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Production of biologically active human interferon-α in transgenic rice

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Abstract Interferon-α (IFN-α) is a principle cytokine that plays a key regulatory role in mammalian immune systems. The recombinant DNA of IFN-α is composed of the signal sequence region for rice 10 kDa prolamin cDNA, the amino-terminal region of β-glucuronidase DNA, and the mature polypeptide region of human IFN-αDNA, and is expected to produce a biologically active form of IFN-α. This chimerical DNA for coding IFN-α under the control of a cauliflower mosaic virus 35S promoter and a 5′-intron of rice cytosolic superoxide dismutase gene (*sodcC1*), was transformed into dwarf rice by an *Agrobacterium*-mediated system. Three lines of transgenic rice plant expressing various levels of IFN-α polypeptide were finally generated. The expression level of the recombinant polypeptide in each line was analyzed by an IFN-α antiviral activity assay and enzyme immunoassay. Higher expression of IFN-α was achieved in developing seed endosperm in two of transgenic rice lines. The replacement of the native signal peptide of IFN-α with the prolamin signal peptide was effective for transporting the IFN-α polypeptide into the ER-derived protein body of developing seed endosperm. These results suggest that rice can be used to produce many biologically active mammalian proteins that accumulate in target organelles such as protein bodies.

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

Recombinant biologically active human proteins produced by *E. coli* or natural-type proteins purified from human cultured cells are already being used as medicines for the treatment of human diseases. Transgenic plants have also emerged recently as attractive expression systems for the production of recombinant biologically active proteins (Goddijn and Pen 1995). There are several advantages to the use of plants as "pharmaceutical factories": (1) the production of biologically active proteins from plants is less expensive than that from traditional microbial or animal cell culture systems; (2) it is easy to accumulate the newly synthesized product into the target organelle; and (3) the risk of contaminating the products by mammalian pathogens is reduced. Moreover, plants can modify newly synthesized proteins, which may be particularly important in the production of plant recombinant mammalian proteins.

Since the first report on the production of a functional full-length mouse monoclonal antibody (mAb) in transgenic tobacco (Hiatt et al. 1989) appeared, many other important therapeutic proteins have been expressed in transgenic plants. These include human diagnostic and therapeutic antibodies, bacterial and viral antigens, and edible vaccines (Giddings et al. 2000; Carter and Langridge 2002).

Human IFN-α is an extracellular signaling protein secreted by body cells corresponding to that cells stimulated by viruses, microorganisms, foreign cells, foreign macromolecules, or various other chemical compounds (Rubinstein et al. 1979 and 1981; Kurane et al. 1986). Then IFN-α enhances the defensive functions in surrounding cells; these functions may regulate virus replication, the immune response, or cell growth (Lee et al. 1982; Attallah et al. 1987). Several cDNA clones of IFN-α have been isolated and expressed in prokaryotic cells and mammalian cells to prepare recombinant IFN-α for clinical use (Maeda et al. 1980; Strander et al. 1975). At present, clinical IFN preparations are used for anticancer and antiviral therapy. However,

Abbreviations: CaMV, Cauliflower mosaic virus; CPE, cytopathic effect; DDBJ, DNA data bank of Japan; EDTA, ethylenediaminetetraacetic acid; EIA, enzyme immunoassay; ER, endoplasmic reticulum; GUS, β-glucuronidase; IFN, interferon; PB, protein body; PCR, polymerase chain reaction; SOD, superoxide dismutase; 2,4-D, 2,4 dichlorophenoxyacetic acid; VSV, vesicular stomatitis virus.

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for the production of pharmaceuticals utilizing bacterial expression systems or mammalian cell culture systems, it is necessary to minimize contamination by foreign proteins and unknown viruses. Therefore, pharmaceuticals produced in cell culture have to be purified from the culture supernatant or extract an expensive process. Moreover, a method for measuring the elimination of potential contaminants must be developed.

