AATGGGAGCT ATTGAAGTTC
 ***** * * * *
 955 CTAATACCTT TTTTCTGAG TTGCTAAAT CCATGCAGAA AATGGGAGCC ATTGAAGTTC

```

1008 TTACTGGAAA TGCTGGAGAA ATCAGGAAGA ATTGCCGCGT CAGAACTAA TTCTTGAAAA
*****
1015 TTACTGGAAA TGCTGGAGAA ATCAGGAAGA GTTGCCGCGT CAGAACTAA TTC-TGAAAA

1068 CCTATGATTA ATTTTATGTC ATATATATGT AATCTCTAAA GTGTACAACA TCTGGTATTT
*****
1074 CCTATGATTA ATTTTATGTC --ATATATGT AATCTCAAAA GTGTACAACA TCTGGTATTT

1128 AGTTTCTGTC TATTTGTTTC TTTTCCTTTT TGAAAGTGAT GTGTTATTG TGTGGAA-A
* * * * *
1132 AATTGTGTC TATTTGTTTC TTTTCCTTTT TGAAAGTGAT GTGTTATTG GGTGGAATA

1187 TGTATTGAC AAATTACTT ATTATTAAT GTATTGATC AAAAGGCTCT TTATTTTC
* * * * *
1192 CGTGCTT--C AA--TAATT -TGAAATAAT GTATCTGAGC ----GGC-CG CGA-----

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Fig. 2 Nucleotide sequences of 40K and 38K cDNA.

The nucleotide sequences of 40K and 38K cDNA are shown. Sequence written in capital letter and small letter correspond to coding and noncoding region respectively. * indicates the matched nucleotide. The underlined sequences are presumed to be polyA site. Hyphen is the gap which is inserted into the sequences to have the best matched alignment.

residues for 40K and 170 residues for 38K). The typical polyA site (AATAAA) was found in 38K cDNA but was not found in 40K cDNA, although similar sequence (AAATTAA) was present at 28 residues upstream of its polyA tail. 40K and 38K cDNA showed high homology (92%) indicating they derived from a common gene. Their similarity to other plant peroxidases have been analyzed; 49-50% to horseradish neutral peroxidase [12], 52-53% to peanut cationic peroxidase [13], and 53-55% to tobacco lignin-forming anionic peroxidase [2].

The deduced amino acid sequences of 40K and 38K are shown in Fig.3. 40K precursor contained mature polypeptide of 305 amino acid residues and signal peptide consisted of 26 residues, confirming that 40K was a secreted protein. There were 4 N-glycosylation sites (position at 33, 75, 132, 190) in mature 40K. I have described previously that 40K and 38K were glycoproteins [8] and the presence of N-glycosylation sites in 40K confirms our data. The mature peptide of 38K consisted of 305 amino acid residues, as did 40K, and signal peptide consisted of 24 residues. Four N-glycosylation sites (position at 75, 132, 190, 202) were found in mature 38K. However, the N-glycosylation site at position 202 in 38K was presumed to be not functional because of the presence of Pro between Asn and Thr [14, 15]. The calculated pI of 40K and 38K was 9.1, which were roughly consistence with the values determined by isoelectric point electrophoresis; 9.8 for both of the isozymes [8]. The

		1	
40K	-24	MGTAQLLLLS--NIFLVFLSIVVCGVSGAGNNVPRKNFYKSTRCPNAEQFVRDITWSKAKND	34
38K	-26	MRTAQLLLLS----FLVFLSIVVCGVSGAGNNVPRKNFYKNTRCPNAEQFVRDITWSKAKND	34
Hp	-28	MKTQTKVMGGHVLLTVFTLCMLCSAVRAQL----SPDIY-AKSCPPLLQIVRDQVKIALKAE	29
Tp	-22	MS-----FLRFVGAILFLVAIFGASNAQL----SATFY-DTTCFNVTSIVRGVMDQRQRTD	29
Pp	-22	MA-----LPISKVDLIFMCLIGLSAQL----SSNFY-ATKCPNALSTIKSAVNSCVAKE	29
		* * * *	
		↓ ↓	
40K		STLGAKLLRLHYHDCFVRGCDASILLDKVGTQFEKEARP NLS -LGGFDVIDDIKRQVEE	93
38K		ATLSAKLLRLHYHDCFVRGCDASILLDKVGTQSEKEARP NLS -LGGFDVIDDIKRQVEE	93
Hp		IRMAASLIRLHFHDCFVNGCDASVLLD--GT-NSEKLAIPNVNVRGFEVIDTIKAIVEN	86
Tp		ARAGAKIIRLHFHDCFVNGCDGSILLDTDGT-QTEKDAPANVGA-GGFDIVDDIKTALEN	87
Pp		ARMGASLLRLHFHDCFVQGCASVLLDDTSNFTGEKTAGPNANSIRGFEVIDTIKSQVES	89
		* * * * * * * * * * * * * * * * * *	
		↓ ↓	
40K		KCPGIVSCADILALATRDVAFRFFKSLWDVATGRKDGNSVLASEVNGNLSPFSDFATL	153
38K		KCFEIVSCADILALAARDVAFRFFKSLWDVATGRKDGNSVFGSEVNGNLSPFSDFATL	153
Hp		ACPGVVSCADILTLAARDSVYLS-GGPQWRVALGRKDGLVANQSSAN-NLSPFPEPLDAI	144
Tp		VCPGVVSCADILALASEIGVLA-KGPSWQVLFGRKDSLTA NRSGANS DIPSPFETLAVM	146
Pp		LCPGVVSCADILAAARDSVVAL-GGASWNVLLGRDRSTTASLSANS DLPA FFNLSGL	148
		** * * * * * * * * * * * * * * *	
		↓ ↓	
40K		QQLFAKKGLNVNDLVALSGAHTIGVAHCGAFSRRLFNFTGKGDVDP SL SSTYAESLKQLC	213
38K		QQLFAKKGLNVNDLVALSGAHTIGVAHCGAFSRRLFNFTGKGDMDP SLNPT YVESLKQLC	213
Hp		IAKFAAVGLNVTDVVALSGAHTFGQAKCDLFSNRLFNFTGAGTPDSTLETTLLSDLQTV	204
Tp		IPQFTNKGMDLTDLVALSGAHTFGRARCGTFEQRLFNFN SG GNPDLTVDATFLQTLQ IC	206
Pp		ISAFSNKGF TTKEL VLTLSGAHTIGQAQCTAFRTRIYNES-----NIDPTYAKSLQANC	201
		* * * * * * * * * * * * * * *	
		↓ ↓	
40K		PNPANPAT-TVEMDPQSSTSFDSNYFNILTQNKGLFQSDAALLTDKKS AKVVQ --LQK-	269
38K		PNPANPAT-TVEMDPQSSTSFDSNYFNILTQNKGLFQSDAVLLTDKKS AKVVQ --LQK-	269
Hp		PIGGNGNKT-APLDRNSTDAFDNNYFKNLLEGKLLSSDQILFSSDLAVNTTKR--LVE-	260
Tp		PQGGNGNTFTNLNDISTNDFNDYFTNLQSNQGLLQTDQELFSTSGSATIAIVNRYAGS	266
Pp		PSVGGDTNLS-PFDVTTNPKFDNAYYINLRNKKGLLHSDQQLFNGVSTDSQVTA--YSNN	258
		* * * * * * * * * * * * * *	
		↓	
40K		TNA-----FFSEFAKSMQKMGAEVL TGNAGE IRKNCRVRN	305
38K		TNT-----FFSEFAKSMQKMGAEVL TGNAGE IRKSCRVRN	305
Hp		AYSRSQYLFFRDFTCSMIRMGSLV--NGASGEVRTNCRVIN	299
Tp		QTQ-----FFDDFVSSMIKLGNISPLTGTNGQIRTDCKRVN	302
Pp		AAT-----FNTDFGNAMIKMGNLSPLTGTSGQIRTNCRKTN	294
		* * * * * * * * * * *	

Fig. 3 Sequence homology of 40K and 38K to plant peroxidases. Amino acid sequences of 40K and 38K were compared with those of horseradish neutral peroxidase (Hp), tobacco lignin-forming anionic peroxidase (Tp) and peanut cationic peroxidase (Pp). Numbers indicate the residue numbers taking first amino acid of the mature protein as 1. * indicates the amino acid conserved among 5 peroxidases. Underlined sequences show the N-glycosylation site and arrow indicates the position of conserved Cys residues. Hyphen is the gap which are inserted into the sequences to have the best matched alignment.

