

# Processing of Human Nerve Growth Factor $\beta$ Proprotein in the Plant Cell Apoplast

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## Research Article

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# Abstract

The Human HNGF- $\beta$  gene encodes a pre-pro-NGF- $\beta$  precursor protein. The signal peptide is cleaved off in the endoplasmic reticulum and the resulting pro-protein is processed in the trans-Golgi network by the Furin enzyme.

The processing of the proproteins in the plant cells is not clearly defined. In the present study, various changes were applied to the HNGF- $\beta$  gene sequence to increase its expression in the plant cells. The construct containing the synthesized Plant NGF- $\beta$  gene was agro-infiltrated into the *Nicotiana benthamiana* leaves. Thereafter, the expression of various forms of the recombinant NGF- $\beta$  and its processing was evaluated using dot blot, western blot, and RP-HPLC analyses. Finally, the biological activity of the mature NGF- $\beta$  purified from the apoplast was assessed on the differentiation of PC12 cells and the expression of tetanus toxin receptors on their surfaces.

Dot blot and western blot results showed that the total soluble proteins extracted from the plant leaves and the apoplast extract contained pro-NGF- $\beta$  and mature NGF- $\beta$  proteins, respectively. The RP-HPLC results reconfirmed the presence of the mature NGF- $\beta$  in the apoplast extract. The amount of the mature NGF- $\beta$  produced in the plant leaves was estimated to be about 39 $\mu$ g/g of the fresh leaves. A gradual increase in the length and number of the neurites of the differentiated PC12 cells was showed upon treatment with the mature NGF- $\beta$ . Immunofluorescence experiments showed that the FITC-labeled tetanus toxin strongly bound to PC12 cells treated with mature NGF- $\beta$ .

This modification shows a very important advantage for plants to produce valuable biosimilar pharmaceutical proteins from precursor without applying biochemical or co-expression of modifying enzymes.

# Introduction

NGF- $\beta$  is the first discovered member of the neurotrophin family [1] and contributes in the growth, maintenance, and survival of the neurons. The perturbation of NGF- $\beta$  synthesis enhances dementia, depression, schizophrenia, Rett syndrome, autism, anorexia nervosa, and bulimia nervosa [2, 3]. It also acts as a key regulator of nociceptive pain and appears to be target molecule in analgesic medication [4].

Therefore, NGF- $\beta$  is considered as a potential pharmacological drug [5]. By far, in the industrial scale, an cost effective expression system capable of producing recombinant NGF- $\beta$  (rNGF- $\beta$ ) in the functional form has not been developed [6].

Plant cells are considered as one of the safest hosts for the production of recombinant proteins due to the lower risk of transmitting animal and human infectious agents. In addition, lower cost of the protein production with the proper post translational modifications (PTMs) as well as higher scalability, offers the plant cells among the most advantageous expression systems, especially in the industrial scale. Recently, the plant-derived recombinant protein H5N1 has entered phase three of the clinical trial as a

vaccine candidate against influenza virus [11]. In spite of these benefits, not all the PTMs is performed in the plant cells including production of active form of proproteins by removal of its prodomain. Serine proteases are enzymes with the serine residue in their active site which triggers cleavage of the peptide bonds. They divided into trypsin-like and subtilisin-like categories. Interestingly, Furin is a subtilisin-like serin protease which resides in the mammalian Golgi apparatus and removes the prodomain from the proproteins [12]. Therefore, production of active form of proproteins such as Bone morphogenesis protein 2, Tissue plasminogen activator, NGF- $\beta$  in the plants cells needs an additional step to be carried out for removal of the prodomain. Galba *et al* have shown that HNGF- $\beta$  could be produced in plant, however, in their study, NGF- $\beta$  protein have not been produced in the form of mature, but produced as Plant pre-pro-NGF- $\beta$ , which eventually the pre-pro sequence was removed manually by addition of enzymes. This process is time consuming and economically expensive and the biological activity of the NGF- $\beta$  protein were not shown.

Recently, existence of the several serine proteases have been demonstrated in different parts of the plant cells like the seeds of barley (*Hordeum vulgare*); latex of dandelion (*Taraxacum officinale*); the flowers, leaves, stems and roots of *Arabidopsis thaliana*; and the leaves of tobacco (*Nicotiana tabacum*) [13]. Moreover, it has been shown that serine proteases as a member of their immune system, are induced following invasion of the pathogens in the apoplast [14] Grosse-Holz *et al.* have shown production of serine proteases from subtilinase family in the tobacco leaves following agroinfiltration [15]. Therefore, apoplast could be an ideal location for detection of a furin processed protein.

It has been shown that a strong promoter, proper regulatory elements and adapting the gene sequence according to the codon adaptation index (CAI) of the host system are the main factors affecting the expression level of a recombinant protein in prokaryotic and eukaryotic hosts [16]. Moreover, the regulatory elements around ATG codon adapted to Kozak sequence are considered as another strategies to improve protein expression [16].

In the present study, various changes were applied to the *HNGF- $\beta$*  gene sequence to increase its expression in the plant cells. Thereafter, the *Nicotiana benthamiana* leaves were transfected by the construct containing the synthesized *PNGF- $\beta$*  gene using agroinfiltration method and its expression was evaluated. More importantly, the processing of the plant pro-NGF- $\beta$  to mature form of NGF- $\beta$  (mNGF- $\beta$ ) in the apoplast of agroinfiltrated plant cells was investigated. Finally, mNGF- $\beta$  was purified from the apoplast using Reversed Phase-High Performance Liquid Chromatographic (RP-HPLC) and its biological activity on the differentiation of PC12 cells and expression of tetanus toxin receptors on their surfaces was assessed.

## Results

### Gene optimization

The sequence alignment of *HNGF- $\beta$*  and its plant optimized counterpart *PNGF- $\beta$*  genes are presented in Fig 1. Other elements such as Kozak sequence, the *HNGF- $\beta$*  sequence and His-tag were also shown.

## Construction of the vector containing *PNGF-β* gene and *Agrobacterium* transformation

To construct the PVX vector containing *PNGF-β* gene, the synthesized *PNGF-β* gene was inserted into the vector by *ClaI* and *SaI* restriction enzymes. The presence of *HmNGF-β* and *PmNGF-β* genes were verified by *ClaI* and *SaI* restriction enzymes digestion showing the bands in approximate sizes of 800 bp confirmed by the sequencing (Fig 2A and 2B). Thereafter, the constructs were transformed into *A. tumefaciens* and the bacteria containing the construct were selected on kanamycin LB agar plate and verified for the presence of the *NGF-β* gene using colony PCR representing itself as a 368 bp band (Fig 2C).

## Identification of *PNGF-β* protein in *N.bentamiana* Leaves

To assess production of the *PNGF-β* in *N.bentamiana* leaf, 25 µg of TSP extract from agroinfiltrated leaves were tested by dot blot assay.

As shown in Fig 3A, the agroinfiltrated leaves carrying *PNGF-β* and *p19* constructs produced more of the *PNGF-β* recombinant protein in dot blot assay A3 versus A4.

The result of western blot analysis on 125 µg of TSP showed a single band with approximate molecular weight of 27 kDa corresponding to pro-*NGF-β* protein in TSPs extracted from agroinfiltrated leaves transfected with the *PNGF-β* gene construct alone or with the additional p19 construct (Fig 3B). These results also confirm the higher amount of production of pro-*NGF-β* in latter. Moreover, Fig 3 B indicates that human signal peptide (pre sequence) could be processed in plant cell.