On the other hand, the genetic modification of plants, which entails the isolation and manipulation of target DNA and the subsequent modified DNA transduction into a plant genome or plant cells, has been utilized to increase yields, reduced production costs, and enhance pest resistance, stress tolerance, and drought resistance (Knight et al. 1991, Wunn et al. 1996). In the last several years, rice transformation mediated by *Agrobacterium tumefaciens* has become the most useful method for introducing foreign genes into plant cells and the subsequent regeneration of transgenic plants. *Agrobacterium* has an exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nuclei of infected cells, where it is then stably integrated into the host genome and transformed (Hiei et al. 1994; Cheng et al. 1998; Bajaj and Mohanty 2005).

In this study, we transformed human IFN-α DNA into the rice genome with the *Agrobacterium* Ti plasmid and obtained a rice seed that produces a biologically active form of IFN-α, toward the goal of developing a more convenient and safer means of orally delivering biologically active proteins as opposed to parental injection in patients. We confirmed that rice seed grown indoors by a dwarf rice cultivar contained IFN-α.

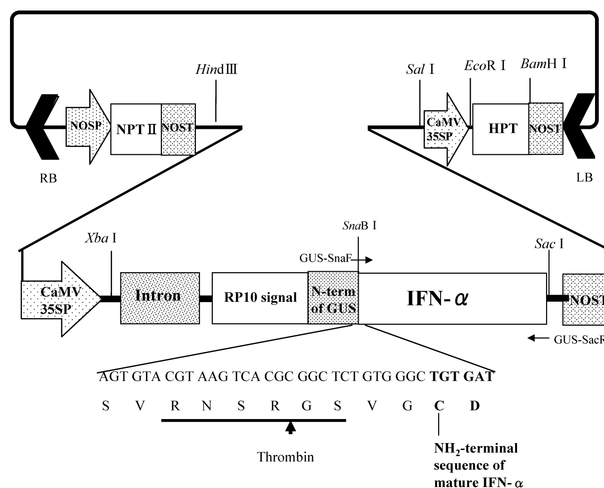

Figure 1. Plant transformation vector for the expression of human IFN-α (pIG-A11). Abbreviations: RB, right border; LB, left border; NOSP, nopaline synthase promoter; NPT II, neomycin phosphotransferase gene; NOST, 3′ termination signal of nopaline synthase; CaMV35SP, cauliflower mosaic virus 35S promoter; Intron, 5′ first intron of rice cytosolic superoxide dismutase gene (*sodcC1*); RP10 signal, signal sequence and NH_2-terminal region of rice 10 kDa prolamin cDNA; N-term of GUS, NH_2-terminal region of GUS gene; IFN-α, mature polypeptide region of human IFN-α 2a DNA; HPT, hygromycin phosphotransferase gene. Thrombin, thrombin recognition sequence; arrowhead points to the thrombin recognition site; The partial amino acid sequence shows the joint region for the partial sequence of GUS, thrombin recognition site, and NH_2-terminal amino acid sequence of IFN-α. Vertical lines are restriction enzyme recognition sites. Horizontal arrows are designed PCR primers.

Materials and methods

Construction of plasmid vector expressing human IFN-α

Human IFN-α 2a DNA (DDBJ, accession No. M11003) was amplified by PCR using specifically designed primers and human blood as template DNA, because genomic IFN-α 2a DNA has no intron sequence. The forward primer (GUS-SnaF) was 5′-CTCCT-GGTGCTCATACGTAAGTCACGCGGCTCTGTGGGCTG-3′ containing an *Sna*BI site, and the reverse primer (GUS-SacR) was 5′-CATTTCCATGTTGGAGCTCTT-TTCATTCCTTACTTC-3′ containing an *Sac*I site. The amplified PCR product was digested with *Sna*BI and *Sac*I, then subcloned into a plasmid vector containing the CaMV 35S promoter, the first intron of the rice cytosolic SOD gene (*SodcC1*), the signal sequence region (24 a.a. of the signal sequence region and 19 a.a. of the mature sequence region) of rice 10 kDa prolamin (Masumura et al. 1989), and a 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 region (8 a.a.) and the mature polypeptide sequence region (167 a.a.) of IFN-α was subcloned into the pIG121Hm plasmid (Ohta et al. 1986). An expression vector for rice transformation was constructed and named pIG-A11 (Figure 1).

