Transgenic Research **13:** 499–510, 2004.

Expression of bioactive human interferon-gamma in transgenic rice cell suspension cultures

Tzy-Li Chen[1], Yi-Ling Lin[2], Yi-Ling Lee[2], Ning-Sun Yang[1] & Ming-Tsair Chan[1,*]
[1]*Institute of BioAgricultural Sciences, Academia Sinica, Taipei 115, Taiwan, Republic of China*
[2]*Institute of BioMedical Sciences, Academia Sinica, Taipei 115, Taiwan, Republic of China*

Received 24 June 2003; accepted 29 May 2004

Key words: antiviral activity, recombinant human interferon-gamma, transgenic rice suspension cells

Abstract

We investigated the possibility of producing the therapeutic recombinant cytokine, Interferon-gamma (IFN-γ), in transgenic rice cell (*Oryza sativa,* cultivar TNG67) suspension cultures. We tested expression of two vector constructs, each harboring an αAmy3 leader peptide and a C-terminus His 6 tag fused to a human *IFN-γ* cDNA, one driven by a sucrose-starvation inducible promoter (rice αAmy3 promoter) and the other by a constitutive maize ubiquitin promoter, in rice cell suspensions, introduced via *Agrobacterium tumefaciens*. There was a significant difference in the amounts of recombinant IFN-γ protein produced by the Ups and Amy cell lines, as cytosolic and secretory proteins respectively. Immunological analysis of IFN-γ recombinant protein conferred a dose-dependent anti-dengue virus activity in human A549 cells, similar to the commercial product. We discuss the attractive attributes of using rice cell suspension system for the expression of therapeutic recombinant IFN-γ.

Introduction

Recent years have witnessed a surge in human and animal ailments worldwide, especially in developing and underdeveloped countries, despite clinical innovations. Hence the drive of present day drug development is economic considerations, with the prime directive of developing economically feasible practices from clinical innovations. Engineered plant suspension cell culture and transgenic plants expressing novel pharmaceutical proteins have been realized as a potential therapeutic source (Ma et al., 1995; Daniell et al., 2001), to improve the quality of human and animal life. For proof-of-concept purposes, tobacco cell suspensions were transformed to express human cytokine, interleukin-2 and interleukin-4 (Magnuson et al., 1998) and IFN-α and tumor necrosis factor-α in potato (Ohya et al., 200l, 2002). Further, it was assumed that limitations encountered in drug formulations produced in microbial or mammalian systems could be improved by the use of plant systems, as they exhibit different patterns of protein glycosylation and N-linked glycan processing (Horton et al., 1998; Ko et al., 2003).

Interferon-gamma (IFN-γ) is a potent cytokine for the diagnosis of human diseases. With the advent of recombinant DNA technology it has been extensively used in clinical trials for the treatment of HIV (Riddell et al., 2001) and other viral infections (Diamond & Harris, 2001; Frese et al., 2002). *IFN-γ* is a potent immunomodulator with 100–10,000 times more activity than its Type I IFN (IFN-α and IFN-β) counterpart (Farrar & Schreiber, 1993). Its mode of action involves upregulation of MHC class I antigen presentation, resulting in an increase of tumor immunogenicity and enhanced tumor recognition,

*Author for correspondence
E-mail: mbmtchan@gate.sinica.edu.tw

through tumor-specific cytotoxic T-lymphocytes, leading to tumor regression (Oshikawa et al., 1999). IFN-γ has also been observed to induce the expression of CXC chemokines resulting in inhibition of tumor angiogenesis (Addison et al., 2000).

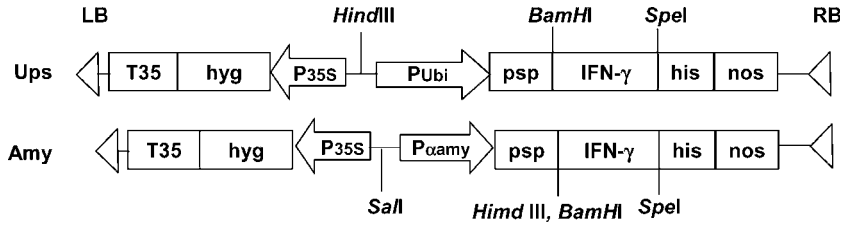
Traditionally, expression of recombinant IFN-γ relies mainly on microbial and mammalian cell systems. Recombinant IFN-γ expressed in these systems exhibit different glycosylation profiles, resulting in susceptibility to proteolysis, shorter survival times in blood (Sareneva et al., 1993) and involves high production costs. Hence, in the present investigation we reasoned to investigate the suitability of plant cell systems as an alternative to microbial and mammalian systems for the expression of recombinant IFN-γ. Since IFN-γ, has a pre-requisite of parenteral delivery to invoke measurable immune response, we opted to use a rice cell culture system as a potentially safer, cost-effective and more efficacious alternative to microbial or mammalian cell-based systems. For proof-of-concept purposes, the expression of human α-1-antitrypsin (Huang et al., 2001) and production of recombinant antibody as a single chain Fv antibody (scFv) (Terashima et al., 1999; Torres et al., 1999; Stoger et al., 2000) in rice suspension cells have demonstrated the practicality of using transgenic rice suspensions as bioreactors for the production of functional pharmaceutical proteins. Further, scale-up in bioreactors (Fischer et al., 1999) for downstream processing, if the cells secrete heterologous protein into the medium, will be based on adaptation of existing technology, which is likely to be inexpensive.

These considerations prompted us to demonstrate the expression of a potent cytokine, recombinant human IFN-γ, in transgenic rice cell cultures. We tested expression of two vector constructs, harboring an αAmy3 leader peptide and a C-terminus His 6 tag fused to human *IFN-γ* cDNA, one driven by a sucrose-starvation inducible promoter (rice αAmy3 promoter) and the other, a constitutive maize ubiquitin promoter for the production of recombinant IFN-γ in rice cell suspension cultures. We demonstrate that the functional therapeutic IFN-γ can be stably expressed in transgenic rice suspension cells and exhibits biological properties similar to the commercially available IFN-γ. To our knowledge, this is the first report describing the production of therapeutic recombinant IFN-γ in a monocot derived suspension cell line.