amino acid sequence similarity between 40K and 38K was so significant that their homology was as high as 94%.

The similarity of 40K and 38K to other plant peroxidases was compared as shown in Fig. 3. The homologies of 40K and 38K to horseradish neutral peroxidase, peanut cationic peroxidase, and tobacco lignin-forming anionic peroxidase were 45, 41 and 43% respectively. The homologies at Arg³⁹-Asp⁶¹ and Val¹⁶⁸-Cys¹⁸¹ of 40K were especially high, in which the distal and proximal His was included respectively. Eight Cys residues (position at 16, 49, 54, 95, 101, 181, 213, 301) were also conserved among the peroxidases. These Cys residues forming disulfide bonds were thought to be crucial in maintaining the proper conformation for plant peroxidase activity [16]. The Ala¹-Leu³⁸ did not show significant homology to other plant peroxidases.

Although the physiological role of 40K and 38K is unknown, I have observed the significant secretion of the isozymes and the induction of their mRNA expression during the cell expansion phase of suspension-cultured BY2 cells [Chapter 1 and ref. 8]. One of the cellular phenomena during the cell expansion phase is the rearrangement of cell wall structure, as the size of plant cell is restricted by cell wall. In short, cell wall must be partially degraded (loosened) for cell to expand and synthesized (tightened) for cell to halt its expansion. The fact that the expression of 40K and 38K mRNA was induced might indicate the involvement of the isozymes in the rearrangement of cell wall structure. I am currently exploring the possible involvement of 40K and 38K in the cell wall rearrangement which occurred during the expansion of the plant cell.

The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL, and GenBank Nucleotide Sequence Data Banks and available under the accession number D42064 and D42065 for 38K and 40K respectively.

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Chapter 3

Expression of human erythropoietin in cultured tobacco cells

INTRODUCTION

Various kinds of recombinant animal proteins such as hormones, cytokines, and interferons have been produced for use in therapy and research [1]. Some recombinant animal proteins are produced by eukaryotic cells including mammalian, yeast, and insect cells, because certain eukaryotic-specific post-translational modifications (*i.e.* modifications of amino acids including glycosylation, specific proteolytic cleavage, and formation of disulfide bonds) are necessary for expressing biological functions of these proteins.

The establishment of foreign gene transfer techniques such as *Agrobacterium*-mediated gene transfer [2, 3], electroporation [4, 5] and the particle gun [6] have made plants and cultured plant cells available for the production of recombinant animal proteins. Plant cells are able to modify the synthesized proteins post-translationally and be cultured at lower cost than animal cells. In certain cases, cultivation of transgenic plant may be suitable for large scale production of useful recombinant proteins. Functional antibody molecules have been constructed in transgenic plants produced by crossing of two transgenic plants, each expressing light or heavy immunoglobulin chains [7]. The transgenic plants transfected with an expression vector containing two cDNAs of light and heavy chains also produced antibody capable of recognizing the antigen [8].

Erythropoietin (Epo) is a principal hormone involved in the regulation and maintenance of a physiological level of circulating erythrocyte mass (see ref. 9 for review). Epo is a heavily glycosylated protein; human Epo isolated from human urine and recombinant human Epo migrates in SDS-polyacrylamide gel with a size of 35~39 kDa, and their size is reduced to 18~20 kDa upon deglycosylation [10~18]. Kidney is a major production site of this hormone in adults [19] and the recombinant human Epo produced in mammalian cells greatly improves anemia caused by renal failure (see ref. 20 for review). In this study, I engineered human Epo cDNA for expression in cultured tobacco cells and obtained one clone producing Epo.

MATERIALS and METHODS

Isolation of protoplasts

Tobacco BY2 cells were cultured in modified Linsmaier and Skoog medium (M-LS medium) [21] as described in Nagata *et al.* [22] except that the pH was 5.6. The culture was maintained by regularly transferring 1 ml of a 7-day-old culture into 75 ml of fresh medium in a 300-ml Erlenmeyer flask. Four-day-old cells, which were in logarithmic growing-phase, were harvested and treated with 1% Cellulase Onozuka RS (Yakult Honsha Co. Ltd. Japan) and 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd. Japan) dissolved in 20 mM MES (2-[N-morpholino] ethanesulfonic acid) buffer, pH 5.6, containing 5 mM MgCl₂, and 0.4 M D-mannitol. After incubating for 4 h at 26 °C in a petridish, protoplasts were collected by centrifugation at 100 x g for 2 min and washed 4 times with electroporation buffer (5 mM MES, pH 5.6, containing 2.5 mM KCl, 0.5 mM MgCl₂, and 0.4 M D-mannitol).

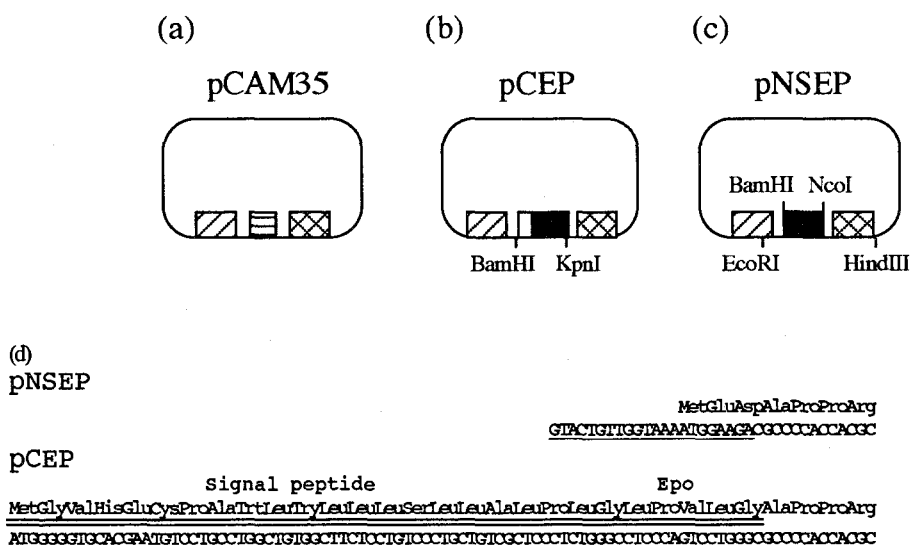
Construction of expression vectors

To obtain human Epo cDNA, a vector, pZIP-Neo(X)1-EPO [14], which was a retrovirus-based expression plasmid containing the Epo gene was transfected into a packaging cell line, ϕ 2 [23]. In the transfectant, introns of human Epo gene were removed by splicing, and a recombinant virus particle that contained an RNA genome coding for human Epo cDNA was recovered. This recombinant virus was transfected into COS cells [24] and the provirus DNA was recovered by the plasmid rescue method [25] on an ampicillin plate using *Escherichia coli* DH1 cells. The sequence of the obtained Epo cDNA agreed with that reported previously [26].

Two types of Epo expression plasmids, pCEP and pNSEP, were constructed based on pCAM35 [27] (a gift from Dr. Furusawa, Kyoto University, Japan), which contained a multicloning site between the cauliflower mosaic virus-derived 35S RNA promoter and terminator (Fig. 1a). pCEP was constructed by inserting the human Epo cDNA fragment containing the whole protein coding region between the *Bam*HI/*Kpn*I sites of the pCAM35 multicloning site (Fig. 1b). To construct the plasmid pNSEP, an expression plasmid for signal sequence-deleted Epo, the signal peptide coding region was deleted from pCEP and a small DNA fragment containing the initiator Met codon of the luciferase gene, which was prepared from pDO432 [28] was inserted into the deleted site (Fig. 1c). Epo encoded by pNSEP has three additional

DNA transfer and selection of transfectants

Protoplasts were electroporated by the method of Fromm *et al.* [29], with some modifications as follows. Protoplasts (3×10^6) and plasmid DNAs pCEP or pNSEP (8 μ g), and pNR35 (2 μ g) were



(a) Structure of pCAM35 having multicloning site between cauliflower mosaic virus-derived 35S RNA promoter and terminator. (b) Structure of pCEP. (c) Structure of pNSEP. Slant-hatched boxes (▨) indicate 35S RNA promoter derived from cauliflower mosaic virus and cross-hatched boxes (▩) indicate terminator derived from cauliflower mosaic virus. Horizontal-hatched box (▧) indicates the multicloning site derived from pUC19. Open box (□) and black boxes (■) indicate the signal peptide-coding region and the matured Epo-coding region, respectively. (d) Comparison of the structures between 5' end region of Epo cDNA in pCEP and pNSEP. The nucleotide sequence with underline indicates the sequence derived from luciferase gene and the nucleotide sequences without underline indicate sequence derived from Epo cDNA. The double underlined sequence indicates signal peptide of Epo. Amino acids encoded by the nucleotide sequence are also shown.

