CLONING, TRANSFORMATION AND EXPRESSION OF HUMAN GAMMA INTERFERON GENE IN TOMATO (LYCOPERSICON ESCULENTUM MILL.)

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ABSTRACT
The demand for recombinant proteins for therapeutic or diagnostic use is expanding rapidly in recent years. Human gamma interferon (Hu-IFN-γ) is one of these proteins against viruses that it has a high value in therapy. Therefore interferon is used against viral infection, cancer and autoimmune diseases. In this study appropriate primers were designed and used for PCR amplification of the Hu-IFN-γ gene which was cloned in the plant expression vector pCAMBIA1304. The created construct was transferred in Agrobacterium tumefaciens strain LBA4404 and used for transformation of tomato cotyledon explants. The presence of the Hu-IFN-γ transgene in the genomic DNA of transgenic plants was proven by PCR. Finally, total protein of tomato was extracted and the presence of Hu-IFN-γ proteins was detected using dotblot assay.

Materials and Methods

Bacteria
Escherichia coli strain DH5α was used for cloning and proliferating the construct and Agrobacterium tumefaciens strain LBA4404 was used for plant transformation.

Vectors
Two vectors were used in this study. One was pRSET (Invitrogen), which carries the Hu-IFN-γ gene and ampicillin resistance gene and the other one was plant expression vector pCAMBIA1304 (CAMBIA Co. Australia) which carries a kanamycin resistance gene for selection of the colonies of the bacteria and a hygromycin resistance gene for the transgenic lines (Fig. 1). This vector carries LB and RB (Left and Right Borders) for integration of foreign genes into the host genome, CaMV35s (Cauliflower Mosaic Virus promoter which induces...
high level of transcription), Ncol and BstEII restriction sites and NOS, (Nopaline Synthase terminator), GFP and GUS gene (as reporter genes) and Histidine tag for purification steps.

Primers

For Hu-IFN-γ gene amplification, the appropriate primers were designed with regard to the restriction sites of pCAMBIA1304 and both sides of the Hu-IFN-γ gene. Forward primers were designed considering the plant high level expression sequence (Kozak sequence), Ncol restriction sites, His-tag sequence to detect expression and for purification, and factor Xa for removing the His-tag sequence. BstEII restriction sites were used in the reverse primers. The nucleotide sequences of primers were the following: Forward primer 5′-CATGCCATGGAACATCATCATCATCATATATGAAAGGTCGTCAGGACCCATATGTAAAAGC-3′ and reverse primer 5′-GTACTAGCGGTCACCTTACTGGGACGTGCTTTTCGACCTC-3′.

Cloning of the Hu-IFN-γ gene in pCAMBIA1304

Hu-IFN-γ gene cDNA fragment had already been cloned in the pRSET vector (accession no. NM_000619.2 GenBank). This vector was used as a template for PCR. The Hu-IFN-γ gene was amplified using the mentioned primers. The reaction mixture was performed in a total of 25 µl final volume that contained the following components: 2.5 mM of each dNTP, 10 pmol of each primer, 1.5 mM MgCl₂, and 2.5 units of High Fidelity DNA polymerase enzyme (Fermentas). Thermocycler (BioRad) was programmed for one cycle at 95 ºC for 5 min (Hot Start), followed by 45 cycles: 30 sec at 95 ºC for denaturation; 45 sec at 59 ºC for primer template annealing; 50 sec at 72 ºC for extension and one cycle at 72 ºC for 10 min as a final extension.

For DNA fragment separation, the PCR products were electrophoresed in 0.7% agarose gel and the resulting bands were purified using the AccuPrep® gel purification kit (BIONEER). The purified Hu-IFN-γ gene and the pCAMBIA1304 vector were digested with BstEII and Ncol (BioLabs). The digested Hu-IFN-γ gene and pCAMBIA1304 vector were electrophoresed in 0.7% agarose gel and purified from the gel using the same gel extraction kit (BIONEER). The purified Hu-IFN-γ gene and the pCAMBIA1304 vector were ligated using T4 DNA ligase (Fermentas) at 16 ºC overnight and the obtained construct (pCAMIFN-γ) was transformed in E.coli DH5α strain using the heat shock method (15). Positive clones were selected on Luria-Bertani Broth (LB) plates containing 50 mg/L kanamycin. The pCAMIFN-γ recombinant plasmid was verified by colony PCR, digestion and sequencing. Consequently, the Hu-IFN-γ gene was inserted in reporter genes region of pCAMBIA1304 after the CaMV35s promoter and before the NOS terminator by ligation process.