## Processing of plant pro-*NGF-β* to mature *NGF-β* in the plant cells

To investigate the processing of *PNGF-β*, the apoplast fluid was isolated from the plant leaves and evaluated by the western blot to identify plant produced *NGF-β* forms. The results showed that the applied anti-*NGF-β* monoclonal antibody bond to the protein in the apoplast with approximated size of 15 kDa corresponding to m*NGF-β* (Fig 4).

## Estimation of the production level of mature *NGF-β* protein and its purification from the apoplast by RP-HPLC

To estimate the level of mature *NGF-β* produced in the plant cells, the apoplastic fluid was analyzed by RP-HPLC. As shown in Fig 5, the apoplast had a peak at retention time of 22.2 min the same as the peak observed for the human *NGF-β* standard reconfirming the presence of the mature *NGF-β* protein in the apoplast. Considering the human *NGF-β* as the standard, the estimated production level of mature *NGF-β* in the plant leaves co-agroinfiltrated with *A. tumefaciens* carrying *PNGF-β* and *p19* genes was 39 µg per g of the fresh leaves. Regarding the concentration of extracted TSP (1033 µg per g of fresh leaves), % 0. 3 of the total soluble proteins produced in the transfected plant leaf cells, were recombinant proteins. The elutes of RP-HPLC containing mature *NGF-β* were collected and used for analysis of the biological activity in differentiated PC12 cells.

## Effect of the purified mature NGF- $\beta$ on the differentiation of PC12 cells

To evaluate biological activity of the purified mature NGF- $\beta$ , its effect on the differentiation of PC12 cells was examined and compared to that of the human NGF- $\beta$  standard. PC12 cells treated with 400 ng/ml of the purified mature NGF- $\beta$  or human NGF- $\beta$  standard started to the differentiation after 24 h of the mature NGF- $\beta$  exposure representing the cells with the extended neuritis while a gradual increase in their neurite length and their numbers was observed during 6-7 days of the NGF- $\beta$  treatment. The number of the cells differentiated with the purified mature NGF- $\beta$  and their neurites length was in a comparable level to the differentiated cells with the human NGF- $\beta$  standard (Fig 6A and 6B). PC12 cells cultured in the absence of the NGF- $\beta$  served as the negative control and demonstrated a rounded shape with the few short projections (Fig 6C). In addition, undifferentiated PC12 cells were smaller in their size than the differentiated cells (Fig 6C).

Since the NGF- $\beta$  differentiated PC12 cells start to increase the expression of tetanus toxin receptors on their surfaces, the effect of the purified mature NGF- $\beta$  on the expression of tetanus toxin receptors in the differentiated PC12 cells was evaluated and compared to that of the human NGF- $\beta$  standard. To this aim, a tetanus toxin binding assay was performed in which binding of the FITC-labeled tetanus toxin to its receptors expressed on the differentiated PC12 cells was evaluated. As shown in Fig 7 A, B, a strong green fluorescence on 15-20% of the PC12 cells differentiated with the purified mature NGF- $\beta$  for 6 days, was observed by Immunofluorescence microscopy. Similar intensity of the fluorescence was visualized on 20-25% of the PC12 cells differentiated with human NGF- $\beta$  standard (Fig 7 C, D). A few number of the undifferentiated PC12 cells which were used as the negative control, showed a green fluorescence under the microscope (Fig 7 E, F).

## Discussion

In this study, human pre-pro-NGF- $\beta$  expressed in *N. Benthamiana* plant cells, and its processing to NGF- $\beta$  was assessed. Several reports show in spite of the production of recombinant NGF- $\beta$  protein in various **eukaryotic systems** such as yeast, insect, mammalian and plant cells, its expression level is low and in some hosts such as yeast is partially or fully non- functional. [6, 17-20]. The advantage of plant as a cheap system with eukaryotic PTM [21], enhanced the research on determination of new molecular pathways for production of active recombinant pharmaceutical proteins. The plant system has already been used to produce NGF- $\beta$ . The plant produced pre\_pro\_NGF  $\beta$  were purified and further chemically processed (Galba *et al* 2011) or the NGF- $\beta$  protein was co-expressed with enzymes such as Furin to process pro-NGF- $\beta$  in the plant cell (Mamedov *et al.*, 2019). Therefore, to find a system with less manipulation process is valuable.

In present study, various strategies used to increase the expression level of the human NGF- $\beta$  protein in *N. benthamiana*, including optimizations of gene and co-agroinfiltration with *A. tumefaciens* carrying *p19* gene silencing suppressor. The results of dot and western blotting analysis of expressed NGF is in

accordance with other studies demonstrating that applying P19 expression cassette improves the expression of recombinant protein from five to 50-folds [22].

The production of NGF- $\beta$  in plant is demonstrated by Galba *et al.* They measured the amount of the recombinant pre-pro-NGF protein as 3-5  $\mu\text{g}$  per kilogram of the fresh *N. bentamiana* leaves using transient expression system. [6]. However, in present study, according to the RP-HPLC results, the amount of the mature NGF- $\beta$  produced in the apoplast of plant leaves was about 39  $\mu\text{g}/\text{g}$  of the fresh leaves.

To identify and pursue processing of the pre\_pro-NGF- $\beta$  in the plant cells, western blot analysis was performed on both the TSPs and the apoplast extracts of *N. bentamiana* leaves. The results showed that the TSPs and the concentrated apoplast extract contained pro-NGF- $\beta$  and mature NGF- $\beta$  proteins, respectively. The two proteins lack human NGF- $\beta$  signal peptides which shows plant could process the pre-domain. Moreover, the two bands with different molecular sizes of 27 kD (corresponding to pro-NGF- $\beta$  in total soluble leaf protein) and 15 kD bands (corresponding to mature NGF- $\beta$  extracted from apoplast) appeared following application of anti-NGF- $\beta$  monoclonal antibody recognizing aa 200-300 of C-terminal of NGF. Interestingly, there were no detectable pro-NGF in concentrated apoplast extract and no mature NGF- $\beta$  in TSP.

The apoplast extract was subjected to RP-HPLC and purified compared to standard mature NGF- $\beta$ . The result also confirmed that the protein obtained from apoplast is a processed NGF- $\beta$  that lacks human signal and pro-domain peptides and functionally is similar to the processed mammalian mature NGF. This suggests an underlined processing mechanism while transferring pro-NGF- $\beta$  into apoplast

Identification of the mature recombinant NGF- $\beta$  in the plant apoplast was unexpected due to previous study by Galba *et al*/2011. They used chemical processing following purification of plant produced pre-pro-NGF to obtain mature and active NGF.

To evaluate the functionality of the HPLC purified mature form of NGF- $\beta$  obtained from apoplast, its effect on the differentiation of PC12 cells was examined. PC12 cell originating from the pheochromocytoma of rat adrenal is a cell line with the ability of the differentiation to the neuron-like cells upon its exposure to hNGF- $\beta$ . So, they have traditionally been used as an *in vitro* model for evaluation of the biological activity of hNGF- $\beta$ . According to results, a gradual increase in the neurites length and number of the differentiated cells was observed by Day 6-7 in the cells exposed to the purified PmNGF- $\beta$  (400 ng/ml). A similar pattern observed using the same concentration of the standard human NGF- $\beta$ . These results suggest that the purified plant processed mature NGF- $\beta$  is a biologically active compound demonstrating its proper post translational modifications in the plant apoplast.

