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Highly efficient production of human interferon-α by transgenic cultured rice cells

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Abstract Interferon-α (IFN-α) is an important antiviral pharmaceutical. A binary vector containing the first intron of the rice cytosolic SOD gene, the signal sequence of the 10 kDa rice prolamin, the amino-terminal region of β-glucuronidase, a thrombin recognition site, and the mature polypeptide region of human IFN-α was constructed, under the regulation of the cauliflower mosaic virus 35S promoter. Here, we report that transgenic rice cells transformed with this fusion protein vector produced a biologically active IFN-α. The vector was introduced into rice calli by *Agrobacterium*-mediated methods. Five lines of transgenic calli were obtained. IFN assay demonstrated that these calli expressed fusion proteins bearing biologically active IFN-α. Liquid-cultured cells exhibited stable growth and the production of active IFN-α during 10 successive generations, i.e. in 10 weeks. The expressed proteins were purified by immuno affinity chromatography and reverse-phase HPLC. Repeated selections of cultured cells that had been obtained by dividing calli into small cell aggregates considerably increased the production of IFN-α. Thrombin protease treatment of the fusion protein yielded the intact IFN-α polypeptide. Thus, transgenic suspension rice cells are expected to be useful for the production of large amounts of biologically active proteins at a low cost; moreover, such a system would be easier to employ than animal cell culture systems.

Key words: Cell culture, Interferon-α, recombinant protein, transgenic rice.

Human IFN-α plays an important role in extracellular signaling pathways in which polypeptides are secreted by somatic cells stimulated by viruses, microorganisms, foreign cells, foreign macromolecules, or various other chemical compounds (Rubinstein et al. 1979 and 1981; Kurane et al. 1986). IFN-α enhances the defensive functions of surrounding cells, which may in turn regulate viral replication, the immune response, and cell growth (Lee et al. 1982; Attallah et al. 1987). Several cDNA clones of IFN-α have already been isolated and expressed in prokaryotic cells and in mammalian cells with the aim of preparing recombinant IFN-α for clinical use (Maeda et al. 1980; Strander et al. 1975). At present, IFN-α medicines are used in the clinical setting as treatments for cancer and various viral infections. However, it remains necessary to minimize the inclusion of foreign proteins and unknown viral contaminants during the production of pharmaceuticals utilizing bacterial expression systems and mammalian cell culture systems. Such cell culture systems, especially animal cell cultures, require nutrient-enriched media, such as serum. Therefore, the production of pharmaceuticals from animal cell cultures necessitates expensive and time-consuming purification from a supernatant or extract. Moreover, it is crucial to confirm that the successful elimination of potential contaminants has taken place when such systems are used.

Whereas recombinant, biologically active human proteins such as IFN-α produced by *E. coli* and naturally occurring proteins purified from cultured human cells are already in clinical use, transgenic plants have emerged as an attractive expression system for the production of such proteins (Goddijn and Pen 1995) for reasons of both cost and purity. There are several advantages to the use of plants as "pharmaceutical factories". The production of biologically active proteins from plant cell cultures is

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; CaMV, Cauliflower mosaic virus; CPE, cytopathic effect; DDBJ, DNA data bank of Japan; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; EIA, enzyme immunoassay; ER, endoplasmic reticulum; GUS, β-glucuronidase; IFN, interferon; NBT, nitro blue tetrazolium; PB, protein body; PCR, polymerase chain reaction; RP-HPLC, reverse phase high performance liquid chromatography; SOD, superoxide dismutase; 2,4-D, 2,4 dichlorophenoxyacetic acid; VSV, vesicular stomatitis virus.
This article can be found at http://www.jspcmb.jp/

less expensive than that of similar proteins from traditional animal cell culture systems, as plant cells can grow under relatively simple culture conditions. Furthermore, the newly synthesized product can easily accumulate in large quantities in target organelles such as the ER, protein storage vacuoles, and plastids, apart from the inherent protease. The risk of contamination of the products by pathogenic compounds contaminating the culture medium is also reduced in plant-based systems as opposed to animal-based systems. In addition, when using plants, it is possible to induce sugar chain modifications of newly synthesized proteins, and such modifications may be particularly important in the context of producing plant-based recombinant mammalian proteins.

