PROGRESS IN BIOLOGICAL SCIENCES

Expression and purification of human interferon gamma using a plant viral vector

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Abstract

A plant viral vector engineered from an *in vivo* infectious clone of zucchini yellow mosaic virus (ZYMV) was used to express the human interferon-gamma (INF-γ) *in planta*. The INF-γ gene was in frame inserted between the P1 and HC-Pro ORFs of the ZYMV vector. The infectious activity of the vector was approved by rubbing the plasmid on *Chenopodium quinoa* and observing local lesions. Individual lesions were mechanically transferred to the systemic host plant zucchini squash at the stage of cotyledonary leaf. The stability of INF-γ expression was assessed by successive passages of recombinant viruses from infected plant and throughout the period of 35 days after inoculating in a single plant. Then, the leaf tissues of inoculated plant were analyzed for the presence of transgene by RT-PCR and western blot analysis. The recombimamt protein was purified using affinity chromatography method. The results showed approximately 1–1.2 mg INF-γ per 100 g tissues were purified from leaves two weeks post inoculation. Also, the vector was remarkably stable in squash after six serial passages and 35 days. The procedure provides a convenient and fast method for production of large quantities of pure INF-γ *in planta*. The system also has a potential for production of other proteins of interest in cucurbits to use as immunogen to produce antiserum or use for other purposes.

Keywords: transient expression, recombinant protein, ZYMV.

Introduction

Biopharmaceuticals present the fastest growing part in the pharmaceutical industry, with an ever widening scope of applications. Whole plants as well as plant cell culture systems are being explored for their potential as a cheap, safe, and scalable production host (Karg and Kallio, 2009). The use of plants as bioreactor for production of biomedical materials was called "green revolution" (Koprowski and Yusibov, 2001). Plant expression system, as a new platform for therapeutic protein production, has many advantages over other expression systems. Firstly, the low cost of crop cultivation will reduces the amount of capital investment required for commercial production. Secondly, it would be safe because generally lacks human pathogens, oncogenic DNA sequences, and endotoxins (Commandeur *et al*., 2003). Although many such proteins are produced through the establishment of transgenic plants, this is a time-consuming and slightly costly process. The use of plant viral vectors for the transient expression of heterologous proteins offers a useful tool for the large-scale production of proteins of pharmaceutical and industrial importance. The plant viral vectors provide a fast and efficient method for production of special proteins in eukaryotic plant cells. Many plant viruses have been successfully engineered for *in planta* expression of heterologous proteins (Gleba *et al*., 2004, Gleba *et al*., 2007).

Zuchiini yellow mosaic virus (ZYMV) causes one of the most devastating diseases in cucurbits including cucumber, squash, melon and water-melon (Desbiez *et al*., 1997). Similar to all

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potyviruses, the ZYMV genome consists of a positive single strand RNA molecule of 9.6 kb encapsidated in a filamentous particle. Viral RNA is translated into a large polyprotein which is proteolytically processed to 10 functional proteins by three virus-encoded proteases: P1, HC-Pro, and NIa (Riechmann *et al*., 1992, Revers *et al*., 1999). Recently, a ZYMV vector containing a cauliflower mosaic virus (CaMV) 35S promoter and the full-length cDNA of ZYMV TW-TN3, an isolate collected from southern Taiwan (Lin *et al*., 1998), was constructed (Lin *et al*., 2002). The green fluorescent protein (GFP) was inserted into the N-terminal region of the HC-Pro of the ZYMV vector. Six histidine residues were added at the C-terminal end of GFP followed by an NIa proteolytic cleavage site to facilitate the purification by nickel nitriloacetic acid (Ni2+-NTA) affinity chromatography and the processing for production of free form proteins, respectively (Hsu *et al*., 2004).

Interferon gamma (INF-γ), or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. The importance of INF-γ in the immune system in part from its ability to inhibit viral replication directly and most importantly from its immunostimulatory and immunomodulatory effects (Schoenborn and Wilson, 2007). Furthermore the INF-γ is used to treat chronic granulomatous disease and osteopetrosis (Todd and Goa, 1992, Key *et al*., 1995). Traditionally, production of recombinant INF-γ has relied mainly upon bacterial and mammalian cell systems. Production in these systems is costly. Furthermore, recombinant INF-γ expressed in bacterial systems is not appropriately glycosylated, leading to susceptibility to proteolysis and shorter survival times in blood (Chen *et al*., 2004). To overcome these problems, some researchers tried to eliminate these restrictions and express INF-γ in plants (Leelavathi *et al*., 2004, Chen *et al*., 2005, Han *et al*., 2009, Bagheri *et al*., 2010). In a research, Chen *et al* (2004) demonstrated that the functional INF-γ can be stably expressed in transgenic rice suspension cells and exhibits biological properties similar to the commercially available one. Similarly, the INF-γ was expressed in tobacco leaves (Han *et al*., 2009), tobacco chloroplasts (Leelavathi *et al*., 2004) and canola seeds (Bagheri *et al*., 2010). These systems are not only time-consuming and costly process but also the results depend on the insertion site of transgene and the recombinant protein expression is not consistence.