To confirm the above results, the differentiation of PC12 cells by increasing the expression of tetanus toxin binding sites on their surfaces were tested [23-25]. Immunofluorescence experiments showed that the FITC-labeled tetanus toxin strongly bound to PC12 cells treated with mature NGF- $\beta$ , in a comparable level to that of the cells exposed to the standard human NGF- $\beta$ .

The plant cell apoplast is a compartment where plant and pathogens interact and it is concerned as the first barrier to block pathogens from entering the cytoplasm. Therefore, plants have developed an immune system in apoplast that recognizes the invading microbes and initiates a set of the responses including secretion of the various proteases [26]. Hou *et al* showed that apoplast space of tobacco plant contains phytaspase, a subtilisin like serin protease (S08.150), which acts as a member of plant immunity system [27].

In addition, it has been shown that filtration of agrobacteria also triggers the plant immune system response resulting in the induction of 6 serine proteases expression (clan SB, family S08). Two of these enzymes are annotated as PF00082 and one is from subtilin family and all are located in the plant apoplast [15]. Moreover, Kinal *et al*. have demonstrated that the subtilisin protease activity also is intrinsic to apoplast as the processing of Kp6 pre-pro toxin in the apoplast of transgenic *N. tabacum* occurs. [28].

Taken together, the present study proposes that the processing of the pro-NGF- $\beta$  to mature NGF- $\beta$  mediated by the serine proteases in the apoplast of the *N. bentamiana* plant cells could be result of both intrinsic characteristics and induced immune response to the agroinfiltration.

Concerning the processing of NGF  $\beta$ , Fig 8 is a comparison between pathways of pre-pro-NGF- $\beta$  protein in mammalian and plant cell following its expression.

In mammalian cells, Fig 8 A, two intra or extra cellular pathways are involved in the Pro-NGF- $\beta$  processing. The intracellular pathway utilizes Furin enzymes such as subtilisin-/kex2p-like endoprotease to cleave pro-NGF- $\beta$  into the mature NGF- $\beta$  protein in the cytoplasm. The extracellular pathway applies Plasmin enzyme which is the active form of Plasminogen obtaining from the action of Tissue plasminogen activator modification of plasminogen in the extracellular space [29]. In the plant cells, the both intracellular and extracellular pathways of pro-NGF- $\beta$  processing have not been recognized previously; however, our results provide evidence on the extracellular-like pathway of Pro-NGF- $\beta$  processing in the apoplast of plant cells. As mentioned before, Grosse-Holtz *et al* confirmed that some enzymes with Furin or Plasmin like activities are induced in the apoplast as a result of the plant immune responses to the biotic stresses such as agroinfiltration procedure [15].

## Conclusion

In conclusion, our results suggest the feasibility of processing of the recombinant pre\_pro-NGF- $\beta$  into the mature biologically active form of NGF- $\beta$  in the apoplast of the *N. bentamiana* plant cells using transient expression. The processing of the pro-NGF- $\beta$  into the mature form is probably mediated by the serine proteases induced in the apoplast as an intrinsic trait and following activation of the plant immune response due to the agroinfiltration. However, further studies are necessary to address this issue.

To our knowledge, this is first report on recruitment of apoplast furin-like intrinsic enzymes for production of mature NGF- $\beta$  with high yield in plant as a pharmaceutical protein with eukaryotic post translational modification.

# Methods

## Plant material, bacterial strains and plasmids

Seeds of *N. benthamiana* were grown in the growth chamber at 25°C with 16 h light (3000 - 4000 Lux intensity) and 8 h dark period. Intact young leaves were used for the agroinfiltration. *Escherichia coli* DH5α was used as a host for maintaining the genes constructs PNGF-β and p19 constructs. *A. tumefaciens* strain GV3101 (pMP90RK) was purchased from DSMZ (Germany, Leibniz Institute).

PVX vector was a donation from Cristiano Lacorteh, Wageningen University [30]. The p19 gene in pCAMBIA 1304 [31] in *agrobacterium tumefaciens* were used for Co-agroinfiltration.

## Gene Optimization, construction of the vectors and *A. tumefaciens* transformation

*HNGF-β* gene (GenBank Accession number: P01138) was optimized according to *N. benthamiana* plant CAI using the online tool [kazusa.or.jp](http://kazusa.or.jp/) ( and Leto software ver. 1.0.11 (Entelechon, Germany). Moreover, ATG codon region was optimized by introducing Kozak sequence (AACA) [32] and inserting purines at the +3 of ATG codon and a GC dinucleotide at the +4 and +5 position [33]. Moreover, following ATG codon at the translation start site, the codons for alanine and serine residues were added. The rare codons in the gene sequence including XCG and XTA were replaced with the abundant codons [1]. His tag sequence for the future possible detection and purification of the recombinant protein as well as the TEV recognition sequence for deletion of His Tag was designed at C-terminal. The *PNGF-β* genes sequences were synthesized by GenScript (Piscataway, USA).

The *PNGF-β* genes were inserted downstream of SGP3 promoter and upstream of a 35S terminator in the PVX binary vector by digesting both the interested genes and vector with the *ClaI* and *SaI* restriction enzymes (Fig 9).

The vector containing *PNGF-β* gene was introduced into the competent *A. tumefaciens* strain GV3101 by electroporation (Gene PulserXcell™ Electroporation System, Bio-Rad, USA). The electroporation conditions were: Capacitance= 25 μF; Resistance= 200 ohm; Voltage= 2.4 kV; Time= 47 ms [34]. The transformed bacteria received the genes constructs were selected on LB medium containing 50 μg/ml kanamycin (Duchefa Biochemie, Haarlem, The Netherlands) and verified for the presence of the *PNGF-β* gene using colony PCR.

The PCR reaction was performed in a mixture with the final volume of 20 μl containing 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.5 μM of each primer and 0.5 U of DNA polymerase (Thermo Fisher Scientific, UK). The sequence of the primers were PNGF-F (5' ACACATATGTCATCATCCCATCCCATCTTC 3') and PNGF-R (5' AAGGATCCCTAGGCTCTTCTCACAGC 3') amplifying the amplicon with 368 bp size. PCR program was as follows: 95°C for 5 minutes, then 30 cycles consists of a 95°C denaturation step for 30 seconds, a 60°C annealing step for 30 seconds, a 72°C extension step for 60 seconds and a final step at 72°C for 10 minutes to complete DNA expansion. PCR products were visualized on a 1% agarose gel [35] and the positive clones were utilized for the plant agroinfiltration.

## **Plant agroinfiltration and protein extraction from the plant leaves**

Each of the *agrobacteria* cultures containing p19 or PNGF- $\beta$  constructs were grown in **Tryptic Soy Broth (TSB)** medium with the appropriate antibiotics to reach the optical density of 2 at 600 nm. The pellet of the bacteria carrying PNGF- $\beta$  and p19 construct were resuspended in induction medium (pH of 5.5), as previously described [35]. Then, the whole plant of *N. benthamiana* was placed upside down in the bacterial suspension under a vacuum pressure at 25 mmHg for 30-60 s. Thereafter, the transformed plant was placed at the plant germinator and maintained at 22°C with a 16 h photoperiod for 4 days [36].

