

Full Length Research Paper

# Expression of human interferon gamma in *Brassica napus* seeds

Khadijeh Bagheri<sup>4</sup>, Mokhtar Jalali Javaran<sup>1\*</sup>, Fereidoun Mahboudi<sup>2</sup>, Ahmad Moeini<sup>1</sup> and Alireza Zebarjadi<sup>3</sup>

<sup>1</sup>Department of Plant Breeding, Faculty of Agriculture, Zanjan University, Zanjan, I.R. Iran.

<sup>2</sup>Department of Biotechnology, Pasture Institute, Tehran, I.R. Iran.

<sup>3</sup>Department of Plant Breeding, Faculty of Agriculture, Razi University, Kermanshah, I.R. Iran.

<sup>4</sup>Department of Plant Breeding, Faculty of Agriculture, Zanjan University, Zanjan, I.R. Iran.

Accepted 8 June, 2010

Expressions of heterologous proteins in suitable plant tissues and targeting it into subcellular compartments using specific signals have been studied. Seed-based platforms are among those that allow recombinant proteins to stably accumulate at a relatively high concentration in a compact biomass. In this study, we used seed specific promoter (Napin) and C-terminal KDEL sequence to express human therapeutic protein, interferon gamma (IFN<sub>γ</sub>) in *Brassica napus* seeds. Kozak sequence was linked to the 5' end of the IFN<sub>γ</sub> gene to increase the level of expression. The constructed cassette was transformed into rapeseed. Presence and expression of the transgene were confirmed in the transformants by polymerase chain reaction (PCR) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Analysis of transgenic plants by enzyme-linked immunosorbent assay (ELISA), dot blot and western blot indicated that IFN<sub>γ</sub> protein is being expressed in *B. napus* seeds and is as active as the standard IFN<sub>γ</sub>. Our results indicate that plant seeds have tremendous potential for production of recombinant proteins as 'natural bioreactors'.

**Key words:** Interferon gamma, KDEL retention signal, seed specific promoter, *Brassica napus*, recombinant proteins.

## INTRODUCTION

Plant-based expression systems have attracted much attention as alternative hosts for the production of recom-

binant proteins and peptides (Twyman et al., 2003). Besides economic advantages, there are qualitative benefits favouring the use of transgenic plants as factories for producing recombinant proteins, in particular for pharmaceutical proteins. Protein synthesis, secretion and post-translational modifications have a lot of commonalities in plant and animal cells (Fischer et al., 2000).

One important factor driving research in this field is yield improvement because of its significant impact on economic feasibility (Abranches et al., 2005). Some of the strategies to increase recombinant protein yield in plants include development of better expression cassettes, improvement of protein stability and accumulation by using specific subcellular targeting signals, and development of downstream processing technologies (Menkhaus et al., 2004). In this respect, seed-based platforms are particularly interesting because they allow recombinant proteins to stably accumulate at a relatively high concentration in a compact biomass, which is beneficial

\*Corresponding author. E-mail: [jalali.mokhtar@gmail.com](mailto:jalali.mokhtar@gmail.com). Tel: +98 21 44196522-3. Fax: +98 21 44196524.

**Abbreviations:** ER, Endoplasmic reticulum; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; IFN<sub>γ</sub>, interferon gamma; IPTG, isopropyl β-D-1-thiogalactopyranoside; Xgal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; CaMV35S, cauliflower mosaic virus 35S; GUS, β-glucuronidase; BAP, benzylaminopurine; IBA, indolebutyric acid; CTAB, cetyl trimethyl ammonium bromide; DAB, diaminobenzidine; PTMs, post-translational modification; TMB, 3, 3', 5, 5'-tetramethylbenzidine; HRP, horseradish peroxidase; PBS, phosphate buffer saline; NOS, nopal synthase; NPTII, neomycin phosphotransferase; TSP, total soluble proteins.

**Table 1.** Primers were designed for IFN $\gamma$  gene amplification.

<b>Forward primer</b>	Additional seq	<i>Bam</i> HI	Kozak seq	ATG seq	5'-CAGGACCCATATGTAAAAG-3'
<b>Backward primer</b>	Additional seq	<i>Sac</i> I	Stop codon	KDEL seq	5'-CTGGGATGCTCTTCG-3'

for extraction and downstream processing (Stoger et al., 2005). The desiccated environment in the mature seed protects the stored proteins from degradation (Stoger et al., 2002), and recombinant proteins remain stable and active after storage at room temperature for more than two years (Ma et al., 2003).