In this study, INF-γ gene was introduced into the ZYMV vector by replacing the GFP reading frame. After inoculation with the recombinants ZYMV, large quantities of the INF-γ was systemically expressed in zucchini squash plants. This procedure provides a convenient and fast method for production of large quantities of pure INF-γ *in planta*. The system also has a potential for production of other proteins of interest in cucurbits to use as immunogen to produce antiserum or use for other purposes.

Materials and Methods

Virus source and amplification of INF-γ gene

A previously constructed infectious plant virus clone, p35SZYMVGFPhis3, that contains the full-length cDNA of an Taiwan isolate of ZYMV (TW-TN3), driven by the cauliflower mosaic virus 35S promoter and a reporter GFP gene between the P1 and the HC-Pro genes (Hsu *et al*., 2004), was engineered as an *in vivo* viral vector to express INF-γ in squash.

Human INF-γ cDNA has been isolated and cloned by Moeen Rezakhanlou *et al*. (2003) (Accession no. AF506749, GenBank). To sub-clone the INF-γ gene, the appropriate primer with *Sph*I and *Kpn*I restriction sites in the forward and reverse primers were designed (Table 1). Polymerase chain reaction (PCR) was performed in a total 50 µl final volume, using 2 mM of each deoxyribonucleotide triphosphate (dNTPs), 0.4 µM of each primer, 1 mM Mg^{2+}, and 0.5 unit of KOD plus DNA polymerase enzyme (ShineGene Molecular Biotech, Inc., China). Thermocycler was programmed for one cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 30 second; 55°C for 30 second; 68°C for 40 second and one cycle at 68°C for 3 min as a final extension. The resulted PCR product was cleaned up using the DNA purification kit (GeneMark, Taiwan).

Construction of recombinant ZYMV-INF-γ vector

Individual INF-γ gene was directly inserted into the cloning site between the P1 and HC-Pro ORFs of the ZYMV vector (named ZYMV-INF-γ) as shown in Fig. 1-A. The purified INF-γ gene was double digested by *Sph*I/*Kpn*I enzymes (Invitrogen, USA). The same restriction enzymes were also used to remove the GFP ORF from the ZYMV vector p35SZYMVGFPhis3, the digested products were separated on 0.8% agarose gel and then was purified using the agarose gel DNA extraction kit (GeneMark, Taiwan). The INF-γ gene was ligated into viral vector and then transformed in *E. coli*-XL1 competent cells by standard method (Sambrook and Russell, 2001). Resulted colonies were screened by colony PCR using INF-γ (pINFG) and ZYMV (mz1155) specific forward and reverse primers (Table 1) that amplify a DNA fragment with 572 bp length (Fig. 1-C). Two positive colonies were selected for plasmid purification. The sequence of the recombinant plasmid was confirmed by restriction enzyme analysis and sequencing.

Infectivity assay of chimeric constructs and RT-PCR confirmation

Plasmids of the individual chimeric constructs were isolated by the mini-prep plasmid purification kit (GeneMark), and mechanically were rubbed with a cotton stick on leaves (10 µg in 10 µl per leaf) of local lesion host *Chenopodium quinoa* previously dusted with 600 mesh carborundum. Individual lesions were mechanically transferred to the systemic host plant zucchini squash at the stage of cotyledonary leaf. Total RNA was extracted with TRIzol reagent (Invitrogene) from the infected squash leaves. Specific primer pairs pINFG and mz1155 (Table 1) were used to prove the presence of the inserted INF-γ gene by RT-PCR. Plants inoculated by p35SZYMV TW TN3 were used as negative control.

Stability assay of the recombinant ZYMV-INF-γ in squash plants

The stability of ZYMV-INF-γ was assessed by successive passages of recombinant viruses from the infected plant tissues and throughout the period of 35 days post inoculation (dpi) in a single plant. Recombinant virus in the infected squash was mechanically transferred to healthy plants at 10 days intervals. After several passages, the leaf tissues collected from the inoculated plants were analyzed by western blotting and RT-PCR with appropriate antisera and primer pairs, respectively. To further monitor the possible deletion of INF-γ gene from ZYMV-INF-γ progeny-infected hosts, the amplified DNA fragment was sequenced and the nucleotide sequence was analyzed by the program of BioEdit (Hall, 1999).