Transformation of rice plant by Agrobacterium-mediated method

Competent cells of an *Agrobacterium* strain (EHA101 or EHA105) were prepared with 10% (w/v) glycerol solution (Chen et al. 1994). The competent cells were transformed with the pIG-A11 plasmid using an electroporation apparatus (Bio-Rad, Hercules, CA, USA) under the conditions of 25 μFD, 200 Ω, and 2.5 kV in a 1 mm cuvette. After selection with kanamycin, the introduced IFN-α DNA was detected by 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 *Agrobacterium*, the calli were co-cultured on N6 agar medium supplemented with 10

$mg\,l^{-1}$ of acetosyringone. Then 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* and select the transgenic tissue (Chu et al. 1975). As the regenerated plants grew, they were transferred to a greenhouse and maintained until maturation.

Southern blot analysis of transgenic rice plant

Integration of the IFN-α DNA into the dwarf rice genome was confirmed by Southern blot analysis. Ten micrograms of genomic DNA was prepared from rice leaves and digested with restriction enzymes (*Hind*III, *Xba*I, and *Sac*I). These DNA fragments were separated on a 0.8% agarose gel and transferred to nylon membranes (Hybond-N+; Amersham Biosciences). The blotted membrane was hybridized with ^{32}P-labeled IFN-α DNA in 6×SSPE, 5×Denhardt's solution, 0.5% SDS, and 100 $\mu g\,ml^{-1}$ salmon sperm DNA at 42°C overnight. It was then washed with 0.1×SSC and 0.1% SDS at 62°C. The washed membrane was then exposed to X-ray film.

Assay for IFN-α in transgenic rice plant

Several tissues (callus, root, leaf, and developing seed) from the transgenic rice plant 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 with a Potter-type Teflon® homogenizer on ice for 1 minute. Each extract was clarified by centrifugation at 8,900×g for 30 min at 4°C, and then both an antiviral assay of IFN-α and enzyme immunoassay (EIA) were performed as described below.

The antiviral activity of IFN-α was determined by the dye uptake method based on cytopathic effect (CPE) using FL cells derived from human amnion and vesicular stomatitis virus (VSV) in 96-well micro-titer plates (Rubinstein et al. 1979). After viral infection, surviving cells were stained with neutral red and extracted with 50% ethanol solution. Dye uptake was estimated by measuring the absorbance at 546 nm using a plate reader (Vmax, Molecular Devices, Sunnyvale, CA, USA). Titer was referenced in international reference units. An EIA was performed with horse anti-human IFN-α polyclonal antibody and its conjugated antibody with horseradish peroxidase by a sandwich method that we reported previously (Shirono et al. 1990). The NIH international standard of human leukocyte IFN-α was used for both the CPE and EIA assays. The protein concentrations of all samples were determined with 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).

Optical microscopic analysis of transgenic rice plant

After being husked, developing rice seeds (14 days after flowering) were cut longitudinally into thirds using a razor blade. The seed blocks were fixed with 100 mM phosphate buffer, pH 7.2, containing 3% glutaraldehyde for 3 h at room temperature. The blocks were then treated with 1% osmium tetroxide in 100 mM phosphate buffer at pH 7.2 for 1 h at room temperature. The fixed tissues were washed six times with 100 mM phosphate buffer, dehydrated through a graded ethanol series, and embedded in epoxy resin using a previously described method (Tanaka et al. 1980). For optical microscopy, 1-μm-thick sections were cut from the embedded block and mounted on glass slides. These sections were stained with CBB staining solution (1% CBB, 40% methanol, 7% acetic acid) and photographed using a light microscope (BH-2; Olympus, Tokyo, Japan).