Materials and methods

Interferon-γ plasmid expression constructs

Human *IFN-γ* cDNA was isolated by polymerase chain reaction (PCR) from a human spleen cDNA library (Stratagene, La Jolla, USA), as described (Chan & Yu, 1998b). Two primers covering the *IFN-γ* coding sequence, but excluding the human leader peptide sequence, were chosen to amplify a 456 bp DNA fragment. The 5′ primer (5′-GGATCCATGTGTTACTGCCAGGACCCA-3′) and the 3′ primer (5′-ACTAGTCTGGGATG CTGTTCGACCTTG-3′) were used to reverse transcribe the bracketed cDNA sequence, located between the 20th amino acid and the end of the *IFN-γ* coding region, excluding the stop codon (TAA). The *IFN-γ* cDNA fragment was then cloned into a pT7 blue (R) vector (Novagen, Madison, USA) with designed restriction sites (*Bam*HI and *Spe*I), to form pT7/*IFN-γ*. ABI PRISM 373 automatic DNA sequencing system was used to confirm the integrity of the *IFN-γ* gene. The 1170 bp rice αAmy3 promoter and the following signal peptide (Acc. No. M59351) were isolated by PCR amplification, from rice genomic DNA, as described (Chan & Yu, 1998b). The 5′ primer (5′-GTCGACGATCTTCAACCACCTG TGCT-3′) and 3′ primer (5′-AAGCTTCTGGA AGAGGACCTGTGTGCTTG-3′) were used to bracket the cDNA coding sequence, located between the 1st amino acid and the end of the signal peptide of the αAmy3 DNA region. This 1170 bp PCR fragment was then cloned into pBluescriptII KS (Stratagene, La Jolla, USA) to generate the pBS3S vector. The pT7/*IFN-γ* was cloned into pBS3S as a *Bam*HI/*Spe*I fragment and designated as pBS3S-IFN. The αAmy3 promoter, the signal peptide and the *IFN-γ* DNA fragment were then digested from pBS3S-IFN DNA and subcloned into the binary vector pCAMBIA1390 (containing a His 6 tag and a nos terminator) as a *Sal*I/*Spe*I fragment, to generate plasmid Amy (Figure 1). The *Hind*III/*Bam*HI DNA fragment, containing the maize ubiquitin (*Ubi*) gene promoter, exon 1, intron 1 and part of the exon 2



psp: M-K-N-T-S-S-L-C-L-L-L-L-V-V-L-C-S-L-T-C-N-S-G-Q-A-Q-V-L-F-N-

Figure 1. Constructs used for rice transformation. P*Ubi*: maize ubiquitin promoter; Pα-amy: rice α-amylase 3 promoter; psp: signal peptide sequence of rice α-amylase 3 gene; his: histidine tag sequence; hyg: hygromycin phosphotransferase cDNA sequence; nos: nos terminator sequence; P35S: cauliflower mosaic virus promoter; T35: cauliflower mosaic virus gene terminator sequence; LB: left border; RB: right border.

from pLAM (Chan & Yu, 1998a), was ligated into pCAMBIA1390 to generate plasmid p1390-Ubi. The 546 bp fragment, containing the rice αAmy3 signal peptide and *IFN-γ* cDNA, was isolated by PCR from pBS3S-IFN, as described (Chan & Yu, 1998b). The 5′ primer (5′-AGATCTATGAA GAACACCAGCAGCTTG-3′) and the 3′ primer (5′-AAGCTTCTGGAAGAGGACCTGTGTGC TTG-3′) were used to amplify the coding sequence located between the 1st amino acid (5′ end) of the signal peptide and the 3′ end of *IFN-γ* gene. The αAmy3 signal peptide and *IFN-γ* DNA fragment were then cloned into a pT7 blue (R) vector (Novagen, Madison, USA) to form pT7/Sp-IFN-γ. The IFN-γ fragment was digested with *Bgl*II and *Spe*I from pT7/Sp-IFN-γ and subcloned into p1390-Ubi plasmid to generate plasmid (Ups) (Figure 1). The 3′ end of the coding region carried a His 6 tag to facilitate protein purification by immobilized metal ion affinity chromatography (IMAC).

Plant transformation

Agrobacterium tumefaciens strains LBA4404 harboring the Amy and Ups (Figure 1) expression cassette was cultured in YEP medium, containing 100 mg/l kanamycin and incubated at 28°C overnight on a rotary shaker (200 rpm). Embryo-derived callus isolated from mature rice seeds (*Oryza sativa* L. cv. Tainung 67, TNG67) was transformed by *A. tumefaciens* as described previously (Chan et al., 1993; Hiei et al., 1994). Infected rice embryogenic callus tissues were cultured at 28°C for 4 weeks under continuous light (1000 lux). Hygromycin-resistant calli, were isolated and transferred to Murashige and Skoog (MS) liquid medium, containing 2 mg/l 2, 4 D and 50 mg/l hygromycin and incubated at 28°C at 110 rpm on a rotary shaker. Suspension cells (0.5 ml) were sub-cultured into 25 ml of fresh medium, every 7 days, in a 125 ml flask and cultured under the same test conditions.

Southern and northern blot analyses

Genomic DNA was isolated from suspension cells according to the method of Dellaporta et al. (Malmberg & Sussex, 1984). Aliquots of 10 μg DNA were digested with *Hind*III (which cuts inside the expression vectors, Figure 1) and fractionated by 1.0% agarose gel electrophoresis. DNA fragments were blotted onto nylon membranes (Hybond-N^+, Amersham, Buckinghamshire, England) and hybridized, as described by Sambrook et al. (1989). A [α-^{32}P]dCTP-labelled hybridization probe, corresponding to the *IFN-γ* coding region was prepared using a random primer labeling kit (Gibco-BRL, Carlsbad, USA). For northern blot analysis, total RNA was isolated from suspension cells using a Trizol RNA isolation kit (Invitrogen, Carlsbad, USA). Aliquots of 10 μg total RNA were fractionated by 1.0% agarose gel and blotted onto nylon membranes (Hybond-N^+, Amersham, Buckinghamshire, England). EtBr staining of ribosomal RNA was used as an internal control. Hybridization was carried out as mentioned above, for Southern blot analysis.

Enzyme linked immunosorbent assay (ELISA)

ELISA was performed to detect IFN-γ protein levels in individual rice suspension cell lines (Ups lines: 1–7 days; and Amy lines with or without sucrose-starvation: 2 days), subjected to time

course experiments. Cytosolic and secretory IFN-γ protein were extracted as follows: the cytosolic IFN-γ proteins were extracted by weighing 1 g packed rice suspension cells, and frozen with liquid N_2. They were then ground with the help of a pestle and mortar, and homogenized in 5 ml of extraction buffer (50 mM Tris buffer, pH 7.0 supplemented with a 0.02% protease inhibitor cocktail, Roche, Mannheim, Germany). Crude cell homogenates were allowed to stand overnight at 4 °C to further extract soluble proteins. Twenty-five millilitres of conditioned media from each expression cell line were concentrated by centricon Plus-20 (MWCO = 8 kDa, Millipore Corporation, Bedford, USA) to 1 ml. Protein concentrations were determined by the Bradford method using a Bio-Rad protein analysis kit, with bovine serum albumin as the standard. An indirect enzyme immunoassay (EIA) was performed using an OptEIA human IFN-γ assay kit according to manufacturer's instructions (Pharmingen, San Diego, USA). The standard curve for EIA, obtained using baculovirus-expressed recombinant human IFN-γ as a control, was linear in the range between 4.7 and 300 pg/ml.