Detection of the expressed INF-γ by immunoassays

Immunoblotting (Gooderham, 1984) and indirect enzyme-linked immunosorbent assays (ELISA) (Wang and Gonsalves, 1990) were used to quantitative detection of the ZYMV-expressed INF-γ and the coat protein of ZYMV (CP) in the infected squash plants at 5, 10, 15, 20, 25, 30 and 35 dpi. For immunoblotting, total proteins were isolated from the recombinant-infected leaf tissues by freezing 0.5 g tissue in liquid nitrogen and macerating into a fine powder, then adding 2 ml dissociation buffer [100 mM Tris–HCl (pH 7.2), 2% β-mercaptoethanol, 10% sucrose, 0.005% bromophenol blue, and 10 mM EDTA] and denatured by boiling for 5 min. After spin down, the supernatant was separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat dry milk powder. For indirect ELISA tests, polystyrene microtitration plates (Greiner Bio-One, Frickenhausen, Germany) were coated with leaf crude extracts at a 40-fold dilution in coating buffer (50 mM sodium carbonate, pH 9.6, containing 0.01% sodium azide). For both immunological assays, the monoclonal antibody to histidine tag (His-MAb) (Amersham Pharmacia Biotech, Buckinghamshire, England) and the rabbit antiserum to ZYMV CP were used at a 1/5000 dilution to detect the histidine-tagged INF-γ and ZYMV CP, respectively. The alkaline phosphatase (AP)-conjugated goat anti- mouse and goat anti-rabbit immunoglobulin (IgG)

Table 1. The primers used for amplification of INF-γ, colony PCR and RT-PCR.

Primer name	Primer sequence (5'-3')	Restriction site (underlined)
pIFNG	GCGCATGCCAGGAGCCATATGTAAAAGAAG	*Sph*I
mINFG	CCGGATCCCTGGGATGCTCTTCGACCTCG	*Bam*HI
mz1155	ACTTTGCACACATGATCTGG	-