Enhancements of these advantages of using plant cells as pharmaceutical factories are achieved by the use of plant-cell culture methods, particularly when studies are coupled with chemical methods of analysis. It is much easier to control plant cell growth conditions to maintain the desired yield and quality, and the growth parameters can be optimized (e.g., pH, changes in nutrient media, temperature, etc.) in order to significantly improved yield. The separation of target compounds is also much easier due to the reduced complexity of the cell materials. Thus, plant cell cultures are expected to be an excellent source of biologically active proteins, and they are moreover likely to be significantly superior to whole plant systems, the productivity and quality of which are often affected by natural disturbances and denature of proteins during isolation.

In this study, we used human IFN-α a2 DNA and the dwarf rice genome. Previously, we established transgenic rice plants that produce rice grain with a biologically active form of IFN-α for the oral delivery to the intestine of biologically active proteins packed into seed protein bodies (Masumura et al. 2006); the aim of this treatment was to induce mucosal immunity. The production of pharmaceutical protein in rice grain required substantial facilities and was also time-consuming. We thus attempted to produce IFN-α in cell cultures and we investigated the characteristics of recombinant IFN-α. Using a callus selection technique, we then attempt to select a transgenic rice callus that express a high level of IFN-α. We then confirmed that transgenic rice cells produced IFN-α in liquid culture with a standard inorganic medium, and the IEN-α produced exhibited long-term stability.

Materials and methods

Construction of plasmid vector expressing human IFN

Human IFN-α 2a DNA (DDBJ, accession No. M11003) was amplified by a PCR method using specifically designed primers and human blood as the template DNA, because genomic IFN-α 2a DNA has no intron sequence. The forward primer (GUS-*Sna*F) was 5′-CTCCTG-GTGCTCATACGTAAGTCACGCGGCTCTGTGGGCT G-3′ containing a *Sna*BI site, and the reverse primer (GUS-*Sac*R) was 5′-CATTTCCATGTTGGAGCTCTTT-TCATTCCTTACTTC-3′ containing a *Sac*I site. The amplified PCR product was digested with *Sna*BI and *Sac*I, and was then subcloned into a plasmid vector containing the CaMV 35S promoter, the first intron of the cytosolic rice SOD gene (*SodcC1*), as well as partial sequence coding for a 24-amino acid (a.a.) signal peptide and a 19-a.a. mature polypeptide of a 10-kDa rice prolamin (Masumura et al. 1989) and the partial sequence (126 a.a.) of the *E. coli* GUS coding region. Subsequently, the *Xba*I-*Sac*I fragment, which consisted of the thrombin recognition sequence (8 a.a.) and a mature polypeptide sequence (167 a.a.) of IFN-α was subcloned into a pIG121Hm plasmid (Ohta et al. 1986). The expression vector (pIG-A11) used for the transformation of the rice was then constructed (Figure 1).

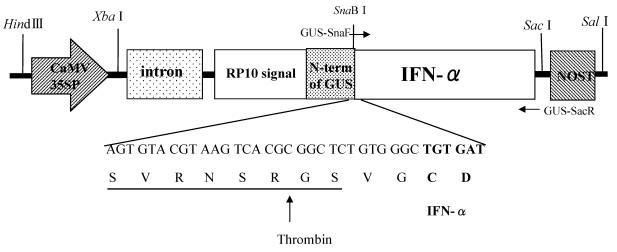

Figure 1. Recombinant DNA for the expression of human IFN-α.

Abbreviations: CaMV35SP, cauliflower mosaic virus 35S promoter; intron, 5′ first intron of cytosolic rice superoxide dismutase gene (*sodcC1*); RP10 signal, signal sequence and NH_2-terminal region of 10 kDa rice prolamin cDNA; N-term of GUS, NH_2-terminal region of GUS coding region; Thrombin, thrombin recognition site; IFN-α, mature polypeptide region of human IFN-α 2a DNA; NOST, 3′ termination signal of nopaline synthase. The partial amino acid sequence shows the joint region for a partial sequence of GUS, the thrombin recognition site, and the NH_2-terminal amino acid sequence of IFN-α; Vertical lines represent restriction enzyme recognition sites. Arrows indicate the designed PCR primers.