Purification of IFN-γ *proteins*

Eight hundred millilitres of suspension medium containing the expressed IFNγ-His protein was concentrated to 100 ml and dialyzed with buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.5). It was then loaded onto a 5 ml Ni-immobilized His-Bind affinity column (Ni–NTA, Novagen, Madison, USA). The column was washed 10 times with void volume of buffer A. Bound proteins were then eluted with 30 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20–250 mM imidazole gradient, pH 7.5) and fractions collected (2 ml/fraction). After being concentrated to 2 ml and dialyzed five times with 50 mM Tris buffer (pH 7.5), the samples were stored at 4°C until use.

Western blot analyses

Total soluble proteins (20 μg of samples loaded per well) were fractionated by 12% bis-acrylamide gel (Bio-Rad, Hercules, USA). Protein bands in the gel were transferred onto a nitrocellulose membrane, using a semi-dry transfer blotting apparatus (Bio-Rad, Hercules, USA). For western blot analysis, test membrane was first blocked by 5% non-fat milk in TBS solution (10 mM Tris, l00 mM NaCl, pH 7.5) and then incubated for 2 h with mouse anti-IFN-γ antibody (US Biological, Swampscott, USA) diluted 1:2500 in blocking buffer (TBS solution, 3% non-fat milk). The secondary antibody (biotinylated goat anti-mouse IgG) was also diluted 1:2500 in blocking buffer. The immuno-blot was developed by addition of a substrate, 3, 3′-diaminobenzidine, (DAB) for histochemical staining of peroxidase, according to instructions provided in the ABC kit (containing avidin/biotinylated peroxidase, Vector Lab, Burlingame, USA).

Antiviral assay for IFN-γ

Wild-type and two highly expressing transgenic rice cell lines, Ups7 and Amy16, were cultured under normal culture conditions as described previously. Aliquots of 25 ml cell suspensions were collected and concentrated by lyophilization, and resuspended in 200 μl of 50 mM Tris buffer (50 mM, pH 8.0 supplemented with 0.02% protease inhibitor cocktail). Tenfold dilutions of the crude concentrated conditioned media were used to perform anti-viral activity assays. Recombinant IFN-γ's capacity to affect dengue virus infection was determined by the infectivity of dengue virus serotype PL046 (Lin et al., 1998) upon human A549 cells. A549 cells (100,000 cells/well) were pre-treated with conditioned culture medium from the wild type or the Ups or Amy transgenic cells overnight, and then infected with dengue virus at multiplicity of infection (MOI) 0.1. Following virus adsorption, A549 cells were replenished with fresh medium, added with test samples and incubated for another 2 days. Viral infection of A549 cells was visualized by immunofluorescence staining with anti-dengue NS1 antibody (red color) for viral antigen and DAPI for cell nucleus (blue color).

Dosage dependent assays were conducted by culturing transgenic cell line Ups7 and Amy16 (without sucrose for 2 days) as mentioned above, followed by collection and purification through Ni–NTA columns. A549 cells were pre-treated with such purified and commercially available IFN-γ (Bio-Rad, Hercules, USA) at various protein concentrations overnight, followed by infection, as mentioned above.

Two days post-infection, the culture supernatants were harvested to determine titers of dengue virus. Plaque assays, were performed by adding various virus dilutions to 80% confluent BHK-21 cells (baby hamster kidney cells) followed by incubation at 37°C for 1 h. After viral adsorption, cells were washed and overlaid with 1% agarose (Sea Plaque, FMC Bio-Products, Rockland, USA) containing RPMI-1640 mammalian cell culture medium supplemented with 1 FCS (fetal calf serum, GIBCO). After seven days incubation, cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet.

Results

Identification and characterization of transgenic rice suspension cell lines

Embryogenic rice callus tissues were transformed with Ups or Amy constructs (Figure 1) by *A. tumefaciens* to evaluate suitability of using rice suspension cells for production of recombinant IFN-γ protein. Four weeks after *Agrobacterium*-mediated transformation, hygromycin resistant rice callus colonies were established as suspension cultures and a total of 36 putative transgenic cell lines were generated. Preliminary screening of putative transgenic cells for detection of *IFN-γ* expression was conducted by ELISA. Further eight to nine putative independent lines were selected for analyses. To confirm the integration of the transgene in rice cell lines, Southern blot analyses were performed using the *IFN-γ* cDNA sequence from the pT7/*IFN-γ* vector as the hybridization probe. Recombinant *IFN-γ* cDNA integration was detected in the genomes of all tested transgenic cell lines (Figure 2(a) and (b)). The *IFN-γ* DNA probe did not hybridize to the genomic DNA of wild-type cells (Figure 2(a), WT and (b), WT). The different patterns in hybridization obtained from different transgenic cell lines strongly suggested that they were independent transformants (Figure 2(a) and (b)).

Expression of recombinant human interferon-γ gene in transgenic rice suspension cells

Transcripts of recombinant *IFN-γ* gene expression in transgenic cell lines were detected by Northern blot analysis. Total RNA was isolated