The endoplasmic reticulum (ER) provides an oxidizing environment and a great deal of molecular chaperons, with few proteases. These are probably the most important factors which affect protein folding and assembly (Nuttall et al., 2002).

As an example of a potentially useful pharmaceutical protein, in this study we investigated the human interferon-gamma (IFN $\gamma$ ). IFN $\gamma$  is a dimerized soluble cytokine which is involved in the regulation of the immune and inflammatory responses. IFN $\gamma$  has antiviral, immunoregulatory, and anti-tumour features. It changes transcription up to 30 genes producing a variety of physiological and cellular responses (Schroder et al., 2004). Traditionally, expression of recombinant IFN $\gamma$  has relied mainly upon microbial and mammalian cell systems. Recombinant IFN $\gamma$  expressed in these systems exhibits different glycosylation profiles, leading to susceptibility to proteolysis and shorter survival times in blood (Chen et al., 2004). On the other hand, this involves high production costs. Therefore, some research to eliminate these restrictions and express IFN $\gamma$  in plants have been done. Chen et al (2004) demonstrated that the functional therapeutic can be stably expressed in transgenic rice suspension cells and exhibits biological properties similar to the commercially available IFN $\gamma$ . In another research, the estimated level of IFN $\gamma$  expression in tobacco chloroplasts was found to be 0.1% of total soluble protein (TSP) (Leelavathi and Reddy, 2003). Recently, Wu et al (2009) reported that chicken IFN $\gamma$  expression in tobacco leaves was 0.02 to 0.04% of TSP. In this study, by using a seed-specific expression cassette based on the seed specific promoter and targeting the recombinant protein to the ER, we endeavored to investigate the suitability of plant seeds for the expression of recombinant IFN $\gamma$ . We witness successful expression of the human interferon gamma in *Brassica napus* seeds.

## MATERIALS AND METHODS

### Plasmids and bacterial strains

The pGEM<sup>®</sup>-T Easy Vector (Promega) and *Escherchia coli* (TOP10F') were used for cloning and sequencing. The plasmid pBI121 (Novagen) and *Agrobacterium tumefaciens* (LBA4404) were used for plant transformation.

### Amplification and cloning of IFN $\gamma$ gene in TA vector

Human IFN $\gamma$  gene cDNA has been isolated and cloned by Moeenrezakhanlou et al. (2002) (Accession no. AF506749, Genebank). For IFN $\gamma$  gene amplification, appropriate primers were designed with regard to Kozak over-expression sequence in the forward and KDEL retention signal (based on plant codon usage) in the reverse primer (Table 1). Polymerase chain reaction (PCR) was performed in a total 25  $\mu$ l final volume, using 2.5 mM of each deoxyribonucleotide triphosphate (dNTPs), 10 pmol of each primer, 1.5 mM Mg<sup>2+</sup> and 2.5 units of Taq DNA polymerase enzyme. Thermocycler was programmed for one cycle at 95°C for 5 min, followed by 25 cycles at 95°C for 1 min; 64°C for 1 min; 72°C for 1 min and one cycle at 72°C for 10 min as a final extension. The resulted band was purified using the agarose gel DNA extraction kit (Roche). The purified IFN $\gamma$  gene was cloned into the TA vector and the transformed colonies were screened by selection on a medium containing 100 mg/l ampicillin plus Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal). Colony PCR was done on white colonies with specific primers. The recombinant plasmids were further analysed by sequencing (in both direction with M13F-pUC and M13R-pUC standard primers). The sequencing results were compared with other sequences deposited in the Genebank using the BLAST software (Altschul et al., 1990) and ClustalW program (Thompson et al., 1994).