Immunocytochemical analysis of transgenic rice plant

Developing rice seeds (9 days after flowering) were embedded in LR White resin (London Resin Co. Ltd., Hampshire, UK). Blocks were polymerized at 55°C for 48 h. Ultra-thin sections were cut with a diamond knife using an Ultracut UCT ultramicrotome (Leica, Heidelberg, Germany) and mounted on nickel grids. For immunocytochemical analysis, the sections were treated with blocking solution of 1% bovine serum albumin in 0.1 M phosphate buffer (pH 7.2) for 30 min at room temperature. The sections were then incubated with horse anti-human IFN-α polyclonal antibody (JCR Pharmaceuticals, Kobe, Japan) diluted 1 : 500 in blocking solution for 1 h at room temperature. After washing with 0.1 M phosphate buffer (pH 7.2), the sections were incubated with a solution of rabbit anti-horse IgG 20 nm gold colloidal particles (EY Laboratories, San Mateo, CA, USA) diluted 1 : 50 in blocking solution for 1 h at room temperature. The sections were washed with distilled water and then stained with 2% uranyl acetate. After staining, all sections were examined with a JEM-1220 transmission electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Results

Transformation of human IFN-α DNA into rice genome by Agrobacterium-mediated method

Two strains of *Agrobacterium* (EHA101, EHA105) were used for the transduction of human IFN-α DNA into the rice genome. In the case of EHA101, 357 calli were recovered under the conditions of infection for 5 min and were co-cultured for 3 days. After selection with hygromycin, 21 calli had survived. However, of the 21 calli, only one callus (No. a-1) expressed IFN-α. In the

Table 1. Transformation efficiency of rice using *Agrobacterium*-mediated method

Bacterial species	Number of original seeds	Number of Calli from seeds	Number of transgenic calli	Efficiency of transformation
EHA101	10	357	1	0.28%
EHA105	25	857	4	0.47%

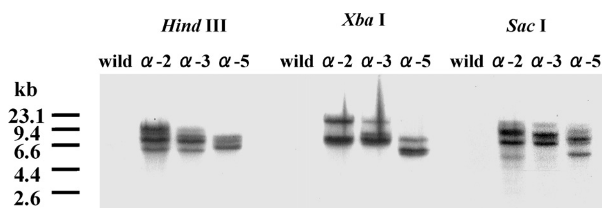

Figure 2. Southern blot analysis of transgenic rice plants. The genomic DNA (10 μg) from transgenic rice leaves was digested with *Hind*III, *Sac*I, and *Xba*I, then the DNA fragments were separated on 0.8% agarose gels. The membrane blotted by separated DNAs was hybridized with ^{32}P-labeled insert DNA from IFN-α a DNA, and was washed with 0.1×SSC and 0.1% SDS at 65°C. The washed membrane was exposed to X-ray film.

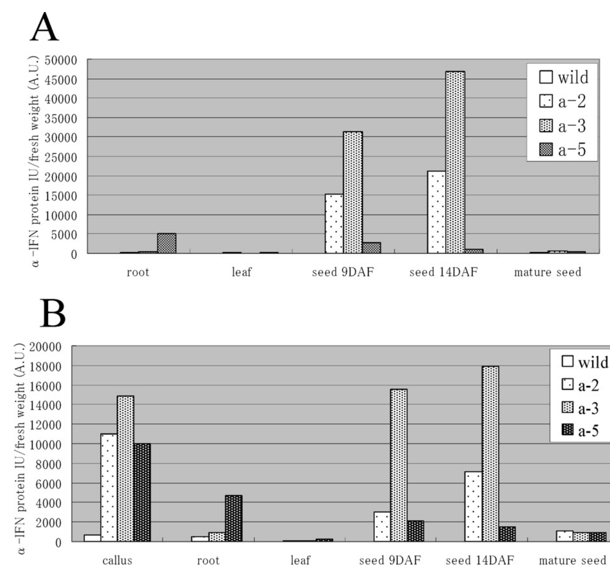

Figure 3. Biological activity of IFN-α from transgenic rice plants. IFN activity of transgenic rice was assayed. Panel A shows IFN biological activity for vesicular stomatitis virus (VSV) in FL cells of transgenic rice root, leaf, and developing seed by CPE assay. Panel B shows IFN values using the extracts from transgenic rice callus, root, leaf, and developing seed by EIA assay. Closed and shadowed bars show IFN activity per 100 mg fresh weight of rice tissues.

case of EHA105, before infection the calli were sonicated for 30 seconds and then infected with *Agrobacterium* for 2 minutes. At the end of this time, 857 calli were recovered, of which 4 (Nos. a-2, a-3, a-4, and a-5) had survived. A total of five callus lines were regenerated to rice plants expressing IFN-α (Table 1).