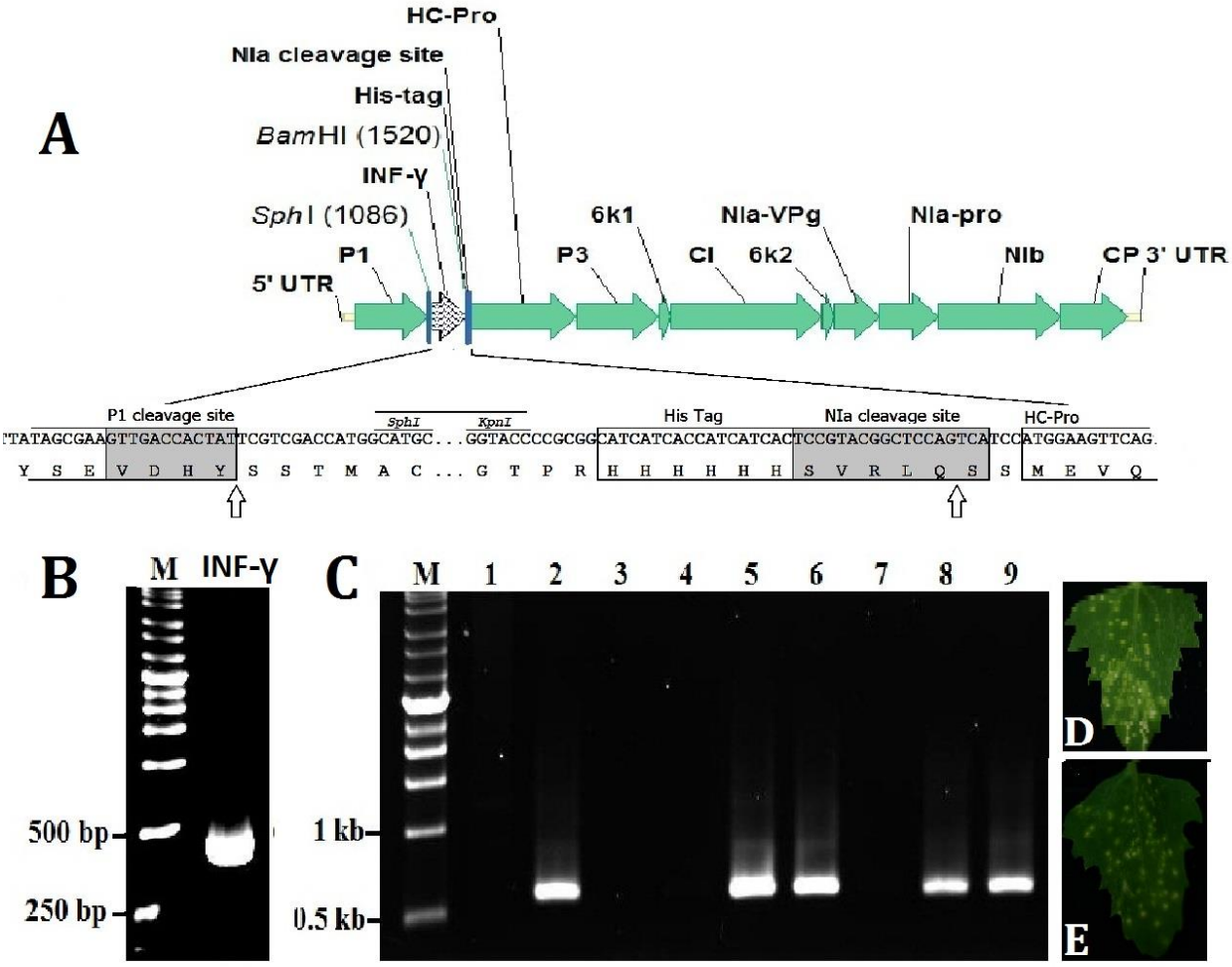

Figure 1. Construction of ZYMV-INF-γ and examining of its infectivity. A. Schematic representation of ZYMV-INF-γ full genome. Viral proteins and relevant restriction sites (with their position in parentheses) for insertion were shown. B. Amplification of INF-γ ORF showing a 448 bp DNA fragment. C. Screening of transformed colonies with primer pairs: pINFG, mz1155. The colonies with a 572 bp DNA fragment were considered as recombinant and selected for plasmid purification. M, 1 kb ladder (GenDirex, USA); lane 1-9: colony numbers. D. The local lesions of ZYMV-INF-γ and of ZYMV TW TN3 on *C. quinoa* (E).

(Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1/5000 dilutions to react with the monoclonal antibody and rabbit antisera, respectively. Reactions were visualized by the addition of chromogenic substrate (nitro-blue tetrazolium/5-bromo-4-chloro-3- indolyl phosphate paratoluidine salt in 100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris–HCl, pH 9.5) for immunoblotting. In indirect ELISA, substrate solution was prepared by dissolving AP substrate tablets (Sigma 104; Sigma-Aldrich Fine Chemicals, Milwaukee, WI) in substrate buffer (9.7% diethanolamine and 0.02% sodium azide, pH 9.8) to a final concentration of 0.5 mg/ml. The absorbance at 405 nm was recorded 1 h after the addition of the substrate using an EL×800 universal microplate reader (Bio-Tek instrument, Winooski, VT, USA).

Purification of the expressed INF-γ

The affinity chromatography procedure described by (Gal-On *et al*., 2000) was modified for purification of the ZYMV-expressed INF-γ from infected plants. Fifteen days after inoculation with the recombinants ZYMV, 100 g infected zucchini squash leaves were harvested and ground in 300 ml of buffer A [50 mM Tris–HCl, pH 8.0, 15 mM $MgCl_2$, 10 m M KCl, 20% (v/v) glycerol, 0.05% β-mercaptoethanol, and 0.1 mM phenylmethylsulphonyl fluoride (PMSF)] in a blender. Extracts were clarified by centrifugation at 20000×g for 10 min, and supernatant filtered through Miracloth (Calbiochem, La Jolla, CA). The filtrate, treated with 2% Triton X-100 at 4°C for 30 min, and approximately 1 ml of Ni^{2+}-NTA resin (Ni-NTA, Invitrogen, USA), pre-equilibrated in buffer B [50 mM Tris–HCl, pH 8.2, 15 mM $MgCl_2$, 20% (v/v) glycerol, 0.05% β-mercaptoethanol, and 0.1 mM PMSF], was added. The mixture was gently stirred for 1 h at 4°C and loaded onto a column. After allowing the resin to settle, the unbound material was collected and the resin was washed with equal volume of buffer B. The proteins bound to the resin were eluted with 6 ml of buffer B containing 250 mM imidazole in three steps. Equal volumes (5 µl for each well) of each fraction obtained from each purification step were analyzed using His-MAbs (Amersham Pharmacia Biotech, Buckinghamshire, England) in immunoblotting. The amounts of purified proteins were estimated by the comparison with bovine serum albumin (BSA) in SDS–PAGE, and estimated by the software Spot Density of Alpha Innotech IS2000 (Alpha Innotech Corporation, San Leandro, CA).

Results

Infectivity of ZYMV- INF-γ and confirmation of the expression of the INF-γ

Chlorotic local lesions similar to those induced by ZYMV TW TN3 developed on inoculated leaves of *C. quinoa* 7–9 days after inoculation with the chimeric constructs (Fig. 1-D). One week after inoculation, systemic symptoms of yellow mosaic were noticed on all squash plants. Successful transcription and translation from ZYMV recombinants in the infected plants were confirmed by RT-PCR and serological assays, respectively (Fig. 2). The specific primer pairs for INF-γ ORF were used to amplify the inserts from total RNAs extracted from the infected plants. The DNA fragments with about 450 bp length corresponding to the inserted INF-γ ORF was amplified till the flowering stage (Fig. 2-A).