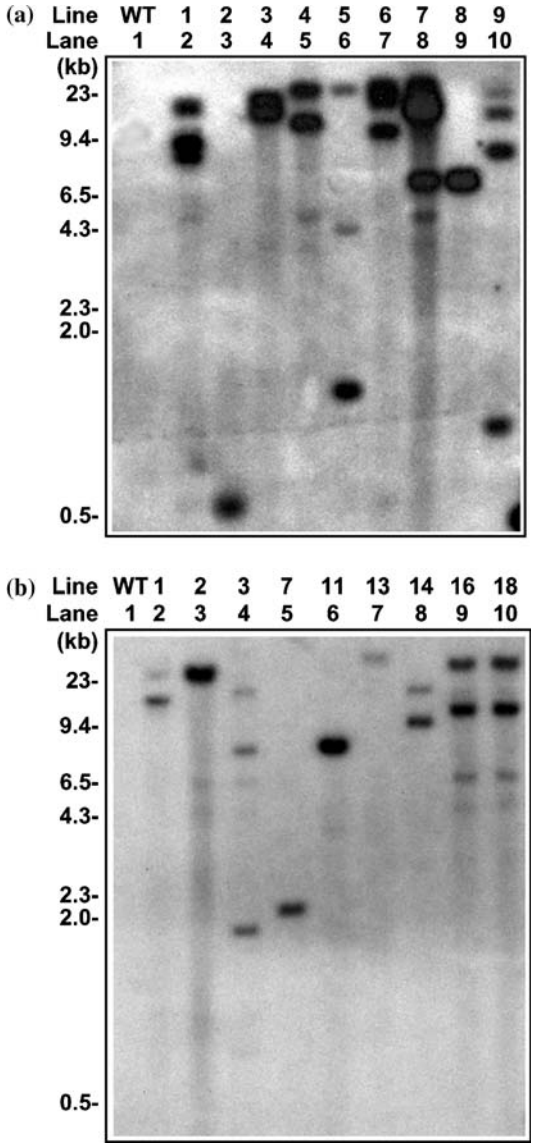


Figure 2. Identification of transgenic rice by Southern blot analysis. (a). Rice cell lines transformed with construct Ups. (b). Rice cell lines transformed with construct Amy. The [^{32}P] radiolabeled coding sequence of the *IFN-γ* gene was used as a probe.

from 7-day-old suspension cell cultures of Ups and 2-day-old cultures of the sucrose-starved Amy transgenic cell lines. Hybridization probe consisted of a 450 bp IFN-γ cDNA fragment. Heterologous, IFN-γ transcript signals were detected in transgenic Ups cell lines (Figure 3(a), lines 1, 3–9) but not in the wild type (Figure 3(a), WT). It was observed that IFN-γ RNA transcripts were not detected in one Ups transgenic cell line (Figure 3(a), line 2). Similarly *IFN-γ*

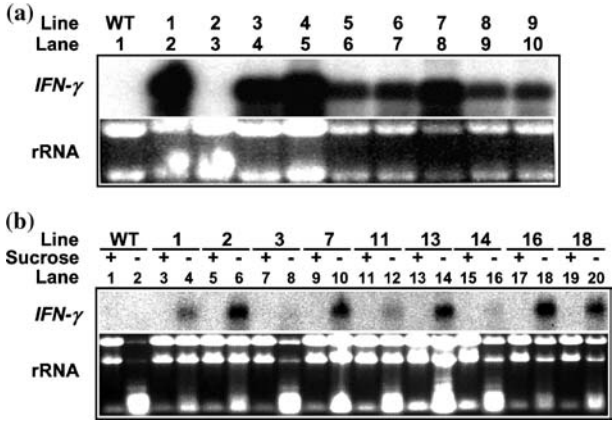


Figure 3. Expression of the *IFN-γ* gene analyzed by Northern blot. (a). Rice cell lines transformed with construct Ups. (b). Rice cell lines transformed with construct Amy. Total RNA was isolated from 7-day-old cultures in the case of Ups lines and from 2-day-old cultures, after sucrose starvation, in the case of Amy lines. The [^{32}P] radiolabeled coding sequence of the *IFN-γ* gene was used as a probe.

transcript signals were detected in transgenic Amy lines under sucrose-starvation conditions, but not when grown in the presence of sucrose (Figure 3(b) lines 1–3, 7, 1l, l3, l4 and 16). IFN-γ mRNA was not detected in wild-type cell lines, irrespective of the presence or absence of sucrose in test culture media (Figure 3(b), WT + and −, respectively).

Expression of recombinant human interferon-γ protein in transgenic rice suspension cells

ELISA was conducted to initially screen IFN-γ protein expression in the conditioned media and cell extracts of transgenic cell suspensions (data not shown). This resulted in identification of 6 'high-producer' lines (Ups lines 4, 5, 7 and Amy lines 2, 11, 16).These six lines were then subjected to time course and sucrose-induction experiments. The results are depicted in Figures 4 and 5. The conditioned culture medium of Ups lines (lines 4, 5 and 7) were tested for secretory protein levels as follows: 0.5 ml of packed transgenic suspension cells were subcultured in 25 ml fresh MS medium and incubated at 28°C at 120 rpm, overnight, The results of time course experiments following cell growth are shown in Figure 4(a). The supernatant was separated from the suspension cells by a microcloth filtration step preceding isolation of total secretory protein from the culture medium. The secretory proteins in culture media were collected at different time points ranging from 0 to 6 days and subjected to ELISA (Figure 4(b)). In addition, intracellular IFN-γ protein levels in suspension cells were also assayed (Figure 4(c)). Secretion of recombinant IFN-γ protein from Ups 7 cells began on the 3rd or 4th day and increased steadily until the 7th day. On day 7, in Ups7 cultures, 11.1 ng/ml media and 699.79 ng/g cell of secretory and intracellular IFN-γ protein, were harvested from the medium and suspension cells, respectively (Figure 4(b) and (c)).

For the Amy cell lines, results of time course experiments following cell growth and recombinant IFN-γ production are depicted in Figure 5. In transgenic Amy cells, the *IFN-γ* gene driven by a rice α-amylase 3 promoter could be induced to express the recombinant IFN-γ protein by sucrose starvation (Chan et al., 1994) and secreted into culture media with the aid of the α-amylase 3 signal peptide. For the induction of *IFN-γ*, the Amy suspension cell lines were cultured in sucrose-containing media for 4 days and then transferred to a sucrose- free media for 48 h. The cell growth rate and time point of media change are shown in Figure 5(a). ELISA was conducted for quantifying the intracellular as well as secreted IFN-γ proteins in the suspension cells. The Amy cell line 16 exhibited the highest expression level of IFN-γ for all tested Amy cell lines. After sucrose starvation IFN-γ protein, in Amy16 culture, was estimated to be 17.4 ng/ml media and 131.6 ng/g cell in culture medium and intracellularly, respectively (Figure 5(b) and (c)).