Transformation of rice callus using an Agrobacterium-mediated method

Competent cells of one of two *Agrobacterium* strains (EHA101 or EHA105) were prepared with 10% (w/v) glycerol solution (Chen et al. 1994). The competent cells were transformed with a pIG-A11 plasmid using an electroporation apparatus (Bio-Rad, Hercules, CA, USA) under the following conditions: 25 μFD, 200 Ω, and 2.5 kV, in 1-mm cuvettes. After selection with kanamycin, bacterial colonies containing IFN-α DNA were detected using PCR. Somatic calli from dwarf rice (*Oryza sativa* L cv. Hosetsu-dwarf, Kurita et al. 2002) seed embryos were infected with pIG-A11 transformed

Agrobacterium in AA medium supplemented with 20 g l^{-1} of glucose, 0.2 mg l^{-1} of kinetin, 2 mg l^{-1} of 2,4-D, and 10 mg l^{-1} of acetosyringone for several minutes (Toriyama and Hinata 1985). After infection with the *Agrobacterium*, the calli were co-cultured on N6 agar medium supplemented with 10 mg l^{-1} of acetosyringone. Small pieces of callus were transferred onto N6 agar medium supplemented with 500 mg l^{-1} of carbenicillin and 20–50 mg l^{-1} of hygromycin in order to remove the *Agrobacterium*.

Selection and cell culture of transgenic rice calli expressing high levels of IFN-α activity

Small fragments of transgenic calli were placed on N6 agar plates supplemented with 2 mg l^{-1} of 2,4-D, 2 g l^{-1} of gelrite, 500 mg l^{-1} of carbenicillin, and 40 mg l^{-1} of hygromycin. The hygromycin-selected calli were incubated for 9 days at 30°C. The levels of expression of IFN-α in the growing transgenic calli were determined. Moreover, transgenic calli were separated into small aggregates of less than 0.5 mm in diameter, and these aggregates were subsequently placed on the grids of Gamborg's B-5 medium agar plates (Gibco BRL, Rockville, MD, USA, Gamborg et al. 1968) supplemented with 10 g l^{-1} of sucrose, 330 mg l^{-1} of ammonium sulfate, 2 mg l^{-1} of glycine, 2 mg l^{-1} of 2,4-D, and 50 mg l^{-1} of hygromycin. After incubation of the hygromycin-selected calli at 28°C for 3 weeks, the levels of expression of IFN-α in the growing transgenic calli were measured.

Growing calli were sieved with 1-mm mesh and those cell aggregates were then suspended in Gamborg's B-5 medium. The cell aggregates were washed with Gamborg's B-5 medium once, then resuspended in Gamborg's B-5 medium supplemented with 10 g l^{-1} of sucrose, 330 mg l^{-1} of ammonium sulfate, 2 mg l^{-1} of glycine, 2 mg l^{-1} of 2,4-D, and 50 mg l^{-1} of hygromycin. The rice cell suspensions were incubated at 30°C at 100 rpm in a reciprocating shaker. Every week, a quarter of the cultured cells was transferred into fresh medium on fresh-weight balls and growth and IFN-α productivity were analyzed for a total more than 10 weeks.

Detection of IFN-α in transgenic rice calli

Transgenic calli were homogenized in 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.1% Triton X-100, and 0.1% sodium N-dodecanoyl sarcosinate using a Potter-type Teflon® homogenizer on ice for 1 min. The extracts were centrifuged at 8,900×*g* for 30 min at 4°C, and were then subjected to antiviral activity measurements and enzyme immunoassay, as described below.