### Construction of seed expression cassette in plant binary vector (pBI IFN $\gamma$ )

The CaMV35S promoter was replaced by the Napin promoter in the pBI121 binary vector. Napin is a promoter of major seed storage protein in *B. napus*, making up 20 to 30% of total protein at seed maturity (Barciszewski et al., 2000). GUS ( $\beta$ -glucuronidase) gene was eliminated with *Bam*HI and *Sac*I restriction enzymes and IFN $\gamma$  gene was subcloned in modified pBI121 vector with the same enzymes. The presence and orientation of gene in recombinant pBI121 (pBIIFN $\gamma$ ) were analysed by PCR and restriction enzyme digestion. All recombinant DNA techniques (DNA digestion by restriction endonucleases, T4-DNA ligase-mediated ligation, plasmid preparation and growth of bacterial cultures) were performed according to Sambrook and Russell (2001).

### *Agrobacterium*-mediated transformation and regeneration of *Brassica* plants

Seeds of *B. napus* (PF4570/91 cultivar) were surface sterilized with 2.5% (v/v) sodium hypochlorite by shaking for 15 min. The seeds were washed 3 times in sterile distilled water and were germinated aseptically on Murashige and Skoog (MS) medium (1962) in glass bottles at 25°C in a 16 h light/8 h dark photoperiod. Plant transformation and regeneration were performed after Moloney et al, (1989). In brief, the 5-day old cotyledons were excised in such a way that they included approximately 3 mm of petiole at the base. Care was taken to eliminate the apical meristem which sometimes adheres to the petioles. The excised cotyledons were placed on MS medium containing 3% (w/v) sucrose and 0.7% (w/v) agar enriched with 4.5 mg/l benzylaminopurine (BAP) as a cytokinin. Single

colonies of the *A. tumefaciens* strain LBA4404 containing the modified binary plasmid pBI IFN<sub>γ</sub> were grown overnight at 28°C in lysogeny broth (LB) medium supplemented with 50 mg/l kanamycin. Then, explants were inoculated with *A. tumefaciens* for 20 - 30 s and the cultivation was continued on the same medium which solidified with 8 g agar/l at 25°C in the dark. After 2 days of co-cultivation, explants were transferred to the same medium containing 15 mg/l kanamycin (for selection of transgenic plant cells) and 200 mg/l cefotaxime (for elimination of *Agrobacteria*). Subculturing was done at 10-15 day intervals and kanamycin concentration increased up to 25 mg/l. Root development often started at this phase as well and/or induced by transferring shoots to a fresh medium supplemented with 2 mg/l indolebutyric acid (IBA). Rooted plants were transferred to perlite and acclimatized in a growth chamber, then transferred to the greenhouse. Seed harvest was about 20 weeks after transfer to soil.

### Molecular analysis of transgenic plants (T0)

#### PCR

Total genomic DNA was extracted from leaves of putative transformed and non-transformed Brassica plants by the cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson (1980). PCR was performed by using IFN<sub>γ</sub> gene-specific primers and amplified DNA fragments were electrophoretically separated on 1% agarose gel.

#### Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

TSP were extracted from seed tissue of transformed and nontransformed plants by grinding 0.1 g dry seeds in liquid nitrogen and resuspended in 3 ml of extraction buffer (Tris-HCl 0.5 M, Glycine 0.4 M, and 10% v/v Glycerol, pH 8.3). The insoluble debris was removed by centrifugation at 10500 Xg (10000 rpm) for 20 min at 4°C. The soluble seed protein was centrifuged one more time to remove the remaining insoluble fractions (at the same speed for 15 min). The total soluble protein concentration in the supernatant was estimated by Bradford protein assay using bovine serum albumin as a standard. Extracted proteins were subjected to 12.5% SDS-PAGE as described by Laemmli (1970) and visualized by Coomassie-blue staining.

#### Enzyme-linked immunosorbent assay (ELISA)

Seed total proteins of transgenic and non-transformed plants were prepared and coated onto the wells at the concentration of 10 µg per well. Wells were incubated at 37°C overnight and washed 3 times with phosphate buffer saline (PBS); 10 mM, pH 7.2 and blocked with 4% (w/v) skim milk, washed, and added to 1:40 of rabbit anti-IFN<sub>γ</sub> antibody and incubated for 2 h, washed, and added to 3:5000 of mouse anti-rabbit labeled with horseradish peroxidase (HRP). The wells were then incubated for 1 h at the same temperature, washed and combined with 50 µl of substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) and incubated for 15 min. The enzyme reaction was stopped using 50 µl of a 2 N HCl solution, and optical density of reaction was read at 450 nm using an ELISA reader.