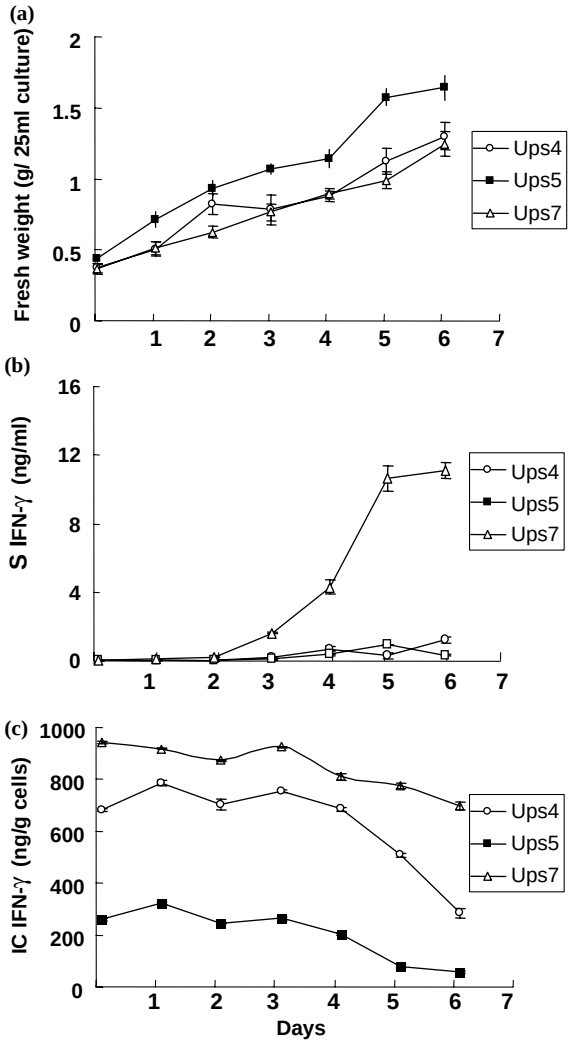


Figure 4. Cell growth (a) and ELISA analyses of IFN-γ protein accumulation in media (b) and in the cells (c) of three Ups transgenic rice cell suspension lines. Samples were assayed every day after subculture until the 7th day. One liter of culture was harvested, separating the media and cells respectively. The IFN-γ expression level in the media and packed cells was estimated. S indicates secreted IFN-γ. IC indicates intracellular IFN-γ. Three repeats were done in each experiment.

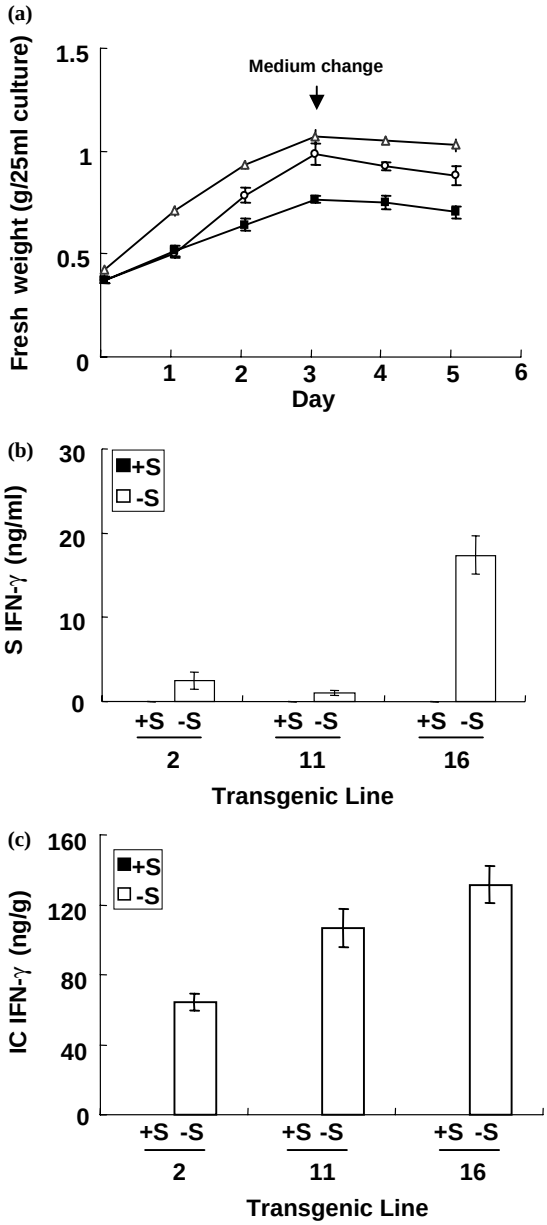


Figure 5. Cell growth (a) and ELISA analyses of IFN-γ protein accumulation in media (b) and in the cells (c) of three Amy transgenic rice cell suspension lines. Samples were assayed after 2 days of sucrose starvation. One liter of culture was separately harvested media and cells, respectively. The IFN-γ expression level in the media and packed cells was estimated. S indicates secreted IFN-γ. IC indicates intracellular IFN-γ. Three repeats were done in each experiment.

Purification of recombinant Hexahistidine-Tagged human IFN-γ with One-step Ni-NTA affinity chromatography

All tested IFN-γ DNA constructs contained a hexahistidine peptide coding sequence introduced at the 3′end of the recombinant *IFN-γ* gene. This peptide confers on chimeric proteins a high affinity for metal ions (e.g., Nickel). Using this property, we were able to partially purify recombinant IFN-γ by Ni–NTA affinity chromatography. After 48 h sucrose-starvation, liquid growth medium of Amy16 cultures were harvested, concentrated and loaded onto the Ni–NTA column. Washed and