The antiviral activity of IFN-α was determined by a dye-uptake method based on cytopathic effects (CPEs) using FL cells derived from human amnion and vesicular stomatitis virus (VSV) in 96-well microtiter plates (Rubinstein et al. 1979). After viral infection, the surviving cells were stained with neutral red, extracted with 50% (v/v) ethanol solution, and the estimation of dye uptake was carried out by measuring the absorbance at 546 nm using a plate reader (Vmax, Molecular device, Sunnyvale, CA, USA). The titers were measured in international reference units. The enzyme immunoassay (EIA) was performed with horse anti-human IFN-α polyclonal antibody and its horseradish peroxidase-conjugated antibody using a previously reported sandwich method (Shirono et al. 1990a). The NIH international standard of human leukocyte IFN-α was used for both the CPE and the EIA assays. The protein concentrations of all samples were determined using a BCA protein assay reagent (PIERCE, Rockford, IL, USA). *E. coli*-derived recombinant human IFN-α 2a (CANFERON®) was purchased from Takeda Chemical Industries (Osaka, Japan)

Extraction and purification of IFN-α from transgenic rice calli

Liquid-cultured cells were harvested with gauze, and were suspended in a 50 mM sodium phosphate buffer solution (pH 7.0) containing 10 mM EDTA, 0.1% Triton X-100, and 0.1% sodium N-dodecanoyl-sarcosinate. Then, the cells were homogenized on ice for 1 min with a Potter-type Teflon® homogenizer. The extracts were centrifuged at 8,900×*g* for 30 min at 4°C, ultra-filtrated, and then were dialyzed in phosphate-buffered saline using a Microsa hollow-fiber module (molecular weight cut-off, 10,000; Asahikasei, Tokyo, Japan). The samples were then subjected to immuno affinity chromatography using anti-human IFN-α polyclonal antibody-immobilized Sepharose (Shirono et al. 1990a, 1990b). The eluate obtained with 0.1 M citric acid, 0.1 M sodium chloride (pH 2.0) was subjected to RP-HPLC using a C18 column (HiPore® RP-318, Bio-Rad). IFN was eluted with a linear gradient of acetonitrile (25 to 70%) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml min^{-1}.

Analysis of purified IFN-α from cultivated transgenic calli

The IFN-α polypeptides purified by HPLC were separated on 10–20% polyacrylamide gradient gels under reducing conditions with 0.1% sodium dodecyl sulfate (SDS). The separated polypeptides were transferred onto nitrocellulose membranes using a semi-dry electro-blotting apparatus (Bio-Rad) and then were detected with mouse anti-human IFN-α monoclonal antibody, alkaline phosphatase-labeled anti-mouse IgG (CHEMICON, Temecula, CA, USA), and NBT/BCIP (Western Blue stabilized substrate for alkaline phosphatase, Promega, Madison, WI, USA), as described elsewhere (Towbin et al. 1979). The NH_2-terminal amino

acid sequence was determined with a model 476A peptide sequencer (PE Applied Biosystems, Foster City, CA, USA). The fusion protein containing the GUS amino-terminal region and IFN was cleaved with thrombin (Amersham Pharmacia Biotech, Piscataway, NJ, USA) under the following conditions: 10 units mg^{-1} fusion protein mixed with PBS at 25°C for 16 h (Guillin et al. 1977).

Results

Establishment of rice callus with human IFN-α DNA by an Agrobacterium-mediated method

Two strains of *Agrobacterium* (EHA101, EHA105) were used for the transformation of rice. In the case of EHA101, 357 calli were recovered and 21 calli survived following selection with hygromycin. IFN-α analysis revealed that only one line of calli (No. a-1) exhibited IFN-α activity. In the case of EHA105, 857 calli were recovered (Masumura et al. 2006), and all four cell lines of calli (No. a-2, a-3, a-4, and a-5) selected on hygromycin medium showed IFN-α activity.

The IFN-induced antiviral activity of these five lines of transgenic calli expressing IFN-α was measured using the abovementioned EIA and CPE methods. The IFN activity (international units: IU in the extracts per mg fresh weight of the calli) ranged between approximately 70 IU and 150 IU, as determined by EIA, and between approximately 120 IU and 280 IU, as determined by CPE assay (Table 1). The expression levels of the five lines of transgenic rice calli differed, but production of biologically active IFN-α was confirmed in all of the cell lines. In particular, the level of expression in cell line a-3 was highest among the 5 lines, and we therefore used this line for all subsequent experiments.