suspended in 1 ml of the electroporation buffer described above. Salmon sperm DNA (40 μ g) was also added to the suspension as carrier DNA.

The suspension was transferred to a glass cubette (inter-electrode distance was 10 mm) and after the cubette was incubated for 10 min on ice, an electrical pulse was applied. The electrical pulse was delivered from a 1.7- μ F capacitor charged at 600 V/cm using an Electric Cell Borer (Wakenyaku Co. Ltd., Japan). The time constant of the pulse was 2.3 msec. After the cubette was incubated for 10 min on ice, the electroporated-protoplasts were washed once with the M-LS medium with 0.4 M D-mannitol. Half of the washed protoplasts (1.5×10^6) were suspended in 3 ml of the M-LS medium with 0.4 M D-mannitol and plated in a petridish. After incubating the petridish for 4 days at 26 °C, 3 ml of the M-LS medium containing 2% agarose and 20 μ g/ml G418 sulfate was added to the petridish and the petridish was further incubated at 26 °C. After incubation for one month, the G418-resistant calli formed in the petridish were transferred to suspension culture in M-LS medium containing 10 μ g/ml G418.

Preparation of DNA and RNA from transfectants

Nucleic acids containing genomic DNA and total RNA was prepared from transfectants as described by Murray *et al.* [30]. DNA and RNA fractions were separated by LiCl precipitation [31]. The genomic DNA was further purified by CsCl density gradient ultracentrifugation.

Southern and Northern blot analyses

For Southern blot analysis, genomic DNA (10 μ g) prepared from transfectants was digested with *Eco*RI and *Hind*III. The resulting DNA fragments were fractionated by agarose (1%) gel electrophoresis, followed by blotting to a nylon membrane (Pall BioSupport Division, USA). Northern blot analysis was done essentially as described by Thomas [32]. Total RNA (20 μ g) was denatured with glyoxal and dimethyl sulfoxide and resolved by agarose gel (1%) electrophoresis in 10 mM sodium phosphate (pH 6.6). The RNA was then transferred to a nylon membrane. The *Bam*HI-*Nco*I fragment of pNSEP (Fig. 1c) was ³²P-labeled by random priming (Multiprime Labeling System, Amersham, UK) and the labeled fragment was used as a probe in both Southern and Northern analyses. The prehybridization was done at 42 °C for 48 h in a hybridization buffer consisting of 50% formamide, 5 x Denhart's, 5 x SSPE (0.15 M NaCl, 12 mM sodium phosphate, pH 7.4, 1 mM EDTA), 0.3% SDS, 250 μ g/ml salmon sperm DNA, and 5% dextran

sulfate. The hybridization was done at 42 °C for 12 h in hybridization buffer with the ³²P-labelled probe. The membranes were washed at 42 °C for 10 min twice in 2 x SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS, and once in 0.2 x SSC, 0.1% SDS.

Preparation of cell extract and concentration of Epo

All procedures for the extraction and the concentration were done at 4 °C. Cells (15 g) cultured in 75 ml medium were harvested and suspended in 15 ml PBS and the cells were disrupted by sonication. The sonicated cell suspension was clarified by centrifugation (10,000 x g for 10 min). The supernatant obtained was used as the cell extract.

To concentrate the Epo in the cell extract, the extract was chromatographed on an affinity column using an anti-Epo monoclonal antibody R2 described previously [33]. The eluate was used as the concentrated Epo fraction. The cell extract prepared from non-transfected BY2 cells was also chromatographed on the affinity column to obtain the control eluate.

Detection of Epo

Epo was identified and measured by a sandwich-type enzyme-linked immunoassay using two anti-Epo monoclonal antibodies, R2 and R6 [34], that recognize different epitopes. This assay has two steps. The first step is a sandwich-type immunoreaction by which Epo is sandwiched between R6 fixed on the surface of the microtiter wells and R2 labeled with alkaline phosphatase [33]. The second step is the colorimetric measurement of the phosphatase activity through the substrate cycling system by the use of alcohol dehydrogenase and diaphorase [34]. All procedures were done at room temperature. The R6 solution (10 µg/ml in 50 mM sodium carbonate buffer, pH 9.6, containing 0.02% NaN₃) was added to the wells (100 µl per well) of microtiter plates and incubated for 2 h. The unfixed R6 was removed by washing three times with PBS containing 0.05% Tween 20 and 0.02% NaN₃ (washing buffer). The sample mixture (mixture of 100 µl of sample and 10 µl of PBS containing 0.5% Tween 20, 10 mM EDTA, and 0.2% NaN₃) was added to the wells, which were incubated for 2 h to form the fixed R6 • Epo complexes. To the wells washed three times with the washing buffer, 100 µl/well of the 2000-fold diluted alkaline phosphatase-linked R2 solution [33] was added and the wells were incubated for 2 h for the formation of ternary complexes (fixed R6 • Epo • enzyme-linked R2). The wells were washed five times with washing buffer and then 100 µl of substrate solution (0.4

mM NADP dissolved in 0.1 M diethanolamine buffer, pH 9.5, containing 2 mM MgCl₂, 0.02% NaN₃) was added to each well. The wells were incubated for 20 min to allow dephosphorylation of NADP to NAD by alkaline phosphatase linked to R2. To each well, 200 µl of substrate cycling solution (3.5 mg/ml *p*-iodonitrotetrazolium violet, 0.2 mg/ml diaphorase, and 0.2 mg/ml alcohol dehydrogenase in PBS containing 0.02% NaN₃) was added. After incubation for 2 min, the reaction was stopped by addition of 50 µl of 0.4 N HCl. The optical density at 490 nm was measured by a micro plate reader (Model 2550, Bio-Rad, USA). A calibration curve was made with recombinant human Epo [14] mixed with the control eluate that was obtained from the extract of non-transfected cells.

RESULTS and DISCUSSION

Plant transfection

Four independent G418-resistant calli were obtained from protoplasts which were electroporated in the presence of pNSEP and pNR35, but no G418-resistant calli were obtained when pCEP and pNR35 were introduced. Of the four G418-resistant calli, only one callus, named 11N, survived in the suspension culture. This clone that might express signal-deleted Epo was further analyzed.

Southern blot analysis of DNA prepared from cultured 11N cells was done using Epo cDNA as a probe (Fig. 2). The probe hybridized to a 1-kbp fragment of *Eco*RI-*Hind*III digested DNA, and this size corresponded to that of the region consisting of Epo cDNA and the terminator in pNSEP (see Fig. 1c). These results indicated that the plasmid pNSEP DNA was integrated into the genome of 11N cells. Comparing the densities of bands in the autoradiogram, I concluded that a single copy of pNSEP has been integrated into the host genome (Fig. 2). The presence of Epo cDNA in the host genome was also confirmed by PCR analysis using primers specific to Epo cDNA (data not shown). Integration of one copy of the NPT-II gene into the 11N cell genome was found by Southern blot analysis (data not shown).

Expression of Epo in 11N cells : RNA and protein analyses

The expression of Epo mRNA was assayed by Northern blot

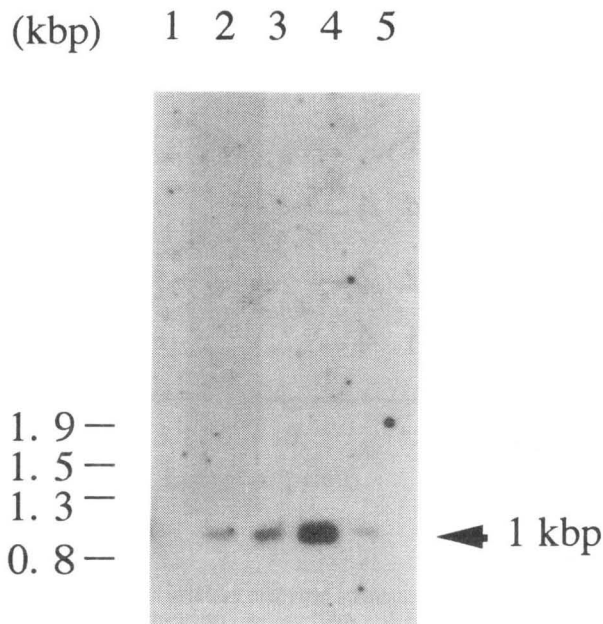


Fig. 2 Southern blot analysis of 11N cell DNA

Genomic DNAs (10 μ g) digested with *Eco*RI and *Hind*III were put on : lane 1, control BY2 cells; lane 5, 11N cells. Positive control pNSEP DNA digested with *Eco*RI and *Hind*III. These positive control DNA had been mixed with control genomic DNA (10 μ g) prior to the restriction enzyme digestion. : lane 2, 15 pg; lane 3, 30 pg; lane 4, 150 pg. Densities of bands in lane 2, 3, and 4 correspond to 1, 2, and 10 copies of pNSEP per genome, respectively. Numbers at the left-hand side show the position of DNA size markers.