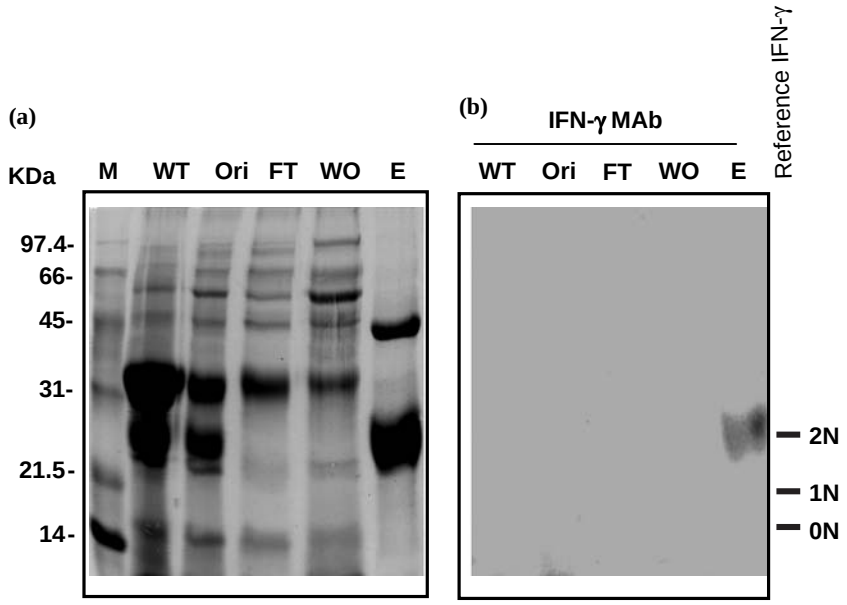


Figure 6. Purified recombinant IFN-γ analyzed by SDS-PAGE and Western blot. (a) 12% SDS-PAGE separation of recombinant IFN-γ with Coomassie blue staining. M: molecular weight marker, lane WT: the concentrated medium of untransformed rice cell line, lane Ori: concentrated medium of transgenic line Amy 16, lane FT: The flow through fraction, lane WO: The wash out fraction, lane E: the eluted fraction. Reference IFN-γ indicate the reference size of secreted recombinant IFN-γ proteins produced by Chinese hamster ovary cells (Hooker & James, 1998; Hooker et al., 1999). IFN-γ can exist in three forms: doubly glycosylate (2N, occupied at Asn_{25} and Asn_{97}), singly glycosylated (1N, occupied at Asn_{25} only), and nonglycosylated (0N). (b). Western blot of (a). Anti-IFN-γ antibodies were used as the first antibody (1:2500). 20 μg/well of total protein were usd for SDS-PAGE.

eluted fractions were collected and analyzed by Western blotting and ELISA. Figure 6 shows the results from SDS-PAGE and Western blot analyses performed on samples during different stages of protein purification The WT fractions, depicting the total protein extract of non-transgenic rice culture media served as a negative control for Western blotting analysis and ELISA. Eluted fraction contained a 45 and a 24–27 kDa protein. ELISA revealed that the 24–27 kDa protein was an IFN-γ protein and the 45 kDa as a non-specific protein, as depicted in Figure 6(a). We obtained a recovery of 36.7 for the IFN-γ protein with a purity of approximately 75%. Western blots hybridized with an anti-IFN-γ antibody confirmed that the 24–27 kDa protein observed in the eluted fraction was recombinant IFN-γ. (Figure 6(b)). No other immuno-staining bands were detected in Western blot analysis.

Recombinant IFN-γ proteins from rice protect human cells from dengue virus infection

The biological activity of recombinant IFN-γ produced in transgenic rice cells was tested with a previously reported 1FN-γ-sensitive dengue virus, (Diamond et al., 2000) on human A549 cells. Results of immunofluorescent assay depicted in Figure 7(a) clearly indicates that the culture medium collected from Ups7 and Amy16 cell lines effectively reduced the infection rates caused by dengue virus from 3 to 20%, (Figure 7(b)) On the contrary cells treated with the culture medium of wild-type non-transgenic cell lines reduced the infection rates by only 45%. The recombinant IFN-γ was purified from the culture medium of Ups7 and Amy16 by Ni–NTA affinity column and further tested for its anti-dengue viral activity. Ups7 and Amy 16 decreased the levels of dengue virus production measured by plaque assays in a way similar to the commercially available IFN-γ (Figure 7(c)) Hence, the recombinant IFN-γ protein expressed in transformed rice cells confer an effective antiviral activity as is evident by the inhibition of dengue virus infection in human A549 cells. These results strongly demonstrate that the recombinant IFN-γ produced from transgenic rice confers protection against dengue virus infection at levels similar to that exhibited by the commercial product.

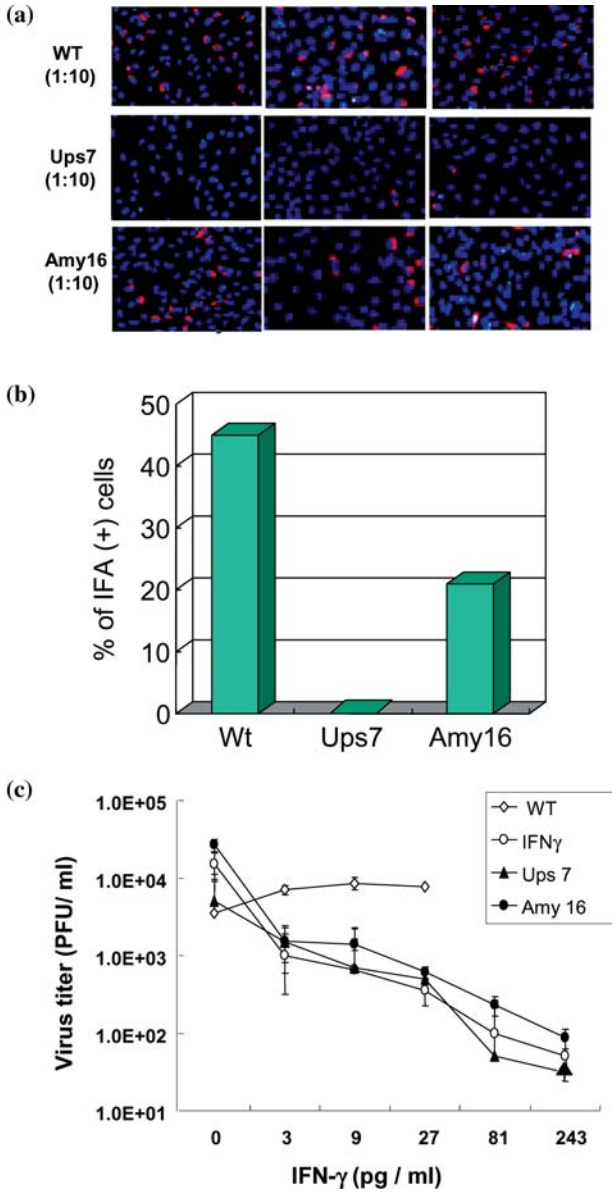


Figure 7. Detection of anti-viral activity of recombinant IFN-γ produced by transgenic rice. (a). Human A549 cells were pre-treated with concentrated media of wild-type (WT) and transgenic lines (Ups7 and Amy16) at a tenfold dilution overnight, then infected with dengue virus (MOI = 0.1). After virus adsorption, cells were replenished with medium, plus test samples, and incubated for 2 days, before the cells were stained for anti-dengue NS1 (red color) and DAPI for nucleus (blue color). (b). The Dengue virus infection ratio calculated from (a). (c). The partial purified IFN-γ of transgenic lines Amy16, Ups7 and the commercial IFN-γ standard were diluted to various concentrations. The virus titers, plaque forming units (PFU)/ml, were determined by plaque assay as described in the Materials and methods.