#### Dot blot analysis

Seed total soluble proteins from two highly expressing T0 transgenic (T2 and T4, based on ELISA result) and non-transformed plants

were spotted onto a nitrocellulose membrane. The membrane was blocked with 5% (w/v) skim milk with gentle shaking for 2 h, followed by three 10 min washes with PBS. Incubation with rabbit anti-IFN<sub>γ</sub> polyclonal antibody was done for 3 h at room temperature (25°C), then washing with PBS was repeated three times. Membrane was incubated with a secondary antibody labeled with HRP for 1 h. Finally, color development of HRP was done by adding the diaminobenzidine (DAB).

#### Immunoblot analysis

Seed TSP were separated on SDS-PAGE gel as mentioned above and transferred from gel to nitrocellulose membrane in transfer buffer at 14 mA for 30 min. After blotting, the rest of the process was carried out as in dot blot.

## RESULTS

### Construction of binary vectors

PCR-amplified fragment (500 bp) was cloned into TA vector. The authentic PCR fragment was sub cloned into a plant binary vector (pBI121) and the resulting clones and orientation of constructs were confirmed by PCR and restriction enzyme digestion (Figure 1). In this construct, the IFN<sub>γ</sub> gene with KDEL sequence in C-terminal was located between the Napin promoter and Nopalinsynthase (NOS) terminator (Figure 2). This construct was transferred to *A. tumefaciens* LBA4404 by the freeze and thaw standard method (Höfgen and Willmitzer, 1988).

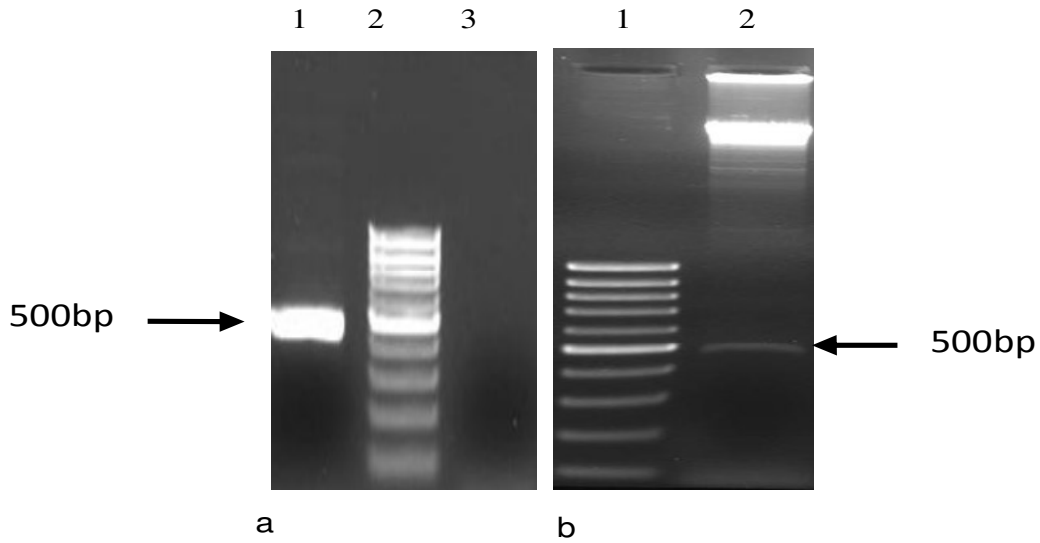
Cotyledon explants from PF cultivar of *B. napus* were co-cultivated with the *Agrobacterium* strain carrying the recombinant binary vector. Two to three times of subculturing resulted in normal shoots that started to elongate. Transformed shoots were first transferred to shoot elongation medium (Figure 3) and then to MS medium containing 2 mg/l IBA and 25 mg/l kanamycin. The transgenic plants had about 29% regeneration frequency in the medium containing 15 mg/l kanamycin.