Transformation of Agrobacterium tumefaciens with the pCAMIFN-γ recombinant plasmid

A. tumefaciens strain LBA4404 was grown overnight at 28 ºC in liquid LB medium containing 80 mg/L streptomycin. The transformation of A. tumefaciens with the pCAMIFN-γ recombinant plasmid was performed according to the thaw and freezing standard method using liquid nitrogen and CaCl₂ (20 mM) (15). Finally, 100 µL of transformed cells were cultured on LB plates containing 50 µg/mL kanamycin and 80 mg/L streptomycin. Plates were incubated for 2 days at 28 ºC. Transformed bacteria were screened by selection on selective medium.

Agrobacterium-mediated transformation and regeneration of transgenic tomato

In this study, the leaf disk method was used for transformation. Seeds of tomato (Cal J cultivar) were surface sterilized by shaking in 70% ethanol for 30 seconds and in 5% (v/v) sodium hypochlorite for 7 min. Then, the seeds were washed 3 times in sterile distilled water for 1 min and they were germinated aseptically on Murashige and Skoog (MS) medium for seedling germination (8). Nine- to 12-day-old cotyledons were selected for transformation. Cotyledons were cut off and the tips were removed. Cotyledon pieces were placed in pre-culture medium (PM) and incubated for 2 days at 22 ºC at a photoperiod of 16:8 (L:D) hours. Single A. tumefaciens colonies carrying the modified binary vector pCAMIFN-γ were grown overnight at 28 ºC in LB medium supplemented with 50 mg/L kanamycin (OD₆₀₀ = 1). Then, explants were inoculated in infection medium (IM) for 5 min at room temperature. Explants were placed on co-culture medium (CM) for 2 days in the dark at 28 ºC. Finally, inoculated explants were transferred into regeneration medium (RM) (Table 1). Then, they were placed in a growth chamber at a photoperiod of 16:8 (L:D) hours at 22 ºC. Subculturing was done every 10-15 days.

Genomic DNA purification and PCR analysis of transgenic plants

Genomic DNA of putative transgenic plants and non-transgenic plants was extracted from 0.5 g (fresh weight) of leaves tissue, using modified CTAB method (20). PCR amplification of
genomic DNA for presence of the \textit{Hu-IFN-\(\gamma\)} gene was carried out using the primers described above.

TABLE 1

Composition of culture media

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<th>IM(^f)</th>
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Sucrose (g/L) 50; Acetosyringone (mM) 0.1; NAA (mg/L) 2; BAP (mg/L) 250; Cefotaxime (mg/L) 7.5; Hygromycin mg/L.

\(^a\)Murashige and Skoog (8);
\(^b\)GM: Germination Medium;
\(^c\)PM: Pre-culture Medium;
\(^d\)CM: Co-culture Medium;
\(^*\)RM: Regeneration Medium;
\(^f\)IM: Infection Medium

Protein extraction and immune blot analysis

The total soluble protein was extracted from transformed and non-transformed plants by grinding 0.5 g of leaves in liquid nitrogen with a pestle and mortar and resuspending in 1.5 ml of extraction buffer (5 ml Tris-HCl 1M, 200 µl EDTA 1M, 0.04% v/v 2-mercaptoethanol for 100 ml). The extracts were centrifuged at 11500 rpm for 15 min at 4 °C. The soluble protein was centrifuged one more time to remove the remaining insoluble fractions (at the same speed and time). The concentration of extracted soluble protein in the supernatant was determined by the Bradford protein assay (23) using bovine serum albumin (BSA) as a standard. After protein extraction from whole leaves, dot blot analysis was carried out. Solution preparation and the methods are described by Roche Company which provides Anti-His6Peroxidase. Ten nanograms of protein samples were directly spotted onto nitrocellulose membrane. After drying, western blocking solution 1X was added and incubated for 1 h at 15-25 °C. Solution was poured out and replaced with Anti-His6Peroxidase. Then it was incubated for 90 min. After the solution was poured out, washing with TBST1x was repeated 3 times for 5 min each time. Antibody and substrate were added. After color development nitrocellulose membrane was washed with water and checked in both transgenic and control plants.

Results and Discussion

Construction of binary vector

The nucleotide sequence of the \textit{Hu-IFN-\(\gamma\)} gene was modified using designed primers via PCR technique. A 500-bp-long fragment of this PCR product was cloned into the CaMV35S in upstream and \textit{NOS} terminator in downstream of pCAMBIA1304 vector between \textit{BstEII} and \textit{NcoI} restriction sites (Fig. 2). Then, this construct was transformed into \textit{E. coli} strain DH5\(\alpha\). The presence of the \textit{Hu-IFN-\(\gamma\)} gene was confirmed by colony PCR (Fig. 3) and digestion reaction which was conducted by \textit{BstEII} and \textit{NcoI} restriction enzymes (Fig. 4). Finally, the designed construct was transformed into \textit{Agrobacterium tumefaciens} strain LBA4404 and, using the colony PCR technique, it was confirmed that those recombinant constructs were transformed into the \textit{Agrobacterium}.