Discussion

In this study, we have characterized the suitability of genetically engineered rice suspension cell culture system for the expression of recombinant human IFN-γ. Two DNA expression cassettes, one containing maize ubiquitin (Ups) and the other a rice α-amylase 3 (Amy), were used to drive an αAmy3 leader peptide and a C-terminus His 6 tag fused to a human *IFN-γ* cDNA. Representative samples, Ups (1–9) and Amy (1–3, 7, 11, 13, 14 and 16), out of 36 transgenic cell lines

screened by ELISA, were analyzed for the expression of IFN-γ by Southern and northern blot analyses. Southern blot analysis revealed unique integration patterns among different lines confirming their independent origin. As can be observed in Figure 2(a), line 2 a low molecular weight fragment was observed in Ups2. Correspondingly, transcriptional signals of *IFN-γ* mRNA were not detected by northern blot analysis (Figure 3(a), line 2) in this line. Further in culture, transgenic cell line Ups2 was found to be only partially resistant to hygromycin. Hence, based on ELISA readings, three highly expressing cell lines were selected from each transformation event (Ups lines 4, 5, 7 and Amy lines 2, 11, 16) for further analysis.

Given that major expression studies normally use the CaMV35S promoter for stable expression of recombinant proteins we opted to use a constitutive Ubiquitin promoter and a sucrose-starvation inducible, α-amylase 3 promoter. Terashima et al. (1999) and Stoger et al. (2000) have demonstrated observable effects in expression of pharmaceutical proteins in plant bioreactor systems, using these promoters. The transgenic rice suspension cells express IFN-γ driven by Ups and Amy promoters which amounted to 17.4 and 11.1 ng/ml of secretory and 131.6 and 699.79 ng/g of intracellular protein. We also found that expressed IFN-γ protein levels differed significantly in the transgenic Ups and Amy cell lines. The Ups lines released and accumulated IFN-γ into media for 7 days. Interestingly, this cumulative level was similar to that of Amy lines sucrose-starved for only 2 days (Figures 4(a) and 5(a)). However, the Ups lines accumulated a significantly higher level of intracellular IFN-γ protein than the Amy cell lines. On the contrary Amy cell lines accumulated more secretory proteins than Ups cell lines. Low secretory protein level observed in the Ups lines maybe due to the instability of the secreted IFN-γ protein in plant cell culture medium which is clearly evident in Figures 4 and 5. Consistent with our findings, previous reports suggests that, when present in plant cell culture medium the stability and accumulation of recombinant proteins may decrease (Sharp & Doran, 2001; Tsoi & Doran, 2002). For this study, improving accumulation of target proteins in cell culture media by the use of protein stabilizing agents such as PVP, gelatin and mannitol (Magnuson et al., 1996) will be further explored in future studies, and we believe the current Amy and Ups cell lines, producing IFN-γ, provide a good experimental system for this aspect of research.

To demonstrate further that the recombinant IFN-γ produced in rice cell system is the target protein and possess biological properties similar to the commercial product, SDS-PAGE followed by western blot analysis was conducted. The IFN-γ antibody hybridized to a 24–27 kDa band indicating that the eluted product was indeed IFN-γ. Further it was interesting to observe that the hybridized signal on the western blot using an IFN-γ antibody was detected only in the purified extract and not in the Ori fraction. This result is in accordance with the previous reports documenting expression of human IFN-α in transgenic potato (Ohya et al., 2001) and in transgenic rice (Zhu et al., 1994). The purified IFN-γ secretory protein derived from transgenic lines Ups7 and Amy16 exhibited highest dosage-dependent protection against dengue virus infection in human A549 cells (Figure 7(a) and (b)), similarly to the commercially obtained IFN-γ. Similarly purified form of the secretory protein from cell line Ups7 and Amy16 demonstrated the ability to confer protection against the dengue virus similar in effect to the commercial IFN-γ. The results of the *in vitro* bioassay system strongly suggest that the recombinant IFN-γ produced in the rice cell culture system is functionally stable, similar to the commercial IFN-γ.

Although glycosylation in plant cells is known to be often different from that of mammalian cells, *in vivo* animal experiments and plantibodies in plants, have been shown to raise a significant serum immune response (Chargelegue et al., 2000). However, the plant-specific complex N-glycan, remains a major concern for applications of recombinant proteins as therapeutics to human diseases (Stoger et al., 2002). Addressing this concern, humanization of plant N-glycans is a recent approach aimed at modification of plant-derived glycoproteins. Using this approach a human β-1,4-galactosyltransferase (hGT) was stably expressed in tobacco plants and used to modify a recombinant immunoglobulin (Bakker et al., 2001). There exists the possibility that hGT may be employed to modify IFN-γ or other pharmaceutical biological agents in future

studies. We suggest, based on this study that the current transgenic rice cell suspension culture system, especially the Amy lines, can be further developed as an experimental bio-reactor system for evaluating and upgrading the production of IFN-γ proteins in plant systems.

In conclusion, the present study demonstrates rice suspension culture as a promising system for production of related cytokines or other high value human cell growth factors, using IFN-γ as a reference or index protein. Attractive attributes include low cost of materials and reagents, and the competence and manipulability in modulating the efficacy, expression and secretion of biologically active proteins with a possibility of adapting this inexpensive technology for downstream processing.

Acknowledgements

We thank Drs Kenrick Deen and Venkatesh Prasad for their helpful suggestions concerning this manuscript. We also thank Dr Su-May Yu for providing the rice α-amylase 3 promoter. We are grateful to The Institute of Molecular Biology, Academia Sinica for providing experimental equipment and facilities. This work was supported by a grant from Academia Sinica and grants 91 Agri-3.1.l-Crop-Z4 from the Council of Agriculture of the Republic of China. Ning-Sun Yang and Ming-Tsair Chan contributed equally to this work.

References

Addison CL, Arenberg DA, Morris SB, Xue YY, Burdick MD, Mulligan MS et al. (2000) The CXC chemokine, monokine induced by interferon-gamma, inhibits non-small cell lung carcinoma tumor growth and metastasis. *Hum Gene Ther* **11:** 247–261.

Bakker H, Bardor M, Molthoff JW, Gomord V, Elbers I, Stevens LH et al. (2001) Galactose-extended glycans of antibodies produced by transgenic plants. *Proc Natl Acad Sci USA* **98:** 2899–2904.

Chan MT, Chang HH, Ho SL, Tong WF and Yu SM (1993) Agrobacterium-mediated production of transgenic rice plants expressing a chimeric alpha-amylase promoter/beta-glucuronidase gene. *Plant Mol Biol* **22:** 491–506.