Stability and detection of INF-γ expressed by ZYMV

ZYMV-expressed INF-γ containing a histidine tag was identified by immunoblotting using the His-MAb (Fig. 2-B). Protein of 18.5 kDa was present in the crude extracts of zucchini squash till 35 days after inoculation with recombinant ZYMV and after 6 passages (Fig. 2-B, Fig. 3-B). Sequenced RT-PCR product sampled from the 6^{th} passage and after 35 dpi did not show any change in nucleotide sequence. Each expressed INF-γ was 1.5 kDa larger than the native INF-γ resulting from the added cloning sites and the histidine tag (Fig. 1-A, 2-B). No serological reactions were detected from extracts of p35SZYMV TW TN3 inoculated squash plants (negative control). The highest level of ZYMV-expressed INF-γ and ZYMV CP was shown by ELISA at 15 and 10 dpi respectively (Fig. 2-D). Immunoblotting using the antiserum specific to CP of ZYMV revealed the presence of a 31.3 kDa protein similar in molecular weight observed in p35SZYMV TW TN3 infected zucchini squash (Fig. 2-C).

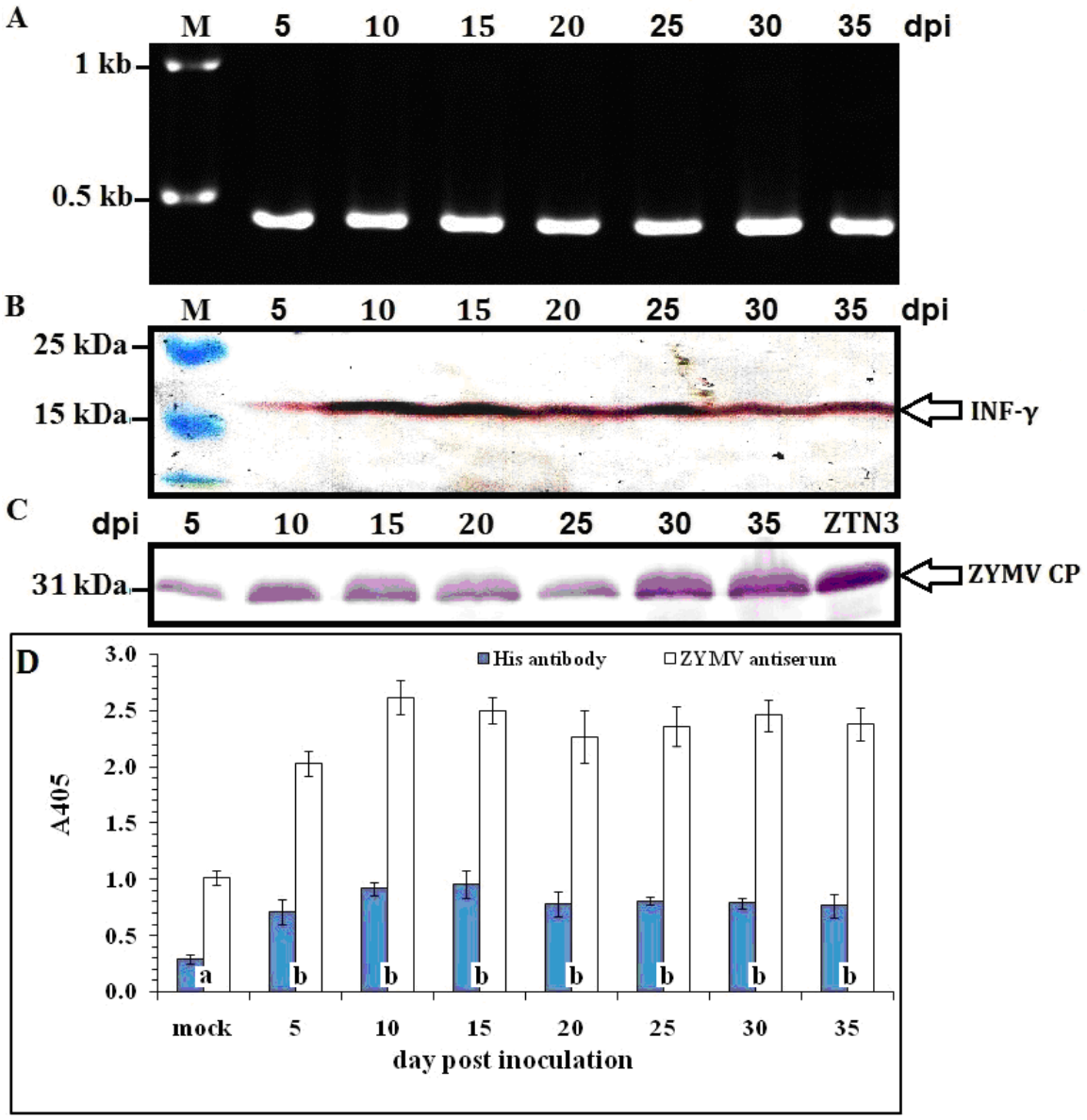

Figure 2. Detection and examining the stability of ZYMV-INF-γ vector in squash. A. The RT-PCR with INF-γ specific primers in the ZYMV genome should yield 448 bp products from ZYMV-INF-γ-infected squash. M: 1 kb ladder (GenDirex, USA); B. Detection of the INF-γ proteins expressed by ZYMV in infected plants of zucchini squash. The His MAb was used to detect individual ZYMV-expressed INF-γ in plants during 35 days after inoculation. M: protein ladder (PageRuller^{TM}Prestained, Fermentas, USA); C. Detection of the ZYMV CP by western blotting using ZYMV antiserum; D. Detection of the INF-γ proteins expressed by ZYMV in infected plants of zucchini squash by ELISA. The His MAb and ZYMV antiserum were used to detect individual ZYMV-expressed INF-γ and ZYMV CP in plants during 35 days after inoculation.

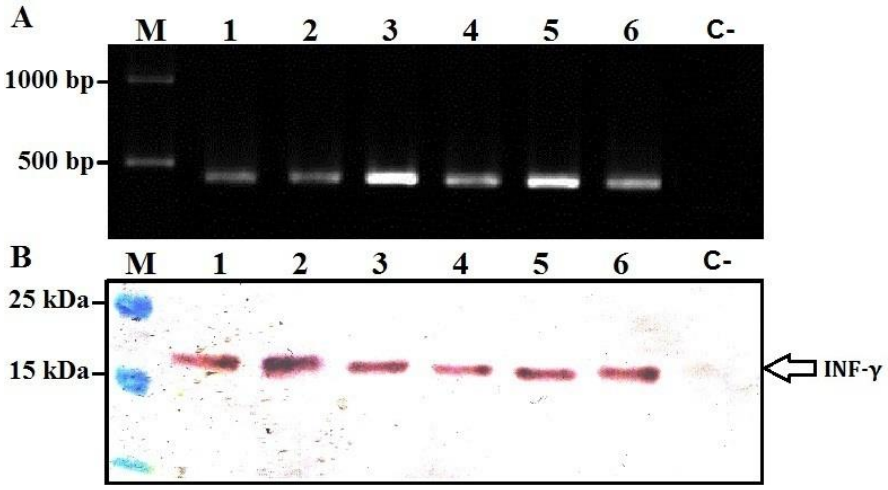

Figure 3. Examining of stability of ZYMV- INF-γ after six passages. A. RT-PCR amplification profile from squash plants infected with ZYMV- INF-γ vectors after 1-6 serial passages corresponding to lane 1-6; lane 7: negative control (ZYMV-WT). B. Detection of the INF-γ proteins expressed by ZYMV in infected plants of zucchini squash. The His MAb was used to detect individual ZYMV-expressed INF-γ in plants after 1-6 serial passages corresponding to lane 1-6; lane 7: negative control (p35SZYMV).