### Screening of the putative transformed plants

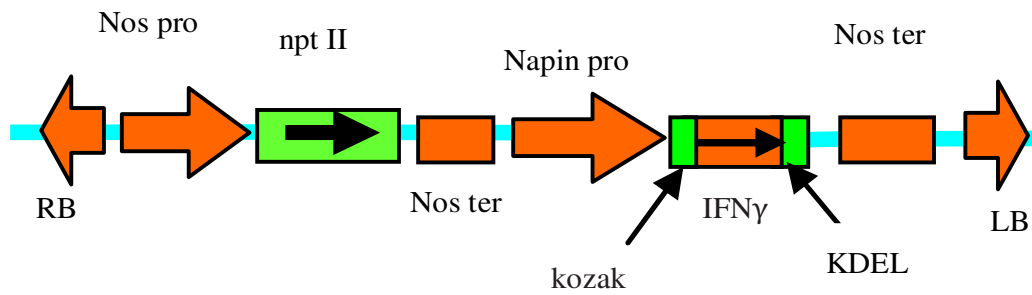
The transformed plants were initially selected by using kanamycin. Later, total genomic DNA of putative transgenic (T0) and non-transgenic plants were analyzed for presence of the gene by PCR using specific primers. PCR amplification produced a fragment of 500 bp in the transgenic plants, whereas no amplification was observed in the control plants (Figure 4).

### Analysis of protein expression by SDS-PAGE

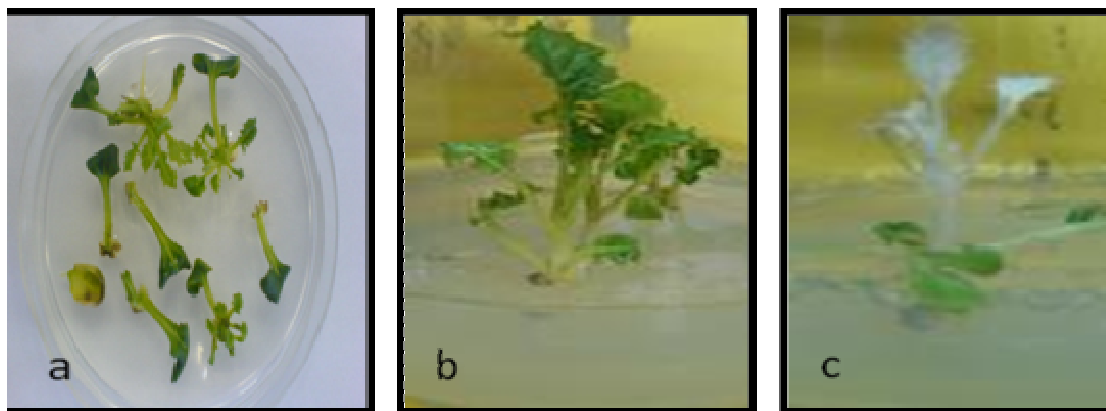
Based on the kanamycin resistance and PCR amplification, selected transgenic lines (T0) were used for SDS-PAGE analysis. Total seed protein from wild-type and transformed plants was loaded onto a SDS-PAGE gel. In the



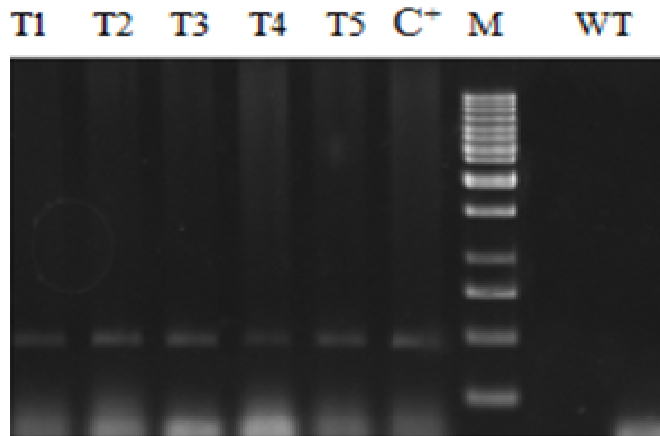
**Figure 1.** Amplification of the IFN<sub>γ</sub> gene and confirmation of cloning by restriction enzyme digestion. a) Lane 1: Amplified IFN<sub>γ</sub> gene (500 bp); lane 2: 100 bp ladder. lane 3: negative control; b) lane 1: 100 bp ladder. lane 2: restriction enzyme analysis of the IFN<sub>γ</sub> gene by *SacI* and *SmaI* digestion (500bp).