Production of IFN-α in suspension cultures

To confirm the stability of IFN-α production in the cell suspension culture, the activity and yield of IFN-α of transgenic line a-3 were assessed under long-term cultivation conditions. Calli with fresh weights of 15–25 g (average weight of 20 g) were sub-cultured on a weekly basis in 400-mL liquid medium in Erlenmeyer flasks, and the IFN-α yields were assayed by EIA. The levels of expression of IFN-α in the transgenic cells fluctuated on a weekly basis due to weekly changes in the growth patterns of the cultivated calli. The amount of IFN content in the extracts per mg fresh-weight of cultured cells ranged between approximately 300 IU and 600 IU, as determined by EIA (Figure 2, solid line). The average estimated IFN yield for a single week was approximately 8 million IU (MIU) per flask (Figure 2, shaded bar). Furthermore, these cultured cells produced IFN-α over a cultivation period of 12 weeks, without exhibiting any remarkable decreases in expression. Moreover, no IFN activity was detected in the liquid culture supernatants.

Table 1. IFN activities produced by transgenic rice calli.

Calli No.	ELISA (IU mg^{-1} callus)	CPE (IU mg^{-1} callus)
a-1	69.30	120.66±15.92
a-2	110.21	187.62±28.38
a-3	148.76	278.90±38.89
a-4	112.19	196.29±18.53
a-5	100.36	165.67±18.00
wild	6.86	1±1

IU: international unit, mean±SD, n=6

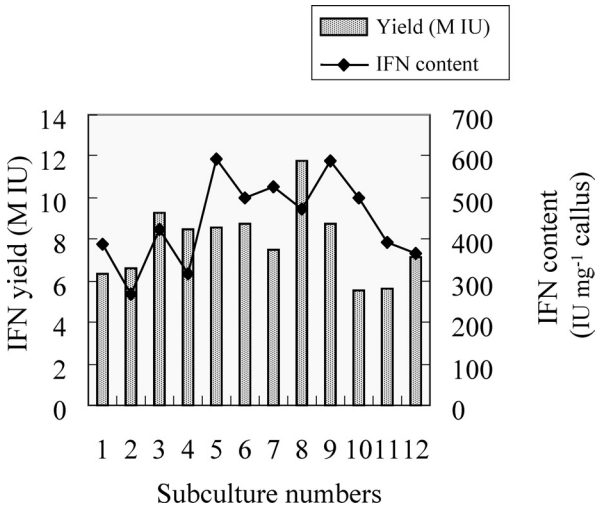

Figure 2. Stability of IFN production in long-term liquid culture.

The liquid suspension culture of rice cells was carried out at 30°C at 100 rpm using a reciprocating shaker. Once weekly, a quarter of the cultured cells were transformed into fresh medium and remaining three quarters of the cultured cells were harvested for IFN analysis. The solid line indicates the content of IFN-α per mg of fresh cell weight, and the shaded bars indicate the total IFN-α yields per week in 400-mL liquid medium. The horizontal axis indicates the subculture number corresponding to the number of weeks after the first inoculation.

Selection of calli producing a high yield of IFN-α

In order to improve the yield of IFN-α products, growing transgenic calli from line a-3 were smashed and split into tiny pieces of less than 0.5 mm in diameter and the samples were incubated on agar plates containing hygromycin. After a 4-weeks incubation of approximately thirty calli samples, the IFN expression levels and the amount of protein were measured. Initially, the IFN expression level of line a-3 calli was approximately 150 IU mg^{-1} fresh weight, but after hygromycin selection, the IFN expression levels of several calli increased. Repeatedly, selected calli (a-3-5, approximately 500 IU mg^{-1} fresh weight) were sieved and cultivated. Hygromycin selections of the growing a-3-5 line enhanced the IFN expression level of selected calli (a-3-5-30, approximately 800 IU mg^{-1} fresh weight). Additional selections further increased the