analysis (Fig.3) using Epo cDNA as a probe. The RNA preparation from 11N cells yielded one positive band with a size of 1.2 kb, while those from the non-transfected cells gave no hybridizable band. The size of Epo coding region expected from pNSEP DNA is about 1 kb and therefore Epo mRNA expressed by 11N cells would contain the entire coding region of Epo and the polyA tail.

To confirm the production of Epo protein in 11N cells, the colorimetric enzyme-linked immunoassay was done as described in "Materials and Methods". When the cell extract was directly assayed with the immunoassay, Epo was undetectable. When known

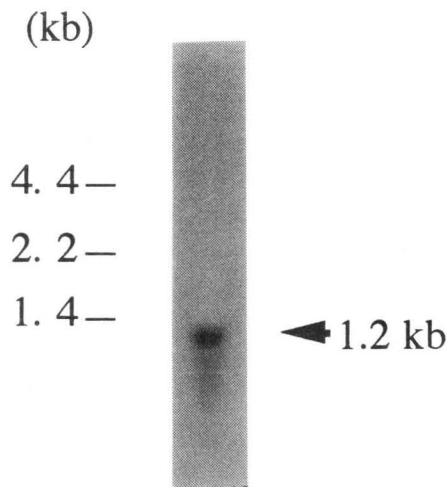


Fig. 3 Northern blot analysis of 11N cell RNA
Total RNA (20 μ g) was put on. Numbers at the left-hand side show the position of RNA size markers.

amounts of recombinant human Epo were added to the cultures of non-transfected cells and the cell extracts were assayed for Epo with the same immunoassay, about 50% of Epo was recovered. This result indicates that the concentration of Epo in 11N cell extract was low beyond the detectable range of the immunoassay. I attempted to concentrate Epo in 11N cell extract by an affinity column containing gel on which anti-Epo monoclonal antibody was fixed. With the fraction concentrated about 40-fold, Epo was detected and its concentration in the concentrated fraction was 7.5 pg/ml (Fig. 4). Based on this value, the production of Epo in 11N was estimated to be 1 pg/g of wet cell. I could not detect Epo in the 7-day-old cultured medium of 11N cells even after concentrating Epo in the medium by affinity column chromatography, indicating that Epo was present inside the cells.

The productivity of human Epo in the cultured tobacco cells was very low. The most likely reason for this low productivity is some instability of the produced Epo caused by the lack of signal peptides. The

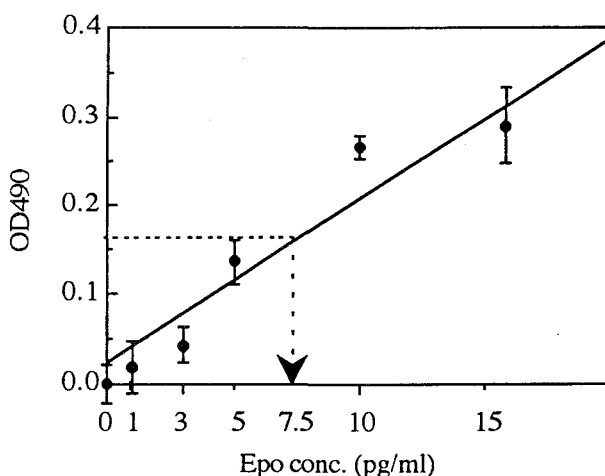


Fig. 4 Determination of epo by colorimetric enzyme immunoassay. Solid line in the graph indicates calibration curve. The calibration curve was made using recombinant human Epo mixed with the control eluate as described in Materials and Methods. Broken line with an arrow indicates the calibration of the concentrated sample of 11N cell extract. Each point is the mean $\pm$ SD of triplicate assays.

Epo produced from the signal sequence-deleted Epo cDNA (pNSEP) could not be glycosylated nor secreted. The deglycosylation of the recombinant human Epo produced in animal cells decreased the stability of Epo [15]. Foreign proteins in cytoplasm of plant cells may be susceptible to intracellular degradation systems. Hiatt *et al.* [7] showed that the expression of antibodies in transgenic plants was low when the signal peptides of the proteins were eliminated. They could not detect a significant difference in the level of mRNA between plants expressing antibodies with or without signal peptides. To obtain efficient production of foreign proteins in plant cells, the subcellular sequestration or secretion of the proteins which are directed by signal peptide seems to be important. In this experiment, I was unsuccessful in isolating cells that produced Epo with signal peptide and the production of the signal peptide-deleted Epo by 11N cells was too low to study of its biological functions. Isolation of the cultured tobacco cells expressing Epo with the signal peptide is currently in progress using *Agrobacterium*-mediated gene transfer.

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Chapter 4

Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells

INTRODUCTION

The establishment of foreign gene transfer techniques such as *Agrobacterium*-mediated gene transfer [1, 2], electroporation [3, 4], and the particle gun [5], have led to the use of plant cells for the production of mammalian proteins. Indeed, several mammalian proteins have been produced in plants, including human serum albumin [6], enkephalin [7], and monoclonal antibodies [8, 9, 10]. In general, plant cells synthesize proteins that are almost indistinguishable from those produced in mammalian cells, confirming that the plant cells can produce functional mammalian proteins.

Plant cells have two features suitable for the production of mammalian proteins. The large scale production of the plant biomass can be achieved easily compared with animal cells and at lower cost. These features are especially important when considering the increasing demands for valuable human proteins in recent therapeutics. In fact, a proposal has been made to use plant-produced hepatic B virus major surface antigen as an oral vaccine [11]. Hiatt et al. [12] have also suggested the possibility of generating secretory IgA by crossing plants expressing the individual components of secretory IgA. Secretory monoclonal IgA antibodies are impossible to obtain by current hybridoma procedures.

Although plant cells are considered to possess suitable features for the production of mammalian proteins used in therapeutics, the *in vitro* and *in vivo* biological functions of such proteins produced in plants have not been analyzed. To evaluate the usefulness of mammalian proteins produced in plants, it is important to analyze their *in vitro* and *in vivo* properties compared with those produced in mammalian cells.

Erythropoietin (Epo) is a principal cytokine involved in the regulation and maintenance of a physiological level of circulating erythrocytes. Epo supports the survival of erythroid precursor cells and stimulates their proliferation and differentiation through interaction with the Epo receptor integrated into the plasma membrane (see ref. 13 for review). Epo is a heavily glycosylated protein; human urinary Epo and

recombinant human Epo produced in mammalian cells migrate in SDS-polyacrylamide gel electrophoresis at 35~39 kDa, which is reduced to 18~20 kDa upon deglycosylation [14, 15, 16, 17, 18, 19]. Mature human Epo has three N-linked oligosaccharides at amino acid positions 24, 38, and 83 in a total of 166 amino acid residues [20, 21, 22, 23]. The N-linked oligosaccharides are not necessary for *in vitro* Epo activity but they play an important role in the *in vivo* activity of Epo through elongation of its half life in the circulation [19, 24, 25, 26, 27]. Recombinant human Epo remarkably improves the anemia caused by renal failure (see ref. 13 for review).

To explore the possibility of therapeutically using recombinant mammalian proteins produced in plants, I produced human Epo in cultured tobacco cells and assessed its biological functions *in vitro* and *in vivo*.

MATERIALS and METHODS

Recombinant human Epo and enzyme-linked immunoassay

Recombinant human Epo (rHuEpo) was produced in and isolated from baby hamster kidney cells [15, 28]. To quantify Epo protein, an enzyme-linked immunoassay (EIA) using two monoclonal antibodies raised against rHuEpo was performed as described [27]. In all EIA, rHuEpo was used as the standard.