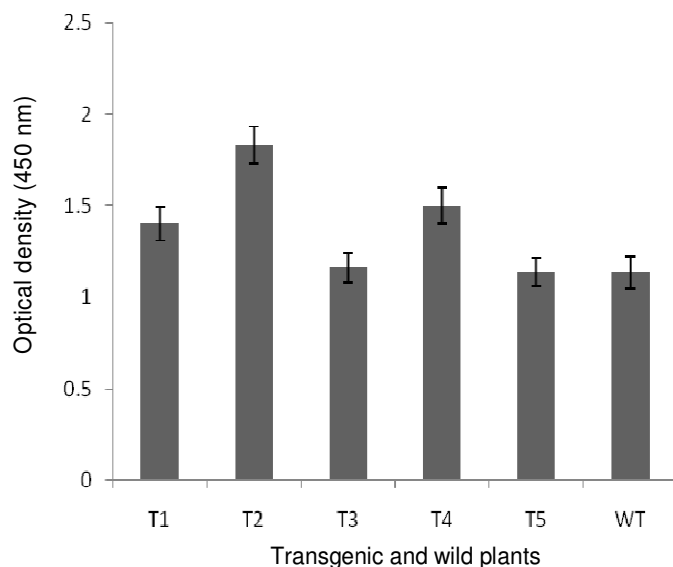
**Figure 2.** Schematic diagram of the T-DNA region in the binary vector pBI121 IFN<sub>γ</sub>. Nos pro, nopaline synthase gene promoter; Nos ter, nopaline synthase gene terminator; nptII, coding sequence of the neomycin phosphotransferase II gene; IFN<sub>γ</sub>, human gamma interferon gene; KDEL, ER retention signal; RB and LB, T-DNA right and left border, respectively.



**Figure 3.** Development and elongation of shoots after transformation. (a) Regeneration of green shoots from cotyledon explants; (b) sample of regenerated shoots on shoot elongation medium; (c) sample of white regenerated shoots on shoot elongation medium.



**Figure 4.** PCR analysis of transgenic plants T1, T2, T3, T4 and T5. Transformed plants (500 bp). C+: positive control (pBI IFN<sub>γ</sub> plasmid). M: 1 kb ladder. WT: wild type plant.

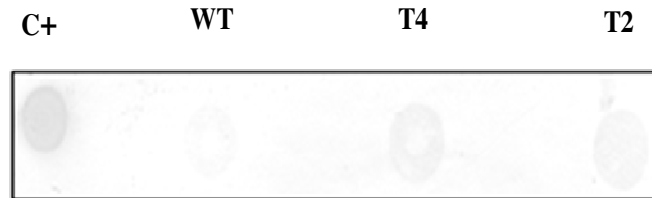


**Figure 5.** ELISA results of seed total soluble protein containing IFN<sub>γ</sub> at OD 450 nm. The results were expressed as optical density (OD). T1, T2, T3, T4, T5, Transgenic plants; WT: wild type plant.

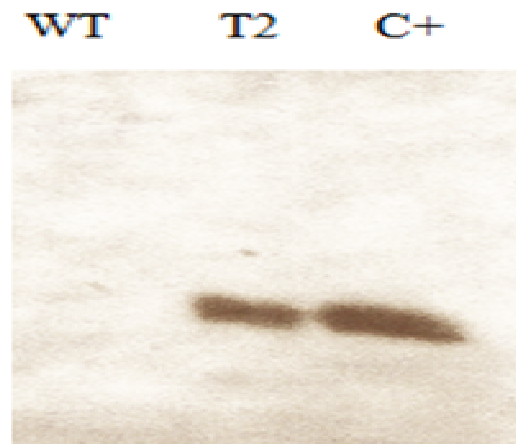
protein extracts of some seed stocks, a new band was detected but not the same band observed in the wild-type plants. The mentioned band had an expected molecular mass of 18 kDa (data not shown).