![Fig. 2](image_url) T-DNA region of pCAMBIA-IFN-\(\gamma\). LB and RB: Left and Right Borders, HYG(R): Hygromycin selectable marker, CaMV35s: Cauliflower Mosaic Virus promoter, \textit{NcoI} and \textit{BstEII}: restriction sites, and \textit{NOS}: Nopaline Synthase terminator.

![Fig. 3](image_url) Proof of the presence of \textit{Hu-IFN-\(\gamma\)} gene in pCAMBIA1304 by colony PCR technique. M: GeneRuler™ 1 kb DNA Ladder (Fermentas), Lane 1: Negative control (pCAMBIA1304 without \textit{Hu-IFN-\(\gamma\)} gene), Lane 2: ddH\(_2\)O, Lanes 3-7: Randomly selected colonies, Lane 8: positive control (pRSET).

![Fig. 4](image_url) Proof of the presence of \textit{Hu-IFN-\(\gamma\)} gene by digestion reaction. M: GeneRuler™ 1 kb DNA Ladder(Fermentas), Lane 1: Negative control (pCAMBIA1304 without \textit{Hu-IFN-\(\gamma\)} gene), Lanes 2 and 3: recombinant vector after digestion by \textit{BstEII} and \textit{NcoI} enzymes.
Plant transformation

Cotyledonary explants from tomato were incubated with the Agrobacterium carrying the recombinant binary vector. After two to three times of subculturing, the cotyledonary leaves regenerated new plants at the cutting edges, while the control plants did not show any regeneration (Fig. 5). The regenerated plants were sub-cultured on the same medium in tissue culture glass bottles (Fig. 6). Eighteen out of 25 transgenic lines were normal. The frequency of transformation was about 19% (data not shown).

Analysis of protein expression by dot blotting

Based on the hygromycin resistance and PCR amplification, selected transgenic plants were used for protein extraction from leaf tissue and dot blot analysis. The comparison of the dot blot results in transgenic and wild type plants confirmed the expression of the Hu-IFN-γ protein (Fig. 8). The expression of Hu-IFN-γ which has a His tag in the C-terminus can be detected by anti His tag peroxidase and changed substrate colour, while the extracted protein from wild type plants did not show changing colour in the dot blot assay.

Some recombinant proteins produced by microorganisms do not have appropriate biological activity in humans. The major disadvantages of microorganisms consist in the inability to carry out many of the post-translational modifications established in eukaryotic proteins, the lack of a secretion mechanism for efficient transport of proteins into the culture media, the production of insoluble forms of proteins and inability to form disulfide bonds in the cytoplasm. Traditional production systems that use microbial fermentation, transgenic animals, insects and mammalian cell cultures have limitations in cost, product safety and credibility. Therefore an inexpensive and simple system that can produce safe and high level recombinant proteins would be demanded (7).

New studies have demonstrated that molecular farming in plants has many advantages in practical, economic and safety aspects. It is estimated that the use of plants can reduce the cost of production compared to other production systems like microbial fermentation systems (2-10% cost reduction) and mammalian cell cultures (0.1% cost reduction) (21). So production of plant-produced proteins is steadily increasing because transgenic plants are a good choice for production of pharmaceutical compounds and complex human proteins.

The purpose of this study was cloning, transferring and expression of Hu-IFN-γ gene in tomato. In this research plant expression vector pCAMBIA1304 was used in plant transformation. This vector carries a kanamycin resistance gene for selection of the bacterial colonies and a hygromycin resistance gene for screening of the transgenic lines. The CaMV35s promoter and NOS terminator were used as transcription elements. In dicot plants, CaMV35s is a suitable promoter because it is potent and constant and it can cause high-level transgene expression in leaves, fruits, tubers, roots and other organs (6, 10). The plant high level expression
sequence (Kozak sequence) was added to the forward primer for enhancing gene expression in the translation step. His-tag sequence was added at the beginning of the Hu-IFN-γ gene and can be used for identification and isolation of our protein from total soluble protein, using Anti-Histidine tags. This strategy is one of the most suitable methods for protein purification. Factor Xa is used for separation of the His-tag from the purified protein.

The production of pharmaceutical recombinant protein in plant expression systems can compete with conventional expression systems. However, a number of problems in biopharmaceutical based plant production such as higher cost of purification and low level expression must be addressed and new technologies have to be established to overcome the problems (14).

According to the therapeutic use of interferon and the benefits of the tomato as a host for recombinant protein production, this expression platform can be an appropriate alternative system for production of human gamma interferon protein.

The present study is, to the best of our knowledge, the first research report for production of human gamma interferon in tomato and can be used as a basis for further research to produce human gamma interferon protein in this plant.

Acknowledgements
The financial and moral assistance from the Center of Biotechnology and Life Science and the support of the Agricultural Biotechnology Lab of SCU are gratefully acknowledged.

REFERENCES