Chan MT, Chao YC and Yu SM (1994) Novel gene expression system for plant cells based on induction of alpha-amylase promoter by carbohydrate starvation. *J Biol Chem* **269:** 17635–17541.

Chan MT and Yu SM (1998a) The 3′ untranslaled region of a rice alpha-amylase gene functions as a sugar-dependent mRNA stability determinant. *Proc Natl Acad Sci USA* **95:** 6543–6547.

Chan MT and Yu SM (1998b) The 3′ untranslated region of a rice alpha-amylase gene mediates sugar-dependent abundance of mRNA. *Plant J* **l5:** 685–695.

Chargelegue D, Vine ND, van Dolleweerd CJ, Drake PM and Ma JK (2000) A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. *Transgenic Res* **9:** 187–194.

Daniell H, Streatfield SJ and Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci* **6:** 219–226.

Diamond MS and Harris E (2001) Interferon inhibits dengue virus infection by preventing translation of viral RNA through a PKR-independent mechanism. *Virology* **289:** 297–311.

Diamond MS, Roberts TG, Edgil D, Lu B, Ernst J and Harris E (2000) Modulation of Dengue virus infection in human cells by alpha, beta, and gamma interferons. *J Virol* **74:** 4957–4966.

Farrar MA and Schreiber RD (1993) The molecular cell biology of interferon-gamma and its receptor. *Annu Rev Immunol* **11:** 571–611.

Fischer R, Drossard J, Commandeur U, Schillberg S and Emans N (1999) Towards molecular farming in the future: moving from diagnostic protein and antibody production in microbes to plants. *Biotechnol Appl Biochem* **30**(Pt 2)**:** 101–108.

Frese M, Schwarzle V, Barth K, Krieger N, Lohmann V, Mihm S et al. (2002) Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. *Hepatology* **35:** 694–703.

Hiei Y, Ohta S, Komari T and Kumashiro T (1994) Efficient transformation of rice *(Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* **6:** 271–282.

Hooker A, Green N, Baines A, Bull A, Jenkins N, Strange P et al. (1999) Constraints on the transport and glycosylation of recombinant IFN-gamma in Chinese hamster ovary and insect cells. *Biotechnol Bioeng* **63:** 559–572.

Hooker A and James D (1998) The glycosylation heterogeneity of recombinant human IFN-gamma. *J Interferon Cytokine Res* **18:** 287–295.

Horton MR, McKee CM, Bao C, Liao F, Farber JM, Hodge-DuFour J et al. (1998) Hyaluronan fragments synergize with interferon-gamma to induce the C-X-C chemokines mig and interferon-inducible protein-10 in mouse macrophages. *J Biol Chem* **273:** 35088–35094.

Huang J, Sutliff TD,Wu L, Nandi S, Benge K, Terashima M et al. (2001) Expression and purification of functional human alpha-1-antitrypsin from cultured plant cells. *Biotechnol Prog* **17:** 126–133.

Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S et al. (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. *Proc Natl Acad Sci USA* **l00:** 8013–8018.

Lin YL, Liao CL, Chen LK, Yeh CT, Liu CI, Ma SH et al. (1998) Study of Dengue virus infection in SCID mice engrafted with human K562 cells. *J Virol* **72:** 9729–9737.

Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P et al. (1995) Generation and assembly of secretory antibodies in plants. *Science* **268:** 716–719.

Magnuson NS, Linzmaier PM, Gao JW, Reeves R, An G and Lee JM (1996) Enhanced recovery of a secreted mammalian protein from suspension culture of genetically modified tobacco cells. *Protein Expr Purif* **7:** 220–228.

Magnuson NS, Linzmaier PM, Reeves R, An G, HayGlass K and Lee JM (1998) Secretion of biologically active human interleukin-2 and interleukin-4 from genetically modified tobacco cells in suspension culture. *Protein Expr Purif* **13:** 45–52.

Malmberg R and Sussex I (1984) Molecular Biology of Plants. A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold spring Harbor, New York.

Ohya K, Itchoda N, Ohashi K, Onuma M, Sugimoto C and Matsumura T (2002) Expression of biologically active tumor necrosis factor-alpha in transgenic potato plant. *J Interferon Cytokine Res* **22:** 371–378.

Ohya K, Matsumura T, Ohashi K, Onuma M and Sugimoto C (2001) Expression of two subtypes of human IFN-alpha in transgenic potato plants. *J Interferon Cytokine Res* **21:** 595–602.

Oshikawa K, Shi F, Rakhmilevich AL, Sondel PM, Mahvi DM and Yang NS (1999) Synergistic inhibition of tumor growth in a murine mammary adenocarcinoma model by combinational gene theraphy using IL-12, pro-IL-18, and IL-1beta converting enzyme cDNA. *Proc Natl Acad Sci USA* **96:** 13351–13356.

Riddell LA, Pinching AJ, Hill S, Ng TT, Arbe E, Lapham GP et al. (2001) A phase III study of recombinant human interferon gamma to prevent opportunistic infections in advanced HIV disease. *AIDS Res Hum Retroviruses* **17:** 789–797.

Sambrook J,Fritsch EF and Maniatis T (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Press, New York.

Sareneva T, Cantell K, Pyhala L, Pirhonen J and Julkunen I (1993) Effect of carbohydrates on the pharmacokinetics of human interferon-gamma. *J Interferon Res* **13:** 267–269.

Sharp JM and Doran PM (2001) strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures. *Biotechnol Prog* **17:** 979–992**.**

Stoger E, Sack M, Fischer R and Christou P (2002) plantibodies: applications, advantages and bottlenecks. *Curr Opin Biotechnol* **13:** 161–166.

Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J et al. (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Mol Biol* **42:** 583–590.

Terashima M, Murai Y, Kawamura M, Nakanishi S, Stoltz T, Chen L et al. (1999) Production of functional human alpha 1-antitrypsin by plant cell culture. *Appl Microbiol Biotechnol* **52:** 516–523.

Torres E, Vaquero C, Nicholson L, Sack M, Stoger E, Drossard J et al. (1999) Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. *Transgenic Res* **8:** 441–449.

Tsoi BM and Doran PM (2002) Effect of medium properties and additives on antibody stability and accumulation in suspended plant cell cultures. *Biotechnol Appl Biochem* **35:** 171–180.

Zhu Z, Hughes KW, Huang L, Sun B, Liu C, Li Y et al. (1994) Expression of human alfa-interferon cDNA in transgenic rice plants. *Plant Cell Tiss Org Cul* **36:** 197–204.