Construction of an Epo expression vector

The DNA fragment which contained full length human Epo cDNA under the control of Cauliflower Mosaic Virus-derived 35S RNA promoter and terminator was obtained by digesting pCEP [Chapter 3 and ref. 29] with *EcoRI* and *HindIII*. The resulting DNA fragment was inserted into the binary plasmid vector pBI121 (Clontech Laboratories, USA) by replacing the *EcoRI-HindIII* region of pBI121, to construct the Epo expression vector pBINCEP. Plasmid pBINCEP was then mobilized to *Agrobacterium tumefaciens* LBA4404 via the tri-parental method [30].

Transformation of cultured tobacco cells and structural analyses of Epo

Tobacco BY2 cells were maintained in suspension culture in modified Linsmaier and Skoog medium [31] as described [32] except that the pH was 5.6. The tobacco cells were transformed by cocultivation with *A. tumefaciens* LBA4404 harboring pBINCEP according to An [33].

The transformed cells were selected with 150 µg/ml kanamycin and the developed calli were transferred to suspension culture for the further analyses.

All procedures for the purification of Epo from C3•5•2, which was the tobacco cell line producing Epo, were performed at 4°C. Four-day cultures of C3•5•2 cells (10 g) were suspended in 10 ml of 2 x extraction buffer (300 mM tricine-KOH, pH7.5, 80 mM EDTA, 2 mM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride, 10 mM ε-amino-*n*-caproic acid, 2 mM benzamidine, and 0.14% Tween 20), disrupted by sonication and the cell extract was collected by centrifugation. The precipitate was washed with 1 x extraction buffer and the wash was combined with the cell extract. The cell extract was applied to affinity chromatography using the anti-Epo monoclonal antibody R2 and Epo was eluted with 0.2 M acetic acid [28]. The chromatography procedure was repeated twice to purify the Epo.

The homogeneity of Epo obtained from tobacco cells was examined by silver staining (Daiichi Kagaku, Japan) SDS-PAGE (12% gel). For Western blotting, Epo (25 ng) was separated by SDS-PAGE followed by blotting onto a nitrocellulose membrane (Schleicher & Schuell, Germany). The blotted membrane was incubated with 2000-fold diluted anti-Epo rabbit antiserum (Genzyme, USA), followed by biotinylated secondary antibody, avidin- biotin- peroxidase complex (Vector Laboratories, USA) and the detected Epo was visualized with the ECL system (Amersham, UK).

Epo was digested with PNGase F (peptide-N⁴-(N-acetyl-β-glucosaminyl) asparagine amidase from *Flavobacterium meningosepticum*) as follows. Epo (25 ng) was lyophilized and the pellet was washed extensively with ethanol to remove Tween 20. The resulting pellet containing Epo was digested with PNGase F according to the manufacturer (Genzyme, USA).

For N-terminal amino acid sequence analysis, Epo produced by tobacco was concentrated by C18 reverse-phase high-performance liquid chromatography (Cosmosil 5C18, Nacalai Tesque, Japan). After removing the solvent, the N-terminal amino acid sequence of Epo was determined using a 477A Protein Sequencer (Applied Biosystems, USA).

The secretion of tobacco-produced Epo

To determine the localization of tobacco Epo, culture medium and cell extract were processed as follows. Four-day cultures of C3•5•2 cells

were separated from the medium by filtration through nylon mesh. The cells (1 g) were then disrupted by sonication in 1 ml of 2 x extraction buffer and the cell extract was collected by centrifugation. Epo protein in the cultured medium and the cell extract were evaluated by EIA.

C3•5•2 cells were treated with 1% Cellulase Onozuka RS (Yakult, Japan) and 0.1% Pectolyase Y-23 (Seishin Pharmaceutical, Japan) dissolved in 20 mM 2-[N-morpholino] ethanesulfonic acid buffer, pH 5.6, containing 5 mM MgCl₂, and 0.4 M D-mannitol for 4 h at 26 °C. The protoplasts were cultured in M-LS medium supplemented with 0.4 M D-mannitol at 26 °C and Epo secreted into the culture medium was measured by EIA.

Assays of the biological activity of tobacco-produced Epo

The *in vitro* biological activity of Epo was assayed by means of its stimulatory effect on erythroid colony formation by erythroid precursor cells in methylcellulose culture [34]. Briefly, erythroid precursor cells prepared from fetal mouse liver were cultured in methylcellulose in the presence of various concentrations of Epo and the number of hemoglobinized erythroid colonies were counted. I also assayed *in vitro* Epo activity using the erythroleukemia line EP-FDC-P2, that requires Epo for growth [27]. The cell growth was measured colorimetrically by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cleavage [35].

Epo activity was assayed *in vivo* using starved rats (4 rats/sample) [36]. Epo (0-80 ng) was injected into the starved rats followed by the administration of ⁵⁹FeCl₃. The stimulatory effect of the injected Epo on the formation of red blood cells was determined by the incorporation of ⁵⁹Fe into nascent hemoglobin. The incorporation of ⁵⁹Fe was calculated from the following formula; ⁵⁹Fe incorporation (%) = radioactivity in 1 ml blood x body weight (g) x 0.05 / total radioactivity of the injected ⁵⁹FeCl₃. The data (Δ% incorporation) were evaluated by subtracting the mean value of the saline-injected rats from that of the Epo-injected rats.

Carbohydrate analysis

Ten nanograms of Epo in PBS containing 0.1% BSA (0.1% BSA-PBS) was applied to a packed column of Concanavalin A (Con A) or wheat germ agglutinin (WGA) (Hohnen Corporation, Japan). After washing with 7 ml of 0.1% BSA-PBS, Epo bound to the lectins was eluted with 8 ml of 0.2 M methyl- α-D-glucoside for Con A, and with 0.2 M N-acetyl-D-glucosamine for WGA. The fractionation was started

immediately after Epo was applied to the lectin columns. Epo in the fractions was measured by means of an EIA.

RESULTS

Production of Epo produced in tobacco cells

Figure 1 shows the structure of the Epo expression vector, pBINCEP, which contains human Epo cDNA encoding 166 amino acids of mature Epo plus a 27 amino acid signal peptide [20, 21]. Plasmid pBINCEP also carried the neomycin phosphotransferase type-II (NPT-II) gene conferring kanamycin resistance upon the transformed tobacco cells. Plasmid pBINCEP was introduced into BY2 cells by cocultivating the tobacco cells with *A. tumefaciens* LBA4404 harboring pBINCEP [33]. I obtained 15 kanamycin-resistant calli and the expression of NPT-II in all of them was confirmed by NPT II assay [37] (data not shown). The cell extracts were prepared from these calli to determine the expression of Epo protein by means of EIA and one callus, designated C3•5•2, was found to produce Epo at a level of 26 pg/mg total protein. Neither the production of Epo protein nor the expression of Epo mRNA was observed in the rest of the calli, although the integration of Epo cDNA

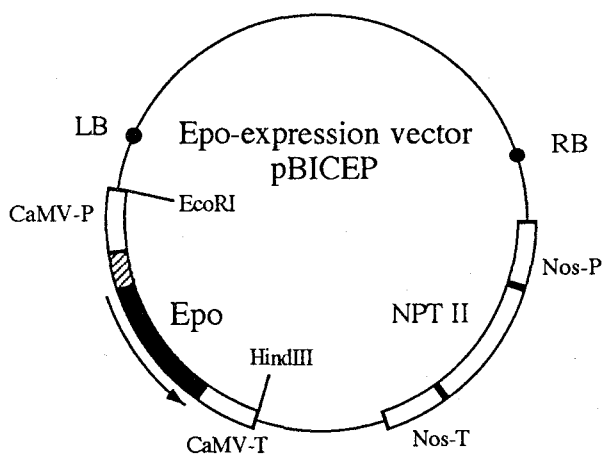


Fig. 1 A schematic representation of the human Epo expression vector pBINCEP. The black and slashed boxes represent Epo protein and the signal peptide sequences, respectively. The arrow indicates the orientation of transcription. Abbreviations: LB/RB, left/right T-DNA border sequence; Nos-P/Nos-T, nopaline synthase promoter/terminator sequence; CaMV-P/CaMV-T, cauliflower mosaic virus 35S RNA promoter/terminator sequence.

into the genome was confirmed in 11 calli by Southern hybridization (data not shown). C3•5•2 was then maintained in suspension culture for further analysis.