The leaves (100 mg) were ground in the liquid nitrogen following addition of 200  $\mu$ l of the freshly prepared protein extraction buffer (100 mM NaCl, 10 mM EDTA (pH= 8), 200 mM Tris-HCl (pH=8), 0.05% Tween-20, 0.1% sodium dodecyl sulfate, 14 mM  $\beta$ -mercaptoethanol, 200 mM sucrose, and 2 mM phenyl methyl sulfonyl fluoride) [37]. Total soluble proteins (TSPs) were quantified using a Bradford-based protein assay (BioRad, Germany) according to the manufacturer's instructions.

## **Isolation of the apoplastic fluid and purification of the mNGF- $\beta$ protein from the apoplast by RP-HPLC**

The leaves of the infiltrated plants were vacuum-infiltrated with the apoplastic extraction buffer (50 mM NaAc, 150 mM NaCl )pH=5.5), %5 mannitol, %5 trehalose, % 0.02 Tween-80) according to the Thermo Fisher Scientific manufacturer's protocol. The vacuum-infiltrated leaves were centrifuged at 3000 g )4 °C( for 10 min and the supernatant was collected as the apoplastic fluid and re-centrifuged at 12000 g (4 °C) for 10 min to be clarified [38]. The fluid was lyophilized until the analytical experiments were performed.

Recombinant NGF- $\beta$  was quantified and purified from the water reconstituted lyophilized apoplastic fluid by RP-HPLC method. 20  $\mu$ l of the human NGF- $\beta$  standard (Sigma, St. Louis, MO, USA) (20 ng/ml) (Sigma-Aldrich) and the plant apoplastic fluid were fractionated on an analytical Eurosil bioselect column (250  $\times$  4.6 mm, 5  $\mu$ m, 100 Å), using a linear gradient from solution A (water with 0.1% trifluoroacetic acid (TFA)) to solution B (acetonitrile with 0.1% TFA) at a flow-rate of 0.8 ml/min at 25°C. Absorbed recombinant protein was eluted by the following procedure: 0–15 min, 90% eluent A; 15–40 min, 90-10% eluent A; 40–46 min, 10% eluent A; 46–48 min, 10-90% eluent A. The absorbance of the elution's peaks was measured at 215 nm using an ultra violet detector.

## **Identification of the recombinant NGF- $\beta$ proteins by dot blot analysis and western blotting**

Dot blot analysis was performed on the recombinant NGF- $\beta$  proteins produced in the plant cells and the standard NGF- $\beta$  as the positive control. 25  $\mu$ g of soluble protein of leave extract was dotted on the nitrocellulose membrane (Sigma, USA). Thereafter the blocking solution, (5% solution of skim milk powder in TBST (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, 0.4% Triton x100, pH=7.5)) was added to the membrane and incubated at room temperature for 1.5 h with shaking. Afterwards, the blocking solution was washed out and horse radish peroxidase (HRP)-conjugated rabbit anti-NGF- $\beta$  monoclonal antibody (EP1320Y, Abcam, USA) (1:2000 dilution) was added to the membrane and incubated at room temperature for 2 h with shaking. After incubation, the membrane was washed with TBST solution for 10

min (3 times. Finally, a solution containing 0.05% 3, 3'-Diaminobenzidine (Sigma, USA), 0.015% H<sub>2</sub>O<sub>2</sub> and 0.01M PBS was added to the membrane to visualize the protein bands.

150 µg of each total soluble protein was used for the sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) on 15% polyacrylamide. For western blot analysis, the proteins were transferred from the gel to the nitrocellulose membrane with the blotting buffer (25 mM Tris, 192 mM glycine, 15% methanol, (pH=8.3). The other steps including blocking, addition of the HRP-conjugated anti-NGF-β antibody and detection were carried out by the protocols and solutions used in dot blot analysis.

### **Analysis of the biological activity of mNGF-β**

The biological activity of the mNGF-β was evaluated using a PC12 cell differentiation assay. Undifferentiated PC12 cells were purchased from Pasture Institute (Tehran, Iran) and maintained in RPMI 1640 (Gibco, California, USA) supplemented with 10% heat inactivated horse serum (Razi Vaccine and Serum Research Institute, Tehran, Iran), 5% heat inactivated fetal bovine serum (Gibco, California, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were incubated in a 95% humidified incubator with 5% CO<sub>2</sub> at 37°C. Thereafter, the cells were seeded at a density of 10000 cells/well on 48-well plates coated by Polyethyleneimine (25µg/ml) and exposed to the mNGF-β at a final concentration of 400 ng/ml of the supplemented medium. The cells treated with the same concentration of the human NGF-β standard were considered as the positive control. The cells fed with the media without any NGF-β, were used as the negative control. Exchange of the medium containing mNGF-β or human NGF-β standard was carried out every 2 days. Morphology of the cells was monitored under an inverted microscope (Olympus, Tokyo, Japan), every day. Cells present at least two neuritis with a length equal to the cell body diameter or more were considered as the differentiated cells. The percent of the differentiated cells was determined from an average of 10 fields per well on day 6.

The biological activity of the mNGF-β was also evaluated by detection of Tetanus toxin receptors expressed on PC12 differentiated cells in an assay in which binding of fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO, USA)-labeled tetanus toxin (Razi Vaccine and Serum Research Institute, Tehran, Iran) was assessed.

FITC labeling of tetanus toxin was performed according to the manufacture's protocol. Briefly, tetanus toxin at a concentration of 1 mg/ml was dialyzed against 0.1 M carbonate-bicarbonate buffer (pH= 9.0) overnight at 4°C. Thereafter, a freshly prepared solution of FITC (1 mg/ml) in 0.1 M carbonate-bicarbonate buffer (pH= 9.0) was added to the dialyzed toxin at a weight ratio of 1:15 and incubated with the toxin for 2 hours at room temperature on the stirrer, protected from the light. The unbound FITC molecules were removed from the solution by an overnight dialysis against PBS at 4 °C.

The FITC toxin (15 µg/ml) was added to the 6-days mNGF-β -treated cells at a final volume of 200 µl of RPMI 1640 medium and incubated at 37 °C for 1 h. Thereafter, the unbound toxins were washed off using sterile PBS. The bound toxins to the cells were visualized under an inverted fluorescent microscope.

Binding of FITC-labeled tetanus toxin to the PC12 cells differentiated with the human NGF- $\beta$  standard (positive control) or those without any NGF- $\beta$  treatment (negative control), was also evaluated.

## Declarations

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### Author Contributions

M.Z. performed the research and prepared the manuscript draft. F.Sh. designed the bioactivity experiments and read the manuscript. S.Gh. performed tissue culture and bioactivity experiments. F.F. drew the figure. P.E. helped in the project process and edited the manuscript. H.O. designed the project, mentoring experiments, analyzed the data, and revised the manuscript.