Purification of ZYMV-expressed INF-γ from the infected squash plants

Using the affinity column chromatography, INF-γ expressed by the ZYMV recombinants were purified from infected squash plants. An example of monitoring purification of ZYMV-expressed INF-γ is presented in Fig. 4. During purification, samples from each step were collected and the presence of expressed INF-γ was monitored by SDS–PAGE coupled with immunoblotting. The 18.5 kDa protein corresponding to the ZYMV-expressed INF-γ obtained in the soluble and eluted fractions was confirmed by immunoblotting using the His-MAb. The expressed INF-γ ranging from 1 to 1.2 mg was purified from every 100 g tissues of squash plants inoculated with ZYMV recombinants as determined by the comparison with BSA in SDS-PAGE and by the software Spot Density of AlphaInnotech IS2000.

Discussion

Plants have been engineered to express foreign genes for agronomic traits since the early 1980s, but only more recently have been used as production vehicles for protein products (Karg and Kallio, 2009). The use of plants as hosts for the over-expression of foreign proteins is still a developing field. Recently there are various approaches used as plant bioreactors, such as transgenic plants (Han *et al*., 2009), agroinfiltration (Li *et al*., 2007) and infection with modified plant viral vectors (Canizares *et al*., 2005). The most successful expression systems today for high accumulation are using viral vectors (Egelkrout *et al*., 2012). The present study was performed in order to investigate whether INF-γ as a model protein could be successfully expressed transiently in a plant-virus expression system. Some studies have shown the importance of having eukaryotic specific modifications and correct folding for proper functioning of recombinant proteins (Han *et al*., 2009; Karg and Kallio, 2009; Egelkrout *et al*., 2012). In this light, we tried to express human INF-γ in the squash plant eukaryotic host using ZYMV as a vector. So, the human INF-γ gene was cloned between P1 and HC-Pro in ZYMV vector and expression analysis was performed. foreign genes could theoretically be introduced in between each of the 10 virally-encoded proteins of a potyvirus (Masuta *et al*., 2000). Nevertheless, we could not detect cucumber green mottle mosaic virus-coat protein expression by immunoblotting when this ORF was inserted between the P1 and HC-Pro genes