Structural analyses of tobacco-produced Epo

To characterize tobacco-produced Epo in detail, I attempted to purify the Epo from suspension cultured C3•5•2 cells by means of immunoaffinity chromatography using an anti-Epo monoclonal antibody [28]. As described below, Epo was not released into the culture medium of C3•5•2. Therefore, I purified Epo from the cell extract of C3•5•2 cells prepared from the whole cell fraction and obtained 13.1 µg of Epo from 6.3 kg of tobacco cells. The final Epo preparation was homogeneous as assessed by silver staining after SDS-PAGE (Fig. 2A).

Epo produced in tobacco migrated in the polyacrylamide gel with a molecular mass of 30 kDa, which was smaller than that of rHuEpo (37 kDa) (Fig. 2B, lane 1 and 3). When produced in mammalian cells, the mass of the rHuEpo varies depending upon the type of the host cells, due to a difference in the N-linked oligosaccharides [15], suggesting that tobacco-produced Epo has N-linked oligosaccharides that are smaller than those attached to rHuEpo. I removed the N-linked oligosaccharides from Epo with PNGase F, which possesses broad specificity for N-linked oligosaccharides [28] to determine the size of oligosaccharides attached to Epo. Upon digestion with PNGase F, the size of both Epo produced in tobacco and rHuEpo shifted to 20 kDa (Fig. 2, lane 2 and 4), which was equivalent to Epo without N-linked oligosaccharides [19, 27], supporting my contention. Although conversion to the deglycosylated form upon PNGase F digestion was complete in rHuEpo, about 60% of the tobacco-produced Epo remained unchanged. A 5-fold increase in PNGase F or a prolonged incubation with the enzyme did not influence the conversion. Thus, about 60% of the Epo produced in tobacco appeared to be resistant to PNGase F and this is probably due to the composition of the carbohydrate residues. The presence of carbohydrate residues such as β 1, 2-xylose linked to β -mannose in plant glycoprotein but not in mammalian glycoproteins, may protect Epo from deglycosylation by PNGase F [38].

The secretion of tobacco-produced Epo

To analyze the production of Epo during the suspension culture of C3•5•2 cells, the culture medium and the cell extract were processed and

A. Silver staining B. Western blotting

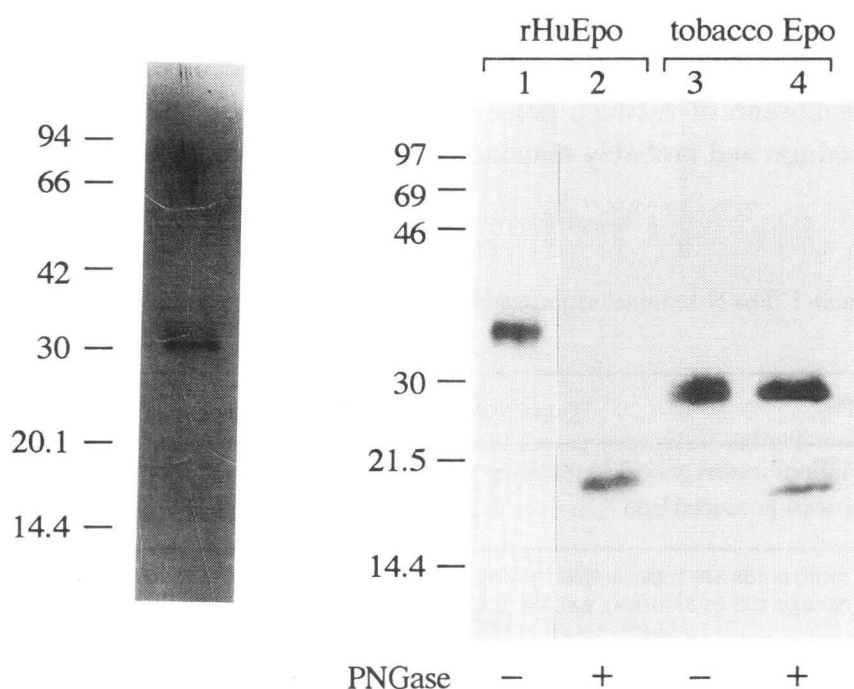


Fig. 2 The molecular mass of tobacco-produced Epo and its glycosylation. A: Silver staining. Tobacco Epo (40 ng) was applied. B: Western blot. Lanes 1 and 2, 25 ng rHuEpo; lanes 3 and 4, 25 ng tobacco Epo; lanes 1 and 3, without PNGase F; lanes 2 and 4, digested with PNGase F. Numbers on the left indicate the molecular mass markers (kDa).

assessed by EIA. As shown in Fig. 3, Epo was detected in the cell extract at levels which increased as the culture proceeded. On the other hand, Epo was not detected in the medium throughout the culture period. As Epo cDNA introduced into tobacco cell encoded intact human Epo including its native signal peptide, it should be secreted into the culture medium through the bulk-flow pathway of protein targeting. To obtain more information, I analyzed the secretion of Epo during the protoplast culture of C3•5•2 (Fig. 4). Epo was detected in the culture medium during protoplast culture, confirming that it could penetrate the plasma membrane assisted by the action of the signal peptide. I also sequenced the N-terminal amino acids of Epo produced in tobacco, to determine the native of the signal peptide processing. As shown in Table 1, the N-

terminal amino acid sequence of tobacco-produced Epo coincided perfectly with that of mature rHuEpo [20], indicating that the signal peptide did function, by targeting Epo into the protein secretory pathway. These results indicate that Epo produced in tobacco penetrated the plasma membrane of tobacco cells, but that it was not released into cultured medium and probably remained attached to the cell wall.

Table 1 The N-terminal sequence analysis of purified Epo produced in tobacco cells.

Epo	N-terminal sequence
rHuEpo	.. LPVLG* APPRLICDSR..
tobacco-produced Epo	.. LPVLG <u>APPRLIXDSR</u> ..

Amino acids are represented by single-letter. A star mark (*) indicates the signal peptide cleavage site of rHuEpo, and the underlined sequence was actually determined.

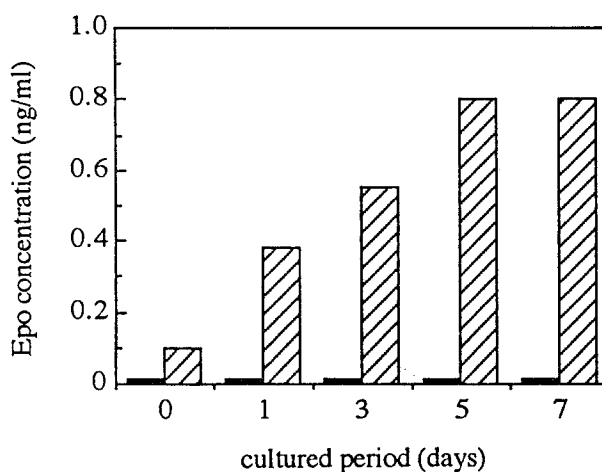


Fig. 3 The production of Epo during suspension culture of C3•5•2. The culture medium and the cell extract were processed as described in "Materials and methods" and the amount of Epo in each preparation was measured by EIA. The vertical axis represents the concentration of Epo assessed by EIA and the horizontal axis represents the culture period in days. The black (■) and slashed (▨) represent the culture medium and cell extract, respectively.

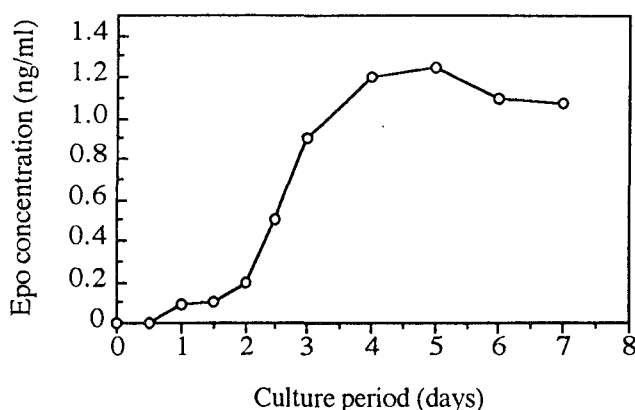


Fig. 4 The secretion of Epo during protoplast culture of C3•5•2. The amount of Epo secreted into the cultured medium during protoplast culture was measured by EIA. The vertical axis represents the concentration of Epo assessed by EIA and the horizontal axis represents the culture days.