Southern blot analysis of transgenic rice plant

The blotted membrane was hybridized with ^{32}P-labeled IFN-α DNA. The probe, which hybridized to several bands of *Hind*III, *Xba*I, and *Sac*I, digested genome DNA fragments with each of these enzymes (Figure 2). Each of the enzymes had one restriction site in the vector DNA sequence. The results showed that two or three copies of IFN-α DNA were inserted into the genomes of three of the transformed dwarf rice strains: as a-2, a-3, and a-5 should be used.

Detection of IFN-α biological activity in transgenic rice plant

Human IFN-α expressed in transgenic rice plants was assayed by the CPE and EIA methods. Tissue extracts from the three lines of transformed rice plants were assayed, and their IFN-α expression levels were different. Three lines (a-2, a-3, and a-5) had biological activity (Figure 3), and two lines (a-2 and a-3) had high expression levels of human IFN in developing seeds and calli. Among developing seeds, line a-3 showed the highest value of IFN activity on the EIA assay. Moreover, line a-3 had biological activity for vesicular stomatitis virus (VSV) in FL cells. The IFN values in developing rice seeds of line a-3 were between approximately 30,000 IU and 45,000 IU by CPE assay (Figure 3A). On the other hand, line a-5 was unique, having slightly higher expression levels in roots than the other lines on the EIA assay (Figure 3B).

Microscopic observation of transgenic rice endosperm tissue

Figure 4 shows an optical micrograph of the endosperm tissues of the transgenic and non-transgenic rice plants 14 days after flowering. The shapes of the rice seeds did not differ remarkably between the transgenic rice plants and the non-transgenic rice plant (*Oryza sativa* L. cv. Hosetsu-dwarf). However, the morphological changes were observed in transgenic rice endosperm tissues. There were fewer small granules, such as PB (CBB stained, small black spheres), in the outer area of the starchy endosperm than in the outer area of the non-transgenic endosperm. The aleurone layer of the transgenic rice had a rough shape and was slightly enlarged. On the other hand, the shapes of starchy granules (unstained, large white spheres in starchy endosperm) were not changed in the transgenic plants. These observations suggest that the recombinant proteins influenced the quantity of authentic seed storage proteins.

The open reading frame of the expression vector (pIG-A11) for rice transformation was composed of