**All authors read and approved the final manuscript, and have no conflict of interests.**

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## Figures

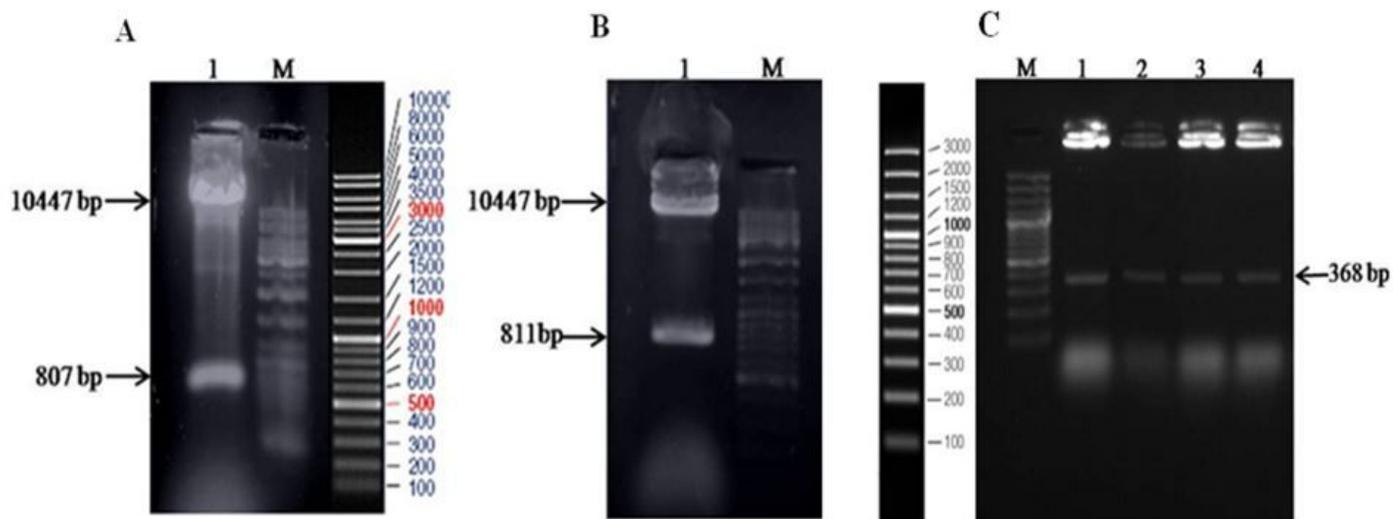
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PNGF-β      ATCGATAAACAATGGCCTCTTCCATGTTGTTCTATACTACTCATTACAGCAITTTCTCATGG
*****  **      *      *****  *      *      *      *      *      *      *      *
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PNGF-β      AATCCAGGCTGAACCACACTCGGAATCTAATGTCCCTGCAGGGCACACAATTCCACAAGC
** ***** ***** **      *****  *      *      *      *      *      *
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PNGF-β      TCACTGGACA AAAACTTCAACATTGCTTGACACTGCACTTCGAAGAGCCCGAAGCGCTCC
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PNGF-β      AGAAGCTGCAGACACGCAGGATCTGGACTTTGAAGTGGTGGAGCTGCACCTTTCACCCG
*****  ***** ***** ***** ***** ***** ***** *****
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PNGF-β      CACTCACAGGAGCAAGCGCTCATCTTCGCATCCGATTTTCCATAGGGGCGAATTCCTCGT
*****  ***** ***** ** ***** ** ***** ***** ***** **
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HNGF-β      GTGTGACAGTGTGAGCGTGTGGGTGGGGATAAGACCACCGCCACAGACATCAAGGGCAA 480
PNGF-β      ATGTGATAGTGTAGCGTGTGGGTGGGAGATAAGACTACCGCTACTGATATCAAGGGGAA
*****  ***** ***** ***** ***** ***** ***** ***** **
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PNGF-β      TGAGACGAAGTGCCGAGACCCAAATCCCGTTGACAGCGGATGCCGGGGCATTGACTCAA
*****  ***** ***** ***** ***** ***** ***** *****
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PNGF-β      ACAITGGAAITCTTATTGTACCACGACTCATACCTTTGTTAAGGCGCTAACAAATGGACGG
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** * ***** ***** ***** ***** ***** ***** ***** **
aa          K Q A W R F I R I D T A C V C V L S R
HNGF-β      GAAGGCTGTGAGAAGAGCCGAGAACCTTTACTTTCAATCCCATCATCACCATCACCATTG 780
PNGF-β      GAAAGCCGCTGAGAAGAGCGGAGAACCTTTACTTTCAATCCCATCATCACCATCACCATTG
*** ** ***** ***** ***** ***** ***** ***** *****
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PNGF-β      AGTCGAC
*****

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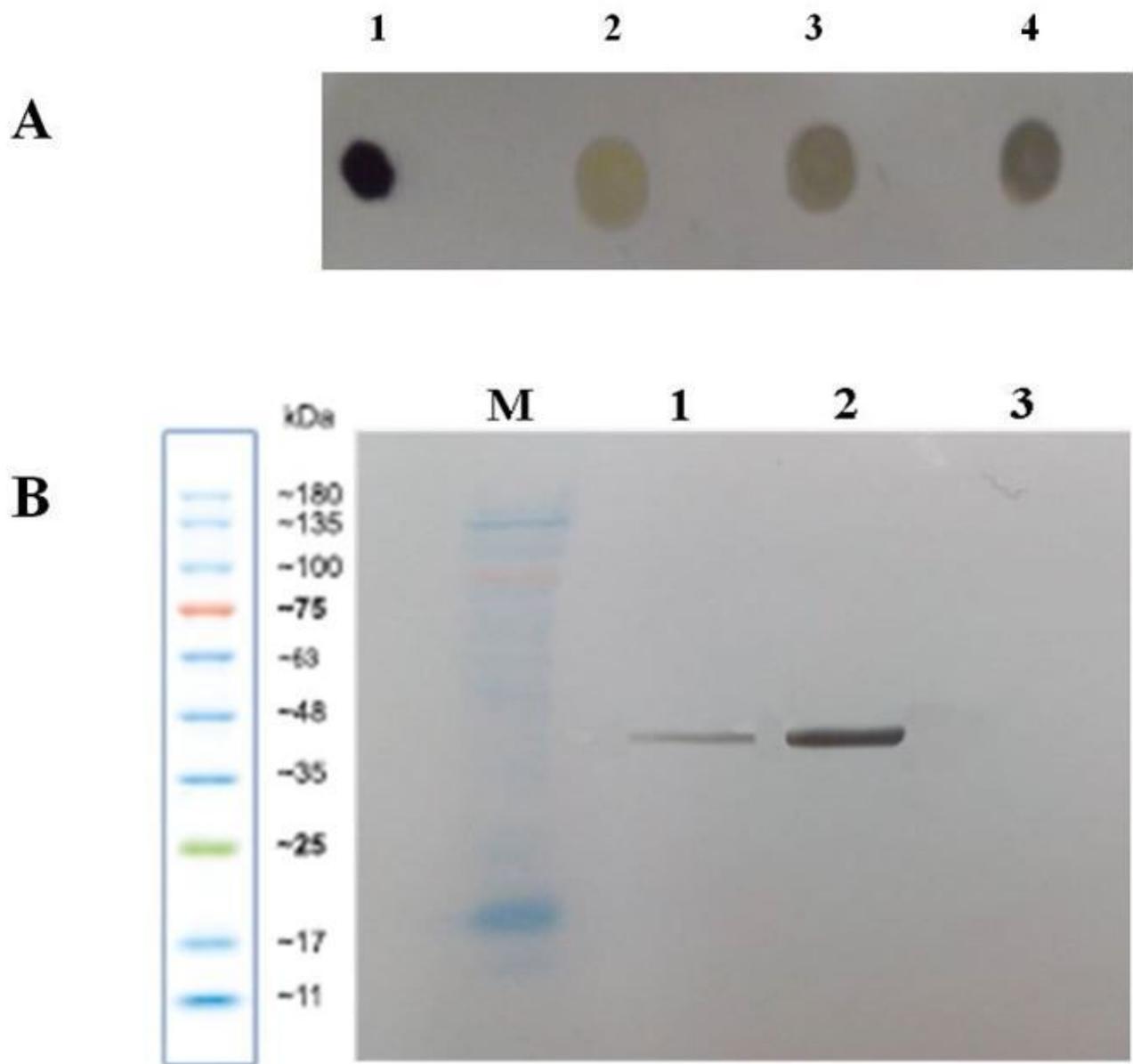
Figure 1

Comparison of native human nerve growth factor (HNGF-β) gene and N. benthamiana codon optimized human nerve growth factor (PNGF-β) containing pre-pro-sequences. The red aa sequence is "pre" domain, the green is "pro" domain, the black is the mature "chain" and the brown is TEV recognition site sequences.