The biological activities of Epo produced in tobacco

The proliferation- and differentiation-inducing activity upon erythroid precursors of Epo produced in tobacco cells, was assayed *in vitro* by measuring the erythroid colony formation of fetal mouse liver cells (Table 2). Upon adding tobacco-produced Epo or rHuEpo to the cell culture, the colony formation was induced dose-dependently upon the Epo concentration. The addition of the anti-Epo monoclonal antibody R6 completely suppressed the effect, indicating that contaminants derived from the tobacco cells were not responsible for this effect. It appeared that the Epo produced in tobacco stimulated the formation of erythroid colonies more effectively than rHuEpo. An *in vitro* assay using an Epo-dependent cell line, EP-FDC-P2, provided more precise data [27, 35], which revealed the unequivocal difference between Epo produced in tobacco and rHuEpo (Fig. 5). At a low Epo concentration before reaching the maximal activity, the former was clearly more active than the latter, indicating that Epo produced in tobacco bound to the Epo receptor with a higher affinity than rHuEpo. The enzymatic removal of sialic acids in carbohydrates attached to rHuEpo yields Epo with an increased affinity to its receptor and consequently, increased *in vitro* activity [19]. The higher activity of Epo produced in tobacco compared with rHuEpo, was probably due to its lack of sialic acids as described below.

Table 2. The *in vitro* biological activity of Epo measured by its stimulatory effect on erythroid colony formation.

Epo added	number of erythroid colonies formed	
	Epo concentration (ng/ml)	
	0.05	0.1
rHuEpo	113 $\pm$ 8.5	162 $\pm$ 50
tobacco-produced Epo	141 $\pm$ 25	180 $\pm$ 20
tobacco-produced Epo + R6	10 $\pm$ 3.5	6 $\pm$ 2.5

Each value is the mean $\pm$ SE in duplicate.

The *in vivo* biological activity of Epo produced in tobacco, was determined using starved rats, whose endogenous Epo level was decreased by starvation and therefore red blood cell formation was enhanced by an injection of exogenous Epo. As shown in Fig. 6, the new red blood cell formation was not stimulated by Epo produced in tobacco, whereas the stimulation was dose-dependent upon rHuEpo and its activity was retarded by the anti-Epo monoclonal antibody R6.

Carbohydrate analysis

As to why tobacco-produced Epo was biologically inactive *in vivo*, I presumed that it was excluded from the circulation due to the structure of the attached N-linked oligosaccharides, and not because the structure of Epo itself was aberrant, since it possessed *in vitro* biological activity. The terminal sialic acids in N-linked oligosaccharides of Epo are reportedly crucial to its *in vivo* biological activity [15, 19]. The difficulty in preparing pure Epo at high levels from tobacco did not permit the structure of its carbohydrates to be determined. I therefore explored their characteristics using the lectins, Con A and WGA. As shown in Fig. 7, Epo produced in tobacco bound to Con A (specific for mannose) [39] and it was specifically eluted with methyl- α -D-glucoside. Tobacco Epo did not bind to WGA (specific for terminal N-acetylneuraminic acids and N-acetylglucosamine dimers) [40, 41] (Fig. 7). On the other hand, rHuEpo, which possesses sialic acids in its N-linked oligosaccharides, bound to WGA, but not to Con A.

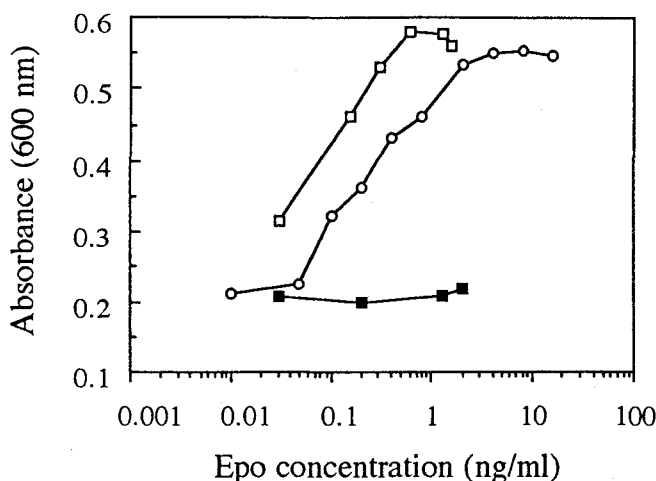


Fig. 5 The *in vitro* biological activity of tobacco-produced Epo. The biological activity of Epo *in vitro* was assayed using its growth-inducing effect on the Epo-dependent cell line, EP-FDC-P2 [27]. The absorbance at 600 nm represents the amount of cleaved MTT [35]. (○) rHuEpo; (□) tobacco-produced Epo; (■) tobacco-produced Epo in the presence of the anti-Epo monoclonal antibody R6 (50 µg/ml). Each point is the mean of duplicates.

DISCUSSION

Human Epo was produced in cultured tobacco cells, with a production level of up to 0.0026% of the total extractable proteins. When human Epo was produced in cultured tobacco cells without its signal peptide, the productivity was extremely low (1 pg/g wet cell) [Chapter 3 and ref. 29]. My results, together with those of other reports describing the production of human serum albumin and monoclonal antibodies [9, 10, 6], confirmed that the signal peptide contributes to the production of the mammalian proteins in plant cells. With regard to secretion, Epo was secreted only during the protoplast culture but not during the suspension culture of C3•5•2, where a cell wall was present (Fig. 3 and 4). This was somewhat unusual, because antibody and human serum albumin produced in plants are secreted into the medium [6, 42], despite their molecular mass being larger than the pore size of the plant cell wall, the exclusion limit of which was reportedly equivalent to a 20 kDa globular protein [43]. The secretion of Epo during protoplast culture reached a plateau on the fourth day, whereas the amount of Epo in the cell extract during suspension culture reached a plateau on the fifth

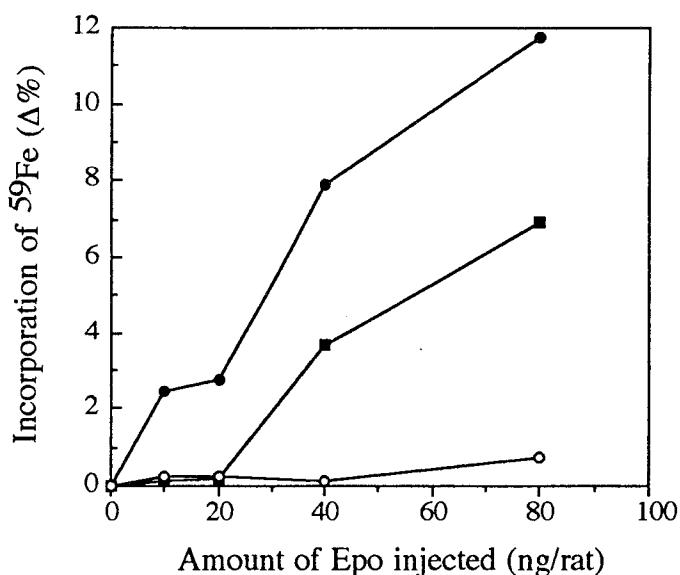


Fig. 6. The *in vivo* biological activity assay of Epo. The effect of injected Epo on the formation of red blood cells was determined by the incorporation of ^{59}Fe into nascent hemoglobin [13]. ($\bullet$), rHuEpo; ($\circ$), tobacco-produced Epo; ($\blacksquare$), rHuEpo in the presence of the anti-Epo monoclonal antibody R6 (50 $\mu\text{g/ml}$). Each point is the mean value of 4 rats.

day. Given that a significant amount of cell wall was reconstructed by the fourth day during protoplast culture (data not shown), the time lag was considered to be the result of the prohibited release of Epo into the culture medium caused by the new cell wall. These results indicated that Epo remained attached to the cell wall components and was not released into the medium during the suspension culture. Actually, I found during the purification procedure, that Epo tends to associate with cell wall components. A high incidence of glycosylation in Epo or the abundance of basic amino acids (18%) might contribute to the association of tobacco-produced Epo to the cell wall.