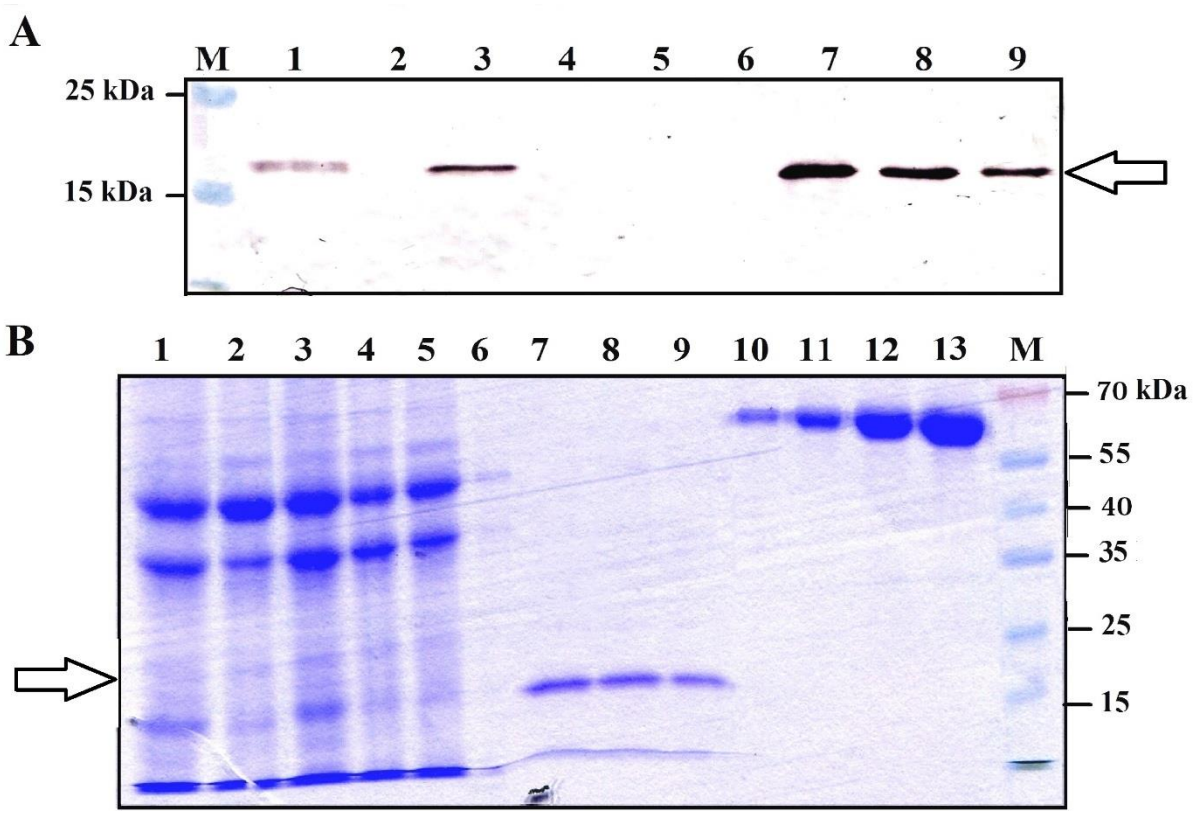

Figure 4. Purification of INF-γ proteins expressed by ZYMV-INF-γ in infected zucchini squash plants by affinity chromatography. A. Monoclonal antibody against the histidine tag was used to detect the presence of ZYMV-expressed INF-γ proteins by western blotting in different fractions during purification by the Ni-NTA affinity chromatography, including the crude (lane 1), the pellet (lane 2) and the supernatant (lane 3) after 20000 × g high-speed centrifugation, the flow-through of the supernatant for two times (lane 4-5), the flow-through of buffer containing 5 mM Imidazole for washing (lane 6), and the eluted fractions of buffer containing 250 mM imidazole (lane 7-9). B. SDS-PAGE of purification process and comparison with BSA; lane 1-9 are the same as above and in lane 10-13 BSA were loaded 1, 2, 5 and 10 µg/5 µl, respectively; M: protein ladder (PageRuller^{TM}Prestained, Fermentas, USA).

of the ZYMV vector; this commonly due to the instability of the protein in plant because it was detected by RT-PCR (unpublished data).