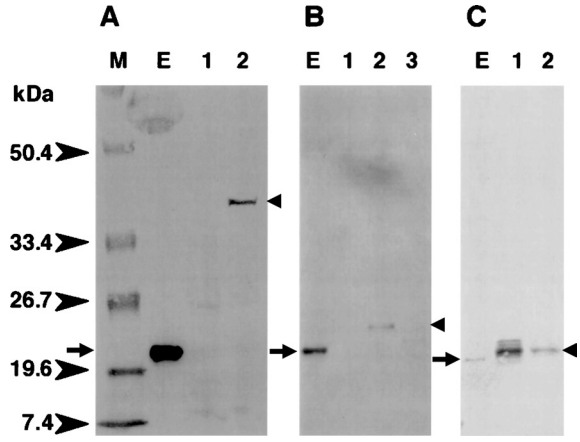

Figure 3. Western blot analysis of proteins expressed in rice calli.

The IFN-α polypeptides purified by RP-HPLC were separated on 10–20% polyacrylamide gradient gel under reducing conditions with 0.1% SDS. The separated polypeptides were transferred onto nitrocellulose membranes and were then processed with mouse anti-human IFN-α monoclonal antibody, alkaline phosphatase-labeled anti-mouse IgG, and NBT/BCIP. Lane M indicates the molecular size markers. Lane E shows *E. coli*-derived recombinant human IFN-α 2a polypeptide (arrow). Panel A: 1, extract from wild-type calli; 2, extract from line a-3 calli (arrowhead indicates the GUS-IFN-α fusion). Panel B: Fractions of RP-HPLC. Lane 1, retention time 47.0–47.5 min (acetonitrile 52.75–53.13%); lane 2, 47.5–48.0 min (acetonitrile 53.13–53.50%). The arrowhead indicates, the truncated GUS-IFN-α protein. The mechanism of this truncation during RP-HPLC remains unknown. Lane 3, 48.0–48.5 min (acetonitrile 53.50–53.88%). Panel C: Fusion proteins digested with thrombin protease. Lane 1, digested for 4 h; lane 2, digested for 16 h; the arrowhead indicates IFN-α of almost the expected size.

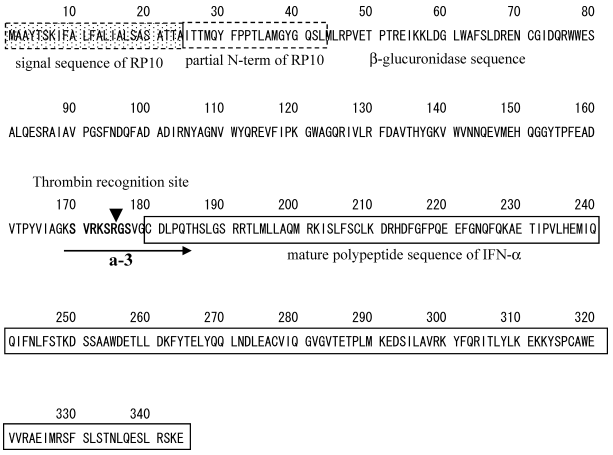

Figure 4. NH_2-terminal amino acid sequence of purified fusion proteins.

The NH_2-terminal amino acid sequence was analyzed using a model 476A peptide sequencer. The NH_2-terminal amino acid sequences of the fusion proteins isolated from a-3 began at the 169th lysine (horizontal arrow). The fusion protein of the GUS amino terminal region and IFN-α was cleaved by treatment with thrombin. The NH_2-terminal amino acid of protein digested with thrombin protease began at the 176th glycine (vertical arrowhead). The shaded dotted box shows the rice prolamin signal peptide, and the dotted box shows the prolamin partially mature polypeptide. The letters in boldface indicate the recognition site for thrombin protease. The region in the box represents the mature polypeptide sequence of human IFN-α 2a.

productivity of IFN-α, i.e., approximately 2,600 IU mg^{-1} fresh weight was observed in the selected calli (a-3-5-30-15). This level of IFN activity of line a-3-5-30-15 calli (2,600 IU mg^{-1} fresh weight) was approximately 17 times higher than the initial level of IFN activity of line a-3 (150 IU mg^{-1} fresh weight).