### ELISA analysis

The reactivity of transgenic seed proteins with anti-IFN<sub>γ</sub> polyclonal antibody was tested by using ELISA. Test results indicated that IFN<sub>γ</sub> protein is expressed such that the difference in optical densities in the case of some



**Figure 6.** The result of dot blotting of transgenic plants (T2 and T4), wild-type plant (WT) and Imukin (C+).



**Figure 7.** Immunological detection of IFN<sub>γ</sub> with anti-IFN<sub>γ</sub> antibody. Wild-type plant (WT), transgenic plants (T) and positive control (C+).

transgenic extracts (T1, T2 and T4) is higher than that of wild type plant. Line T2 showed significant difference compare to other samples. T3 and T5 extracts were the same as wild type plants (Figure 5).

### Detection of IFN<sub>γ</sub> by dot and western blotting

Some T0 lines (T2 and T4) were further tested for the detection of IFN<sub>γ</sub> protein by dot and western blot analysis, using rabbit anti-IFN<sub>γ</sub> polyclonal antibody. Imukin (interferon gamma-1b) was used as positive control (C+). Comparison of dot blot results in transgenic and wild-type plants confirmed the expression of the IFN<sub>γ</sub> protein (Figure 6). Proteins extracted from the putative transgenic plants were separated on 12.5% SDS-PAGE gel. As seen in Figure 7, a band corresponding to a molecular mass of standard IFN<sub>γ</sub> was recognized by anti-IFN<sub>γ</sub> antibody in transformed plant (T2) but anti-IFN<sub>γ</sub> antibody did not recognize any protein from wild-type total seed protein.

### DISCUSSION

The present study was performed in order to investigate

whether IFN $\gamma$  protein could be successfully expressed in a plant seed system. Due to the importance of subcellular localization and N-glycosylation on the stability, correct folding, and biological activity of recombinant proteins, we targeted IFN $\gamma$  protein away from the cytosol to the more favourable environment of the endoplasmic reticulum by adding the tetrapeptide KDEL.

Several reports indicated that the KDEL retention signal, recognized by a salvage receptor, was probably well exposed (Tang et al., 1994), hence enabling the scFv antibody to be retained in the ER. Compared with the plants expressing the secreted scFv, the retention in the ER resulted in a 100-fold increase in the amount of detectable scFv antibody. It seems that the high level of scFv antibody accumulation is because of its strict localization in the ER and consequently is protected from proteolytic activity (Schouten et al., 1996). On the other hand, to inhibit some undesirable plant-specific post-translational modification (PTMs), one strategy to prevent the addition of immunogenic glycans to PMPs involves the storage of the therapeutic protein within the ER, that is, upstream of the golgi cisternae where immunogenic glyco-epitopes are added to plant N-glycans (Gomord and Faye, 2004).

Because of the importance of these factors, the construct containing human IFN $\gamma$  gene was prepared and cloned in common plant expression vector (pBI121 IFN $\gamma$ ). Given that major expression studies normally use the CaMV35S promoter for stable expression of recombinant proteins, we used a seed specific promoter and constructed a seed expression cassette containing Napin promoter, Kozak sequence, start codon, IFN $\gamma$  sequence, KDEL, stop codon, NOS terminator and nptII (neomycin phosphotransferase) gene in T-DNA which was used in order to perform agrobacterium-mediated transformation of *Brassica* plants. Our results show that a Napin gene promoter of *Brassica* can be used to express recombinant proteins in seed. Expression of recombinant IFN $\gamma$  in transgenic seeds was detected by SDS-PAGE, ELISA, dot blot and confirmed by western blot analyses, however transgenic lines differed concerning the level of protein expression. This difference in the IFN $\gamma$  expression may be due to the difference in the position of the gene in plant genome or different copy numbers of transferred gene. In some transformed plants, the protein expression was not seen even though IFN $\gamma$  gene was detected by PCR analysis. One reason may be that the level of expression of recombinant protein had been less than the detection threshold of the aforementioned techniques. The second reason may be due to comparatively short half life of IFN $\gamma$  protein (Leelavathi and Reddy, 2003). The third explanation can be a recombinant event within T-DNA, which has altered IFN $\gamma$  gene structure and has prevented its expression (Prasad et al., 2004). Western blot result shows that the conformation of plant-made IFN $\gamma$  protein is correctly achieved. The results demonstrated that the *Brassica* seeds would be the choice for production of recombinant proteins; low costs of seed production and

compatibility with existing agricultural processing procedures make it an attractive alternative to bacterial and yeast fermentation system. However, more studies should be conducted in order to optimize IFN $\gamma$  production in plants and the extraction/ purification protocols which have a substantial influence on final yields.