Based on our results, INF-γ protein can be produced using ZYMV vector whitin two weeks without problems commonly raised by working on transgenic plants. Also, the stability assay of INF-γ gene indicated that this ORF could be stable throughout a plant infection and even after six passages; so infected plant can be used to inoculate other plants again. Although the protein was detected at 35 dpi, ELIAS results indicated that the highest expression level of recombinant INF-γ was at 10-15 dpi. Regarding this finding, we harvested leaves at 15 dpi to purify INF-γ protein. Due to comparatively short half-life of INF-γ protein (Leelavathi *et al*., 2004), it had better to harvest leaves after emerging symptom and producing enough biomass. Stability assay also showed RNA recombination was not occurred to knock out the insert during a period of 35 days and also after six passages. This may be because sequences flanking this site do not promote a high frequency of recombination events or may be short foreign genes insertion between P1 and HC-Pro are not susceptible for recombination events as was shown for other potyviruses (Gal-On *et al*., 1998).

ELISA result using ZYMV antiserum indicated the highest titer of virus at 10-15 dpi, so it seems that expression level of INF-γ protein is related to virus titer. Because while virus expressed its polyprotein in high amount, the INF-γ protein was also co-expressed in polyprotein and it was released by P1 and NIb cleavage sites at N- and C-terminal, respectively. The fusion forms of P1-INF-γ, INF-γ-HC-Pro or P1- INF-γ-HC-Pro were not detected. Also, in purification process, recombinant protein was always in soluble fractions, so we used Ni-NTA affinity chromatography to purify His-tagged protein. Our analysis showed approximately 1-1.2 mg INF-γ protein per 100 g tissues were purified from inoculated leaves. There is no unique system to compare the expression level with other systems but this amount is more than recombinant protein expression in transgenic plants (Egelkrout *et al*., 2012).

While some reports have been suggested that orally administrated interferon gamma can be use as an efficient drug in animals and humans (Cummins *et al*., 1999, Marcus *et al*., 1999), but other reports suggested injecting INF-γ (Auluck *et al*., 2008). It would be possible that the INF-γ which is expressed by ZYMV in cucurbit fruits might be applied orally to treat patients. However, further studies are needed to determine whether the expressed INF-γ is active in fruits.

Bacterial expression systems were used to produce the INF-γ (Moeen Rezakhanloum *et al*., 2003); but differences between the prokaryotic and eukaryotic cell systems for producing INF-γ may affect the post-translational modification and correct folding of the proteins. In comparison to bacterial expression system, using viral vector for pharmaceutical protein expression in plant is an efficient and inexpensive recombinant protein production while minimizing the risk for contamination with endotoxins and maximizing potential for large-scale manufacturing of biopharmaceuticals to meet the global demand (Yevtushenko and Misra, 2012). Also, this system does not have prokaryotic limitation including the presence of endogenously produced endotoxins and pyrogens, plasmid instability, the lack of capability for post-translational modifications such as the proper formation of disulfide bonds and glycosylation of the designated sites, often leading to misfolded proteins (Bagheri *et al*., 2010). In addition, it is estimated that the cost of producing a recombinant protein in plants could be 10-50 fold lower than producing the same protein in *E. coli* fermentation, depending on the crop (Giddings *et al*., 2000).

Some transgenic plants were engineered to express INF-γ in different parts of plants (Leelavathi *et al*., 2004, Chen *et al*., 2005, Han *et al*., 2009, Bagheri *et al*., 2010); but producing transgenic line compare to using plant viral vector is time–consuming and costly process while the result depends on the position of transferred transgene. In comparison to transgenic plants, this plant-viral expression system showed some advantages: the major advantages of a transient expression approach are time efficiency, high levels of target protein expression, uniformity and consistency of target accumulation, fewer environmental concerns due to contained facility production (Mett *et al*., 2008). The results of this study showed that INF-γ gene was stable even after six passages, there is

no concern for mutation and recombination but it had better to use original plasmid or frozen local lesion host leaves to infect new plants. In addition, the squash plant used in this study is a fast growing plant in which produce large amount of biomass in a short period of time and also squash can be inoculated with plasmid directly and easily. Furthermore, in controlled conditions, virus could not escape and also insertion of a pharmaceutical gene like INF-γ could not make virus strain more severe, because it does not have any role in virus life cycle and we did not see severe symptom after inserting INF-γ gene.

In summary, in the present work, we have demonstrated the feasibility of using attenuated ZYMV as an expression vector in cucurbits. We now have a tool to produce recombinant proteins at high levels making the future very promising for the role of plant viral vectors as a vehicle for industrial and pharmaceutical protein production. This vector may also be useful as a tool for *in planta* genomic studies, as it can express a gene with effective protein expression. The large diversity in plants and viral vectors provides solutions for recalcitrant proteins, particularly for pharmaceutical and industrial proteins. However, many options available in plant-virus expression system also complicate the likelihood of finding the optimal combinations. It is not known what the upper practical limit of recombinant protein expression is in the plant cell but the future for reaching even higher levels of protein accumulation appears hopeful.

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