The major structural difference between Epo produced in tobacco and mammalian cells, is the carbohydrates attached to the peptides. The apparent molecular mass of Epo produced in tobacco, was 30 kDa compared with the 37 kDa of that produced in mammalian cells (Fig. 2). PNGase F digestion revealed that the difference was due to the size of the attached N-linked oligosaccharides. Although I did not digest tobacco

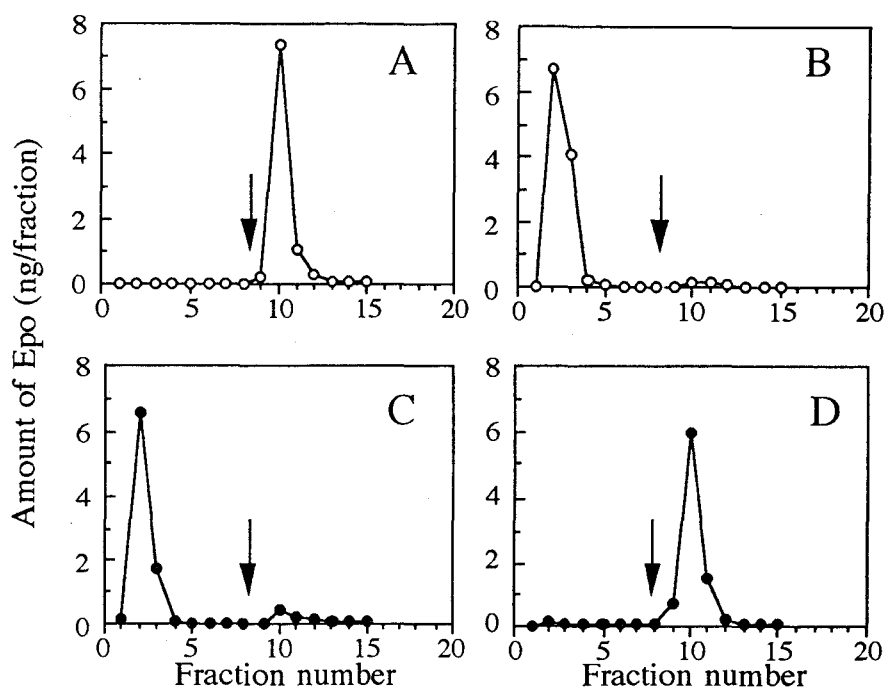


Fig. 7 The binding profile of Epo to ConA and WGA. Panel A, the binding profile of rHuEpo to WGA; panel B, tobacco-produced Epo to WGA; panel C, rHuEpo to ConA; panel D, tobacco-produced Epo to ConA. The vertical axis represents the amount of Epo in each fraction measured by EIA and the horizontal axis represents the fraction number. The arrows in the panels represent the starting position of elution by hapten sugars.

Epo with endo- β -*N*-acetylglucosaminidase H (Endo H), resistance to which is characteristic of complex type oligosaccharides [44], the results of the lectin binding assay coincided with that of mouse monoclonal antibody produced in tobacco [42]. Thus, the possibility remains that Epo produced in tobacco possesses complex type oligosaccharides.

My main interest was whether human Epo produced in tobacco cells could express biological activity *in vitro* and *in vivo*. I described in this chapter that Epo produced in tobacco was functional *in vitro*, but not *in vivo* (Fig. 5 and 6). As tobacco-produced Epo was capable of expressing biological activity *in vitro*, the failure to do so *in vivo* was not attributable to aberrant protein folding that might occur in the tobacco cells. I presumed that the cause lay in the stability of tobacco-produced Epo in the circulation, because desialylated rHuEpo loses *in vivo* biological activity [15, 19]. Lectin binding assays indicated that tobacco

Epo does not possess terminal sialic acids in its N-linked oligosaccharides (Fig. 7), which is reasonable, since sialic acid has not been identified in plants [38]. The predominant terminal sugar residues in mammalian glycoproteins are sialic acids, the removal of which causes the rapid clearance of the glycoproteins from the circulation by the action of the asialoglycoprotein receptor on hepatocytes [45]. Epo produced in tobacco cells, which lacked terminal sialic acids in its N-linked oligosaccharides, was probably trapped by the asialoglycoprotein receptor on hepatocytes and excluded from the circulation before it could express biological activity.

Although the results in this chapter indicated a reason for the *in vivo* instability of the plant-produced mammalian glycoproteins in the circulation, it also showed that plant cells could produce a cytokine which functions quite effectively *in vitro*. This property indicates that plant-produced mammalian glycoproteins could be used as *in vitro* pharmaceuticals, avoiding the problems associated with *in vivo* usage. One possibility is the use of tobacco-produced Epo as a growth factor in the *in vitro* propagation of erythrocytes. Obtaining adequate amounts of erythrocytes from a patient's erythroid precursor cells by *in vitro* propagation could avoid the dangers associated with the blood transfusion, which may be contaminated with pathogens such as human immunodeficiency virus. It is possible that tobacco-produced Epo will fulfill the requirements necessary for use as a growth factor in the *in vitro* propagation of erythrocytes.

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Summary

Chapter 1

Three glycoproteins, designated as 34K, 38K, and 40K were isolated from the spent medium of suspension cultured BY2 tobacco cells. The 38K and 40K were highly cationic peroxidases with indistinguishable enzymatic properties but their structural difference was confirmed by the sequence analysis of the amino terminal regions and the recognition specificity of the monoclonal antibodies. The 34K was a moderately cationic peroxidase with enzymatic properties quite different from those of the 38K and 40K enzymes. They were undetectable in the spent medium during the cell proliferation phase but became abundant in the medium during the cell expansion phase. This was confirmed quantitatively with the 40K using anti-40K monoclonal antibody. The mRNA expression for 40K was at a constant basal level in the cell proliferation phase but increased in the cell expansion phase.

Chapter 2

Two cDNAs encoding novel cationic peroxidase isozymes, 40K and 38K, that were abundantly secreted into the cultured medium of suspension cultured BY2 tobacco cells were isolated. The deduced amino acid sequence of 40K and 38K consisted of 331 and 329 amino acid residues, respectively, and a high homology (94%) was observed between them. 40K and 38K showed similarity to other plant peroxidase with 41-45% homology.

Chapter 3

Human Epo cDNA was engineered for expression in suspension cultured BY2 tobacco cells. Two plasmid DNAs were constructed: pCEP, which contained Epo cDNA under control of the cauliflower mosaic virus-derived 35S RNA promoter and terminator, and pNSEP, which contained signal sequence-deleted Epo cDNA under control of the 35S RNA promoter and terminator. By using the electroporation method, each of these plasmid DNAs was transferred into the protoplasts of BY2 cells together with a plasmid, pNR35, which conferred G418-resistance on the cells. Four G418-resistant clones were obtained from protoplasts transfected with pNSEP and pNR35, and only one of them,

named 11N, survived in suspension culture. Integration of pNSEP DNA into the genome of 11N cells was confirmed by Southern blot and PCR analyses. Production of Epo mRNA was shown by Northern blot analysis. Epo protein was shown to be expressed in 11N cells by colorimetric enzyme immunoassay. The productivity of Epo in the 11N cells (1 pg/g of wet cells) was very low.

Chapter 4

Epo, a glycoprotein that regulates the formation of erythrocytes in mammals, was produced in cultured tobacco BY2 cells by introducing human Epo cDNA via *Agrobacterium*-mediated gene transfer. Epo was correctly processed and subsequently penetrated the plasma membrane of tobacco cells. However, it remained attached to the cell wall and was not released into the culture medium. Although Epo produced by tobacco cells was glycosylated with N-linked oligosaccharides, these carbohydrates were smaller than those of the recombinant Epo produced in mammalian cells. Epo produced in tobacco exhibited *in vitro* biological activities by inducing the differentiation and proliferation of erythroid cells. However, it had no *in vivo* biological activities. A lectin binding assay indicated the lack of sialic acid residues in the N-linked oligosaccharides of Epo, suggesting that Epo was removed from the circulation before it reached erythropoietic tissues.

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List of Publications

1. Narita Hiroshi, Asaka Yukiko, Ikura Koji, Matsumoto Shinya and Sasaki Ryuzo : Cationic peroxidase isozymes released into the medium of cultured tobacco cells; isolation, characterization, and expression. (1995) Eur. J. Biochem. in press
2. Matsumoto Shinya, Narita Hiroshi, Ikura Koji and Sasaki Ryuzo : Nucleotide sequence of cationic peroxidases that are abundantly secreted by cultured tobacco cells. in preparation
3. Matsumoto Shinya, Ishii Atsushi, Ikura Koji, Ueda Masatsugu and Sasaki Ryuzo : Expression of human erythropoietin in cultured tobacco cells. (1993) Biosci. Biotech. Biochem., 57, 1249-1252
4. Matsumoto Shinya, Ikura Koji, Ueda Masatsugu and Sasaki Ryuzo : Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells. (1995) Plant Molecular Biology in press