## ACKNOWLEDGEMENTS

We would like to thank Dr N. Maghsoudi at Shahid Beheshti University for providing anti-IFN $\gamma$  antibodies and Dr A. Rajabi for reading of this manuscript.

## REFERENCES

- Abranches R, Marcel S, Arcalis E, Altmann F, Fevereiro P, Stoger E (2005). Plants as bioreactors: a comparative study suggests that medicago truncatula is a promising production system. *J. Biotechnol.* 120(1): 121-34.
- Altschul SF, Gish W, Miller W, Myers EW (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
- Barciszewski J, Szymanski M, Haertlé T (2000). Minireview: Analysis of rape seed napin structure and potential roles of the storage protein. *J. Protein Chem.* 19(4): 249-254.
- Chen TL, Lin YL, Lee YL, Yang NS, Chan MT (2004). Expression of bioactive human interferon-gamma in transgenic rice cell suspension cultures. *Transgenic Res.* 13: 499-510.
- Fischer R, Hoffmann K, Schillberg S, Emans N (2000). Antibody production by molecular farming in plants. *J. Biol. Regul. Homeost. Agents.* 14: 83-92.
- Gomord V, Faye L (2004). Posttranslational modification of therapeutic proteins in plants. *Curr. Opin. Biotechnol.* 7: 171-181.
- Leelavathi S, Reddy VS (2003). Chloroplast expression of His-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors. *Mol. Breed.* 11: 49-58.
- Ma JKC, Drake PMW, Christou P (2003). The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet.* 4(10): 794-805.
- Menkhaus TJ, Bai Y, Zhang CM, Nikolov ZL, Glatz CE (2004). Considerations in the recovery of recombinant proteins from transgenic plants. *Biotechnol. Prog.* 20(4): 1001-1014.
- Moeenrezakhanlou A, Maghsoudi N, Mahboudi F (2002). Homo sapiens interferon-gamma mRNA, complete cds. <http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=20805895>.
- Moloney MM, Walker JM, Sharma KK (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep.* 8: 238-242.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiol.* 15: 473-497.
- Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321-4325.
- Nuttall J, Vine N, Hadlington JL, Drake P, Frigerio L, Ma LK (2002). ER-resident chaperone interactions with recombinant antibodies in transgenic plants. *Eur. J. Biochem.* 269: 6042-6051.
- Prasad V, Satyavathi VV, Sanjaya VKM, Abha K, Shaila MS, Lakshmi SG (2004). Expression of biologically active Hemagglutinin-neuraminidase protein of *Peste des petits ruminants* virus in transgenic pigeonpea [*Cajanus cajan* (L.) Mills]. *Plant Sci.* 166: 199-205.
- Sambrook J, Russell, DW (2001). *Molecular cloning: a laboratory manual*. 3<sup>rd</sup> edition, Cold Spring Harbor Press, New York, USA.
- Schouten A, Roosien J, Engelen FA, Jong GAM, Borst-Vremsen AWM, Zilverentant JF, Bosch D, Stiekema WJ, Gommers FJ, Schots A, Bakker J (1996). The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be

- targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol. Biol.* 30(4): 781-793.
- Schroder K, Hertzog PJ, Ravasi T, Hume DA (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukocyte Biol.* 75: 163-189.
- Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, Christou P, Fischer R (2002). Practical considerations for pharmaceutical antibody production in different crop systems. *Mol. Breed.* 9(3): 149-158.
- Stoger E, Ma JKC, Fischer R, Christou P (2005). Sowing the seeds of success: pharmaceutical proteins from plants. *Curr. Opin. Biotechnol.* 16: 167-173.
- Tang BL, Wong SH, Low SH, Subramaniam VN, Hong WJ (1994). Cytosolic factors block antibody binding to the C-terminal tail of the KDEL receptor. *Eur. J. Cell Biol.* 65: 298-304.
- Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003). Molecular farming in plants: host systems and expression technology. *Trends Biotechnol.* 21(12): 570-578.
- Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.
- Wu Y, Zhao D, Song L, Xu W (2009). Heterologous expression of synthetic chicken IFN- $\gamma$  in transgenic tobacco plants. *Biology*, 64(6): 1115-1122.