Characterization of IFN-α purified from rice calli

The IFN-α extracted from cultured rice cells was purified with Sepharose 4B immobilized with anti-IFN-α polyclonal antibody on a C18 RP-HPLC column. IFN was eluted with a linear gradient of acetonitrile (25 to 70%) in 0.1% TFA at a flow rate of 1.0 ml min^{-1}. The elution pattern of fusion proteins was monitored at 220 nm. Highly active IFN-α with specific activity of more than 1.2×10^8 IU mg^{-1} protein was obtained after this purification processes with a yield of approximately 70%. As Figure 3A illustrates, the extracts of line a-3 calli contained the complete fusion protein at the predicted molecular size (37 kDa). However, the RP-HPLC fractions of fusion protein purified with immuno affinity chromatography yielded proteins of a molecular size (24 kDa) smaller than that of the complete fusion protein (37 kDa, Figure 3B), but thrombin protease treatment gave proteins of the expected molecular size (22 kDa, Figure 3C). The amino terminal sequence of purified GUS-IFN fusion proteins showed that the NH_2-terminal amino acid sequence of the purified fusion protein from line a-3 calli began with a lysine at a.a. 169 (horizontal arrow), and no amino acid residue encoded by the cDNA sequence of the GUS fusion portion was detected. Additional NH2-terminal amino acid analysis revealed that the protein digested with thrombin protease had the correct amino acid sequence (vertical arrowhead), thus indicating the usefulness of this IFN-α producing system.

Discussion

In recent years, numerous researchers in the biopharmaceutical field have been actively developing vaccines and therapeutic proteins utilizing transgenic plants (Parmenter et al. 1995, Tacket and Mason 1999; Ma et al. 2005). For the purpose of oral administration and immunization, cereals such as rice can be utilized for the production of edible vaccines.

In the present study, human IFN-α a2 DNA was introduced into the dwarf rice genome via an *Agrobacterium*-mediated method. Five lines of transgenic rice calli showed the production of a biologically active form of IFN-α. When these calli were cultivated in liquid culture medium, the levels of expression of fusion protein were stably maintained without any diminution of activity for more than 10

culture generations (12 weeks). The extraction of IFN-α from the calli was easy and did not require any denaturing reagent such as a high concentration of urea. The purification of the fusion protein from the extract was easily achieved by polyclonal antibody affinity chromatography. Whereas RP-HPLC produced a truncated amino terminal at the 169th position (lysine), the thrombin protease treatment produced a single band corresponding to the predicted size of the intact IFN-α protein. In fact, the NH_2-terminal amino acid sequence showed that the almost complete IFN-α protein was practically produced by thrombin treatment.

Cell cloning systems involving mammalian cells stably transfected with expression vector and hybridomas that produce monoclonal antibody have frequently been used to obtain clones that express large amounts of protein in an efficient manner (Mao and France, 1984, Rohrer et al. 1995, Schinkel et al. 2005). Previously, we attempted to clone rice calli using a similar approach, but no callus could be recovered from the single-cell cultures. Calli growing on agar plates were separated into tiny aggregates of about 0.5 mm in diameter, and these cell aggregates were incubated on agar plates for 4 weeks. After 3 successive selections, IFN expression levels of the calli increased by approximately 17-fold, as compared to the starting levels of expression in the initial calli. It is possible that the rice calli possessing the ability to produce high levels of IFN were enriched during the selection process. These results strongly suggest that repeated selection would be useful to improve the ability of transgenic cells to produce IFN in an increasingly efficient manner.

Recently, many biopharmaceutical researchers have been actively developing vaccines and therapeutic proteins utilizing transgenic plants (Kumagai et al. 1993, Parmenter et al. 1995, Dieryck et al. 1997, Tacket and Mason 1999). In this study, we demonstrated that IFN, a biologically active human protein could be obtained in a stable manner from cultivated calli using a simple inorganic liquid medium. Our method is simple and rapid in comparison with system involving animal-based sources, it does not require any specific serum, and there is no risk of contamination by infectious agents such as viruses. Rice cell could be obtained from various useful transgenic crops, since such cells can be cultured under completely controlled environments.

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