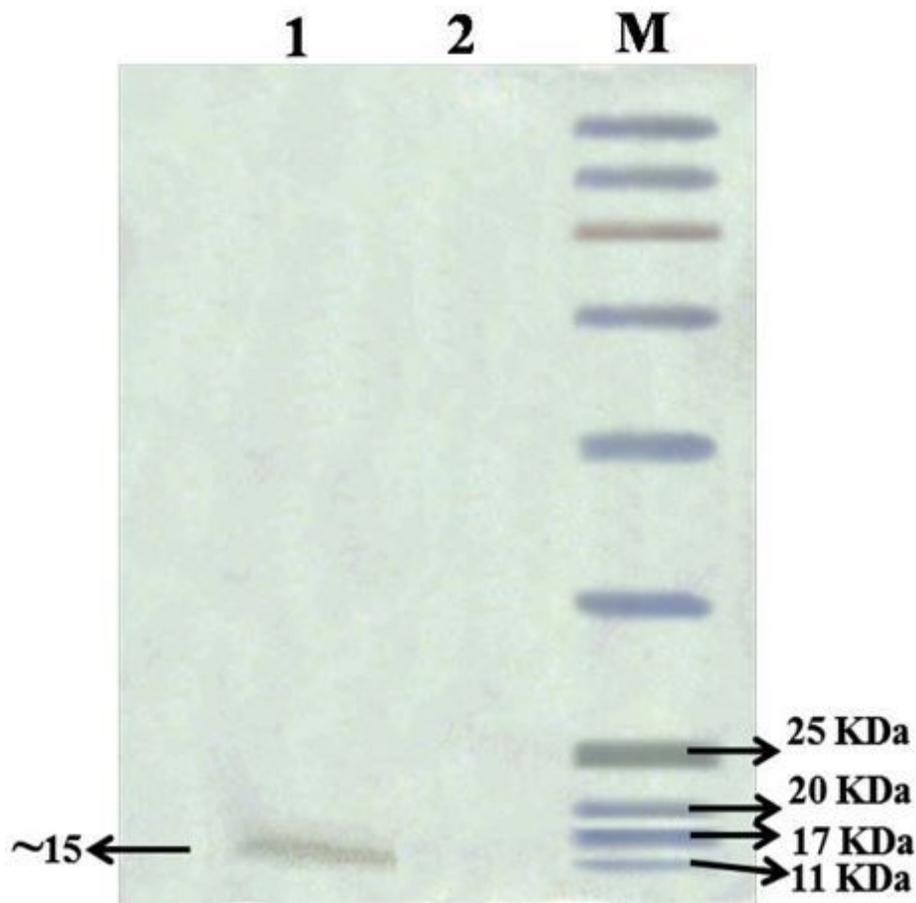
**Figure 2**

Molecular analysis of NGF- $\beta$  expression vectors. A) Lane 1; PVX-HmNGF- $\beta$  following Clal and Sall digestion, Lane M, MW marker. B) Lane 1; PVX-PNGF- $\beta$  after Clal and Sall, digestion, Lane M; MW marker. C) Colony PCR for confirmation of agrobacteria grown on selective media. Lanes 1 and 2; positive PNGF- $\beta$  clone, Lanes 3 and 4; positive HNGF- $\beta$ , Lane M; MW marker.