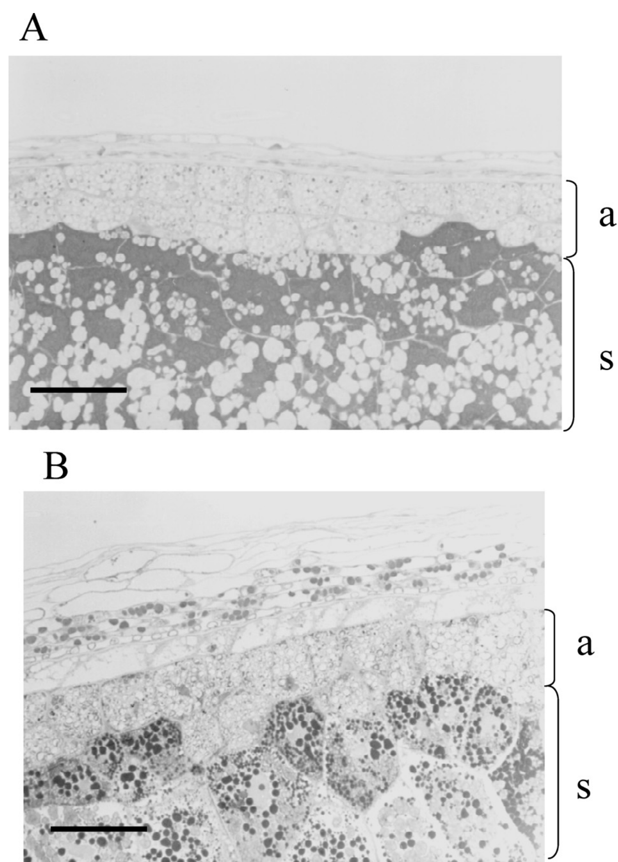

Figure 4. Optical microscopic analysis of the transgenic rice seed. The optical micrograph of the ventral side of the endosperm tissues from the transgenic rice plants at 14 days after flowering. The sections were stained with CBB staining solution (1% CBB, 40% methanol, 7% acetic acid) and photographed using an optical microscope. Panel A shows the section of non-transgenic rice plant (cv. Hosetsu-dwarf). Panel B shows the section of a transgenic rice plant (a-3). a, aleurone layer; s, starch endosperm cells. Scale bar indicates 100 μm.

the signal sequence region of rice 10 kDa prolamin and a partial sequence of the GUS coding region; this open reading frame led to the mature polypeptide of human IFN-α (Figure 1). Consequently, this chimerical polypeptide was expected to translate on PB-ER and transport into PB-I, in which the 10 kDa prolamin originally accumulated. To examine whether or not the recombinant IFN-α polypeptides accumulate in PB-I in rice endosperm cells, we performed an immunocytochemical analysis using specific antibodies raised against human IFN-α. No gold particles for human IFN-α antibody were detected in the PB-I or PB-II of non-transgenic rice (Figure 5A), whereas they were found in the transgenic rice endosperm, mainly in the PB-I and partially in the PB-II (Figure 5B, C, D). These results indicate that the biologically active human IFN-α polypeptides, including the targeting signal sequence for ER-derived PB, accumulated mainly in the PB-I of the developing rice endosperm.

Discussion

Although the *Agrobacterium*-mediated transformation method has been utilized for dicotyledonous plants, monocotyledonous plants, including rice, were thought to be resistant to this method, as the plant species and bacterial host range were related. Generally, direct gene transfer techniques, such as electroporation, particle gun, and polyethylene glycol methods, are the methods chosen to transform foreign DNA into dicotyledonous and monocotyledonous plants (Seki 1991; Lazzeri 1995). Several normal rice cultivars, such as Nipponbare and Kitaake, were easy to transform, but many other cultivars, including Hosetsu-dwarf and Koshihikari, were comparatively difficult. Recently, some modifications of rice transformation have been carried out, and the *Agrobacterium*-mediated transformation method has been popular for rice plants (Hiei et al. 1994; Cheng et al. 1998; Bajaj and Mohanty 2005). However, the conditions for infection and transformation is still need to be optimized for the DNA integration into rice cells.

Also in this study, we verified the high efficiency of transformation using dwarf rice. Dwarf rice is a very useful material for molecular biology and genome research, because large numbers of individual rice plants that have short generation times can be maintained within a limited space. Because dwarf rice is also normally grown in an artificial environment and its life cycle is very short, it facilitates the screening of a transgenic line many times in a single year. These characteristics make it an efficient system for screening large numbers of transgenic plants and treating them under various artificial environments. As no transgenic calli were recovered from long-term sub-cultured calli, host calli should be used between 17 and 31 days after induction from scutella. The optimum time of infection to bacteria was 5 minutes, and the optimum time of sonication was 30 seconds before infection with calli. These infection times were longer than those under the usual condition, but the long exposure and wounding by sonication brought about a high survival rate among infected calli. After selection with hygromycin, all surviving calli expressed the GUS-IFN-α fusion protein, which showed antiviral activity. When these calli were regenerated in hormone-free medium, the expression level of the fusion protein was maintained stably without diminution of activity.