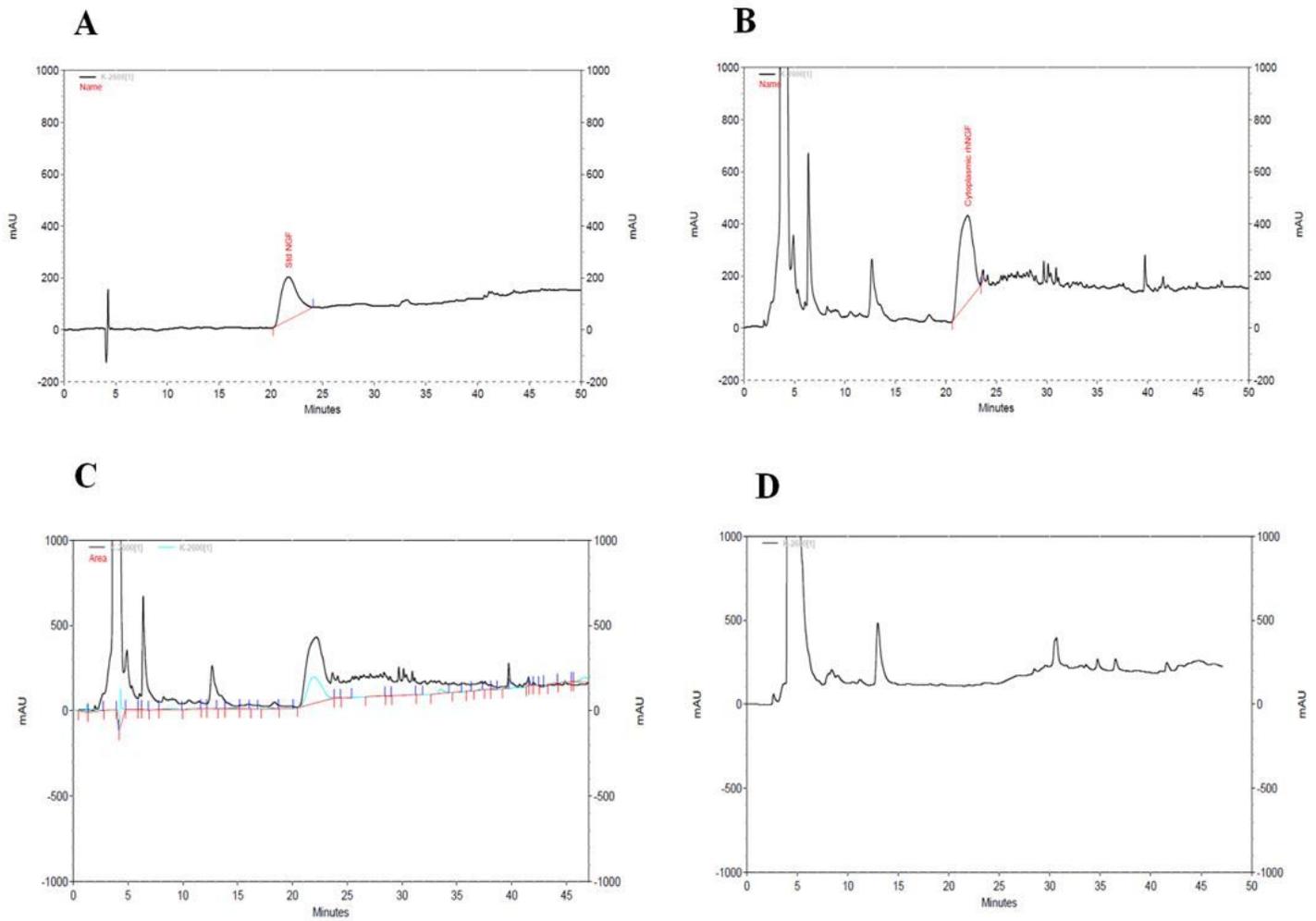
**Figure 3**

Analysis of production of PNGF- $\beta$  in agroinfiltrated plant leaves. A) Dot Blot analysis of plant TSPs extracts. Dot 1: Standard NGF- $\beta$  (positive control); Dot 2: TSP of the plants infiltrated by *A. tumefaciens* containing PVX vector without the inserted PmNGF- $\beta$  gene (negative control); Dot 3: TSPs of the plants infiltrated by *A. tumefaciens* containing PVX vector with the inserted PNGF- $\beta$  gene; Dot 4: TSPs of the plants co-agroinfiltrated with *A. tumefaciens* carrying PNGF- $\beta$  and p19 genes. B) Western blot analysis of plant TSPs extract. 27 KDa band related to pro-NGF- $\beta$  is indicated. Lane M: Protein molecular weight marker; Lane 1: TSPs of the plants by *A. tumefaciens* containing PVX vector with the inserted PNGF- $\beta$  gene; Lanes 2: TSPs of the plants co-agroinfiltrated with *A. tumefaciens* carrying PNGF- $\beta$  and p19 genes Lanes 3: TSP of the plants infiltrated by *A. tumefaciens* containing PVX vector without the inserted PNGF- $\beta$  gene (negative control).



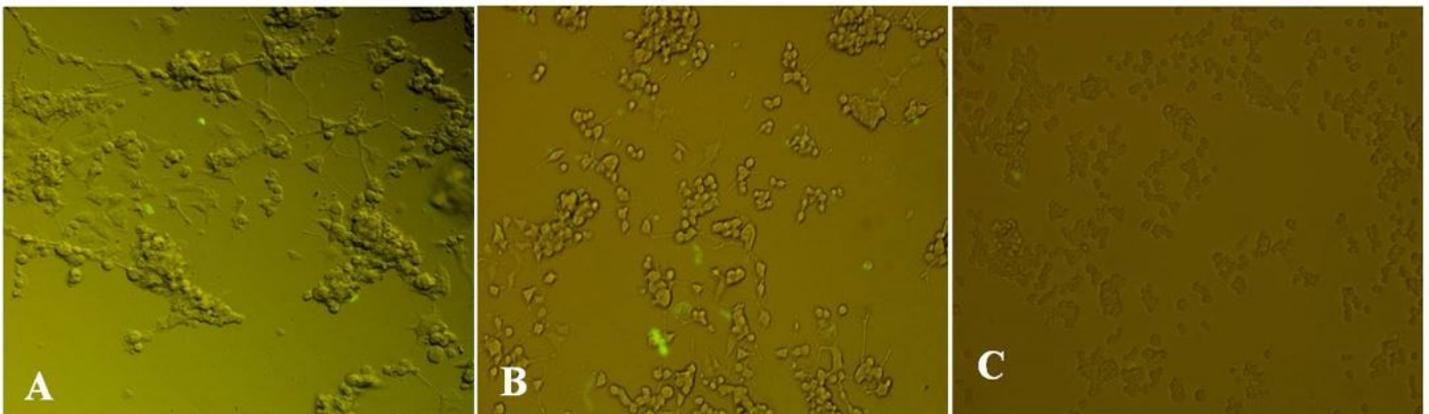
**Figure 4**

Western blot analysis of apoplast extract of the plant leaves co-agroinfiltrated with *A. tumefaciens* carrying PNGF- $\beta$  and p19 genes. Lane 1: Apoplastic fluid of plant leaves co-agroinfiltrated with *A. tumefaciens* carrying PNGF- $\beta$  and p19 expressing vectors, Lane 2: Apoplastic fluid of plant leaves infiltrated by *A. tumefaciens* containing p19 expressing vectors, Lane M: Protein molecular weight marker. The mature PNGF- $\beta$  protein (a 15 kDa band) is shown by arrow.



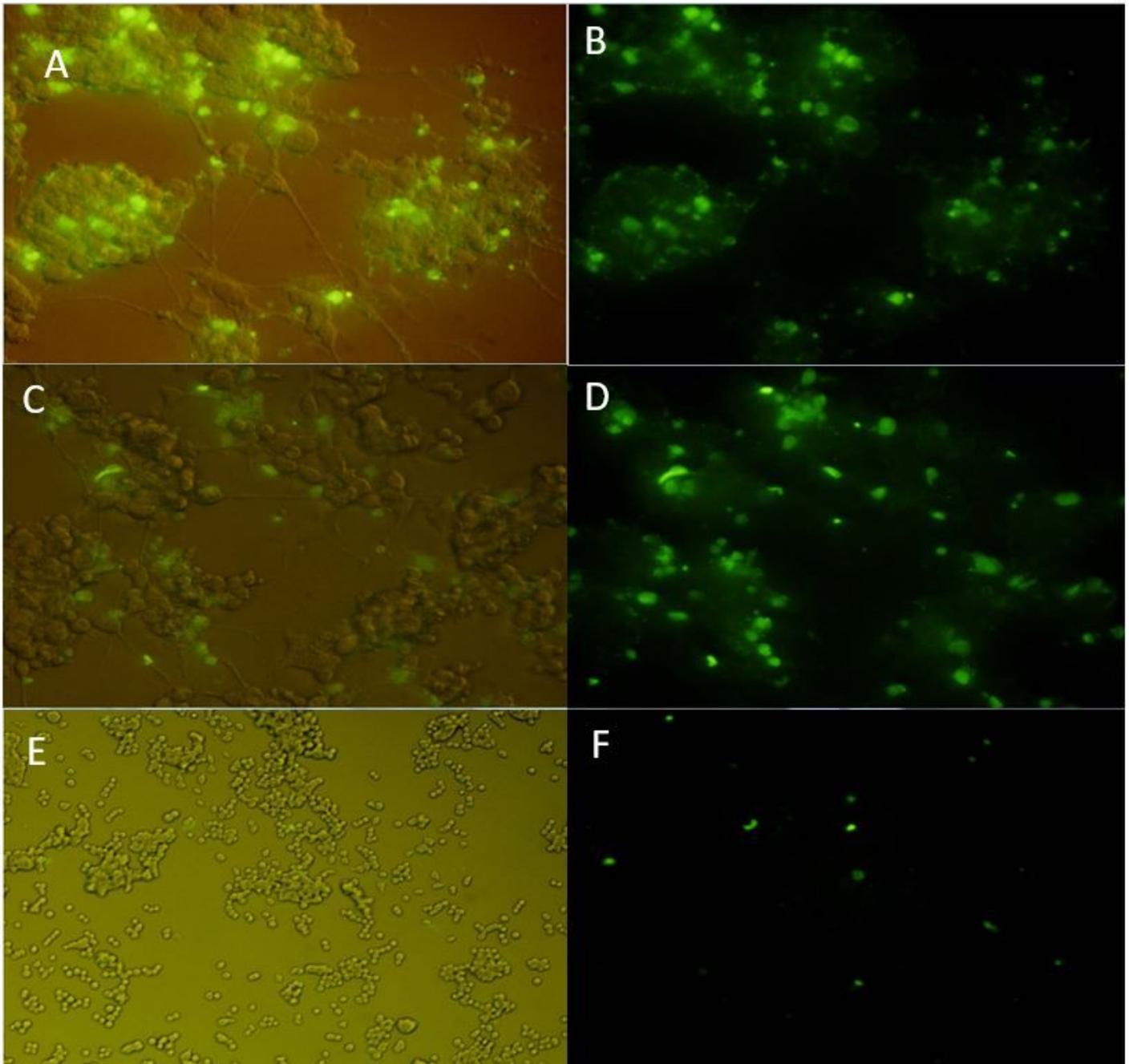
**Figure 5**

RP-HPLC chromatogram of apoplast extract of the plant leaves co-agroinfiltrated with *A. tumefaciens* carrying PNGF-β and p19 genes against human NGF-β standard A) human NGF-β standard B) the apoplast extract of the plant leaves co-agroinfiltrated with *A. tumefaciens* carrying PNGF-β and p19 genes C) The chromatograms of A and B were overlaid D) Negative control (*A. tumefaciens* containing PVX vector without the inserted PmNGF-β gene).