In a recent study, transgenic plants were evaluated for their potential as an alternative and economical source of human biologically active proteins. Recently, many biopharmaceutical investigators have begun to actively develop vaccines and therapeutic proteins utilizing transgenic plants (Kumagai et al. 1993; Parmenter et al. 1995; Dieryck et al. 1997; Tacket and Mason 1999; Ma et al. 2005). Considering the applications for oral

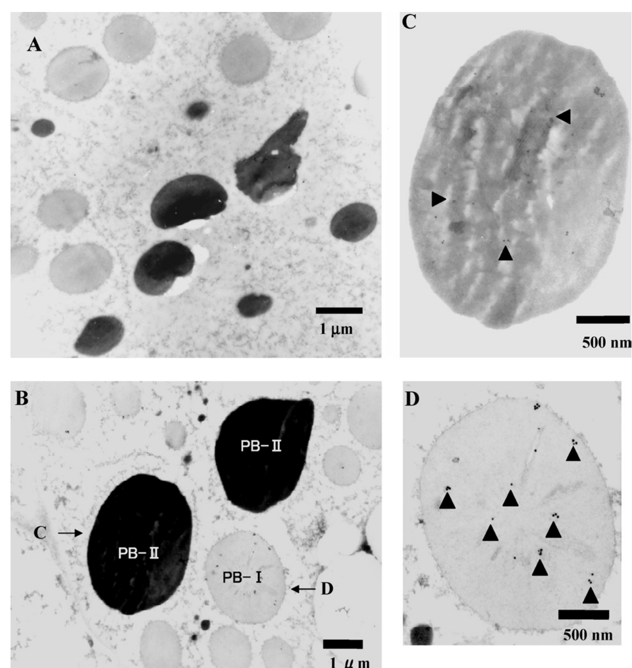

Figure 5. Immunocytochemical analysis of transgenic rice endosperm with specific antibody for human IFN-α. Transmission electron microscopic analysis of transgenic rice seed using specific antibodies raised against human IFN-α was performed at 9 days after flowering. The gold particles (arrowheads) show the localization of human IFN-α polypeptides in transgenic rice endosperm. Panel A is a wide image of the endosperm for non-transgenic rice (cv. Hosetsu-dwarf). Panel B is wide a image of the endosperm for transgenic rice (a-3). Panel C is a magnification of PB-II for transgenic rice, the same as in panel B. Panel D is a magnification of PB-I for transgenic rice, the same as in panel B. Scale bars indicate 1 μm (A, B) and 500 nm (C, D).

administration and immunization, cereals such as rice will be utilized to produce these proteins. For this purpose, transgenic rice plants carrying the IFN-α constructs (Figure 1) were generated and the levels of IFN-α expression were compared in order to determine which factors could be manipulated to enhance the expression of IFN-α in rice. Transgenic rice plants, transformed with pIG-A11 that contained the PB-ER targeting signal sequence of 10 kDa prolamin, displayed higher expression of IFN-α in developing seed endosperm than in leaf or root lacking the PB-ER. This may suggest that the PB-ER targeting signal sequence is important for the stable accumulation of IFN-α into ER and PB-I of endosperm cells.

Our results agree with several previous reports, which showed that the accumulation of recombinant polypeptides in transgenic plants was enhanced several-fold when they were accumulated within the ER (Haq et al. 1995; Artsaenko et al. 1998; Peeters et al. 2001). However, it has also been reported that the addition of a C-terminal ER-retention signal has little effect on the accumulation of foreign polypeptides (Chikwamba et al. 2002). The enhancing effect of the ER-retention signal KDEL may be explained by its ability to efficiently retain foreign polypeptides in the ER. The extended stability of foreign polypeptides could promote correct folding, leading to higher accumulation in the target organelle. Without ER retention, several foreign polypeptides would presumably be directed to the plant extracellular space (apoplast), which does not offer protection from proteolytic activity (Fiedler et al. 1997). Since IFN-α is originally composed of a small secreted protein, some portion of IFN-α polypeptides were carried through a secretory pathway in the rice endosperm cells. Then they may have accumulated in the PB-II secreted from the ER.

In this study, we demonstrated that IFN-α as a human biologically active protein was produced from monocotyledonous plants. Moreover, the products were stably packaged mainly in the ER-derived protein body (PB-I) of developing seed endosperm. Such products would be safer than those derived from animal-based sources, because there is no contamination by human infectious viruses. Proteins stored in seeds become desiccated and could remain intact for a long time, making seeds an advantageous method of storing, distributing, and administering biopharmaceuticals. To further increase IFN-α accumulation in transgenic rice systems, an additional approach will be necessary.

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