**Figure 6**

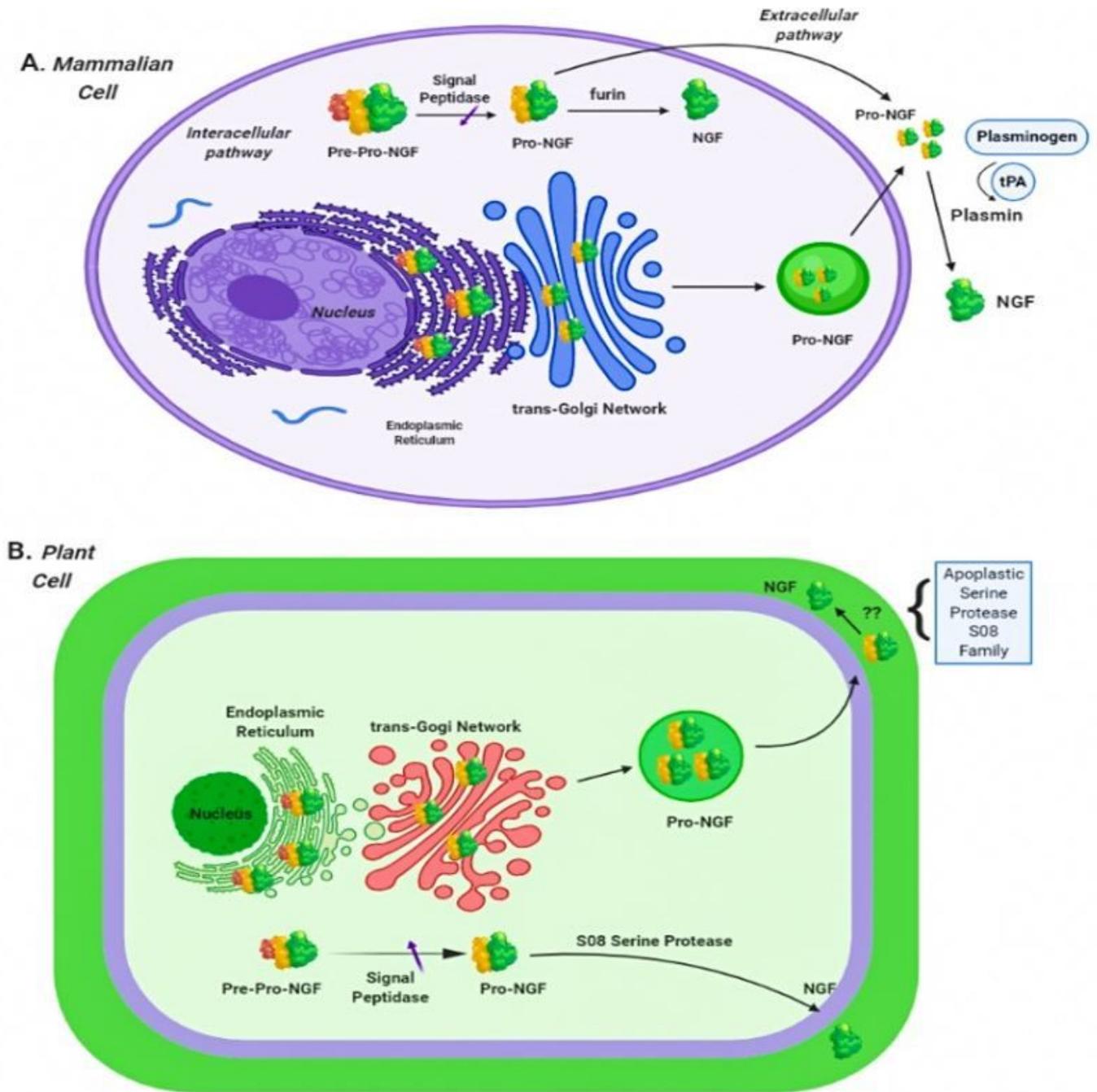
NGF- $\beta$  differentiated and undifferentiated PC12 cells under an inverted microscope; A) Differentiated PC12 cells under the treatment of the purified mature NGF- $\beta$  (400 ng/ml) on day 6, B) Differentiated PC12 cells under the treatment of the human NGF- $\beta$  standard (400 ng/ml) on day 6, Undifferentiated PC12 cells cultured in the absence of NGF- $\beta$  (C).



**Figure 7**

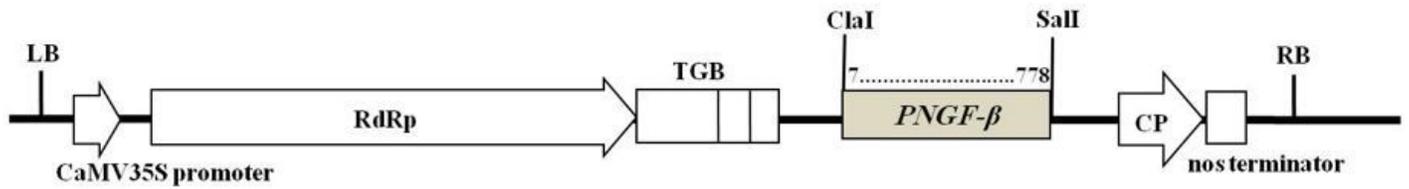
Immunofluorescence microscopy of the NGF- $\beta$  differentiated and undifferentiated PC12 cells after the binding of FITC-labeled tetanus toxin. A) Representative overlay of the fluorescent signal on the light microscopy image of the PC12 cells treated with the purified mature NGF- $\beta$  (400 ng/ml) on day 6; B)

Fluorescence image of the cells in A; C) Representative overlay of the fluorescent signal on the light microscopy image of the PC12 cells treated with the human NGF- $\beta$  standard (400 ng/ml) on day 6; D) Fluorescence image of the cells in C; E) Representative overlay of the fluorescent signal on the light microscopy image of the undifferentiated PC12 cells in the absence of NGF- $\beta$ ; F) Fluorescence image of the cells in E.



**Figure 8**

Processing pathways of pre-pro-NGF- $\beta$  protein into the mature NGF- $\beta$  in (A) Mammalian cells and (B) in the plant cells.



**Figure 9**

Schematic design of location of the PNGF- $\beta$  gene in PVX vector. RdRp, RNA dependent RNA polymerase; TGB, Triple gene box; CP, Coat protein, LB and RB, left and right borders of Transfer DNA; PNGF- $\beta$ , Plant optimized NGF- $\beta$  gene.