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Utility of P19 Gene-Silencing Suppressor for High Level Expression of Recombinant Human Therapeutic Proteins in Plant Cells

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Abstract

Background: The potential of plants, as a safe and eukaryotic system, is considered in the production of recombinant therapeutic human protein today; but the expression level of heterologous proteins is limited by the post-transcriptional gene silencing (PTGS) response in this new technology. The use of viral suppressors of gene silencing can prevent PTGS and improve transient expression level of foreign proteins. In this study, we investigated the effect of p19 silencing suppressor on recombinant human nerve growth factor expression in *Nicotiana benthamiana*.

Materials and Methods: The p19 coding region was inserted in the pCAMBIA using NcoI and BstEII recognition sites. Also, the cloned synthesized recombinant human *NGF (rhNGF)* fragment was cloned directly into PVX vector by ClaI and SalI restriction enzymes. The co-agroinfiltration of rhNGF with p19 viral suppressor of gene silencing was evaluated by dot-blot and SDS-PAGE. The amount of expressed rhNGF protein was calculated by AlphaEaseFC software.

Results: Co-agroinfiltration of *hNGF* with *P19* suppressor showed about forty-fold increase (8% total soluble protein (TSP)) when compared to the absence of P19 suppressor (0.2%TSP).

Conclusion: The results presented here confirmed that the use of P19 gene silencing suppressor derived from tomato bushy stunt virus (TBSV) could efficiently increase the transient expression of recombinant proteins in *Nicotiana benthamiana* manifold.

Keywords: Nerve growth factor; *Nicotiana benthamiana*; P19 suppressor of gene-silencing; Recombinant therapeutic human protein; Tomato bushy stunt virus

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Introduction

Plants are advantageous systems for the expression of valuable therapeutic recombinant proteins (1). In particular, it has been extensively confirmed that plants are ideal bioreactors compared to classical exogenous protein expression systems for the production of functional monoclonal antibodies, vaccine components and enzymes (2). The major advantages of plants over traditional bacterial and mammalian cell expression systems are minimizing the risk of contamination by human pathogens and reducing production costs, especially for large-scale production (3). The main drawback of this novel technology is that the expression level of heterologous proteins is limited by the PTGS response that may occur in the infiltrated plant tissue (4). To overcome this limitation, the use of viral suppressors of gene silencing can prevent PTGS and improve transient expression level of heterologous proteins (5, 6). This was verified in *N. benthamiana* using the TBSV P19 suppressor of gene silencing, which was able to increase expression yields of a variety of proteins up to 50 folds (3). RNA silencing is an ancient eukaryotic surveillance system that occurs in a variety of eukaryotic organisms including fungi, animals and plants. RNA silencing

acts as an antiviral defense in plants; thus, successful virus infection requires evasion or suppression of gene silencing (7).

The process of RNA silencing response is initiated through the production of the viral double-stranded (ds) RNA (8). Accumulation of double-stranded RNA in eukaryotic cells could act as a trigger for RNA silencing, so the dsRNAs are converted to small interfering (siRNAs) which direct the degradation of homologous single-stranded (ss) mRNAs (9). As a whole, RNA silencing is a self-defense mechanism acting against transposons and viruses (10).

A number of plant viral suppressors of silencing have been identified so far such as P21, P22, P14, P15, P25, HC-Pro, P1, and P19 (11).

Recent findings show that in particular P19 protein, as a potent suppressor, binds to short-interfering RNA (siRNA), suppresses RNA silencing with high selectivity and has a widespread use in various plant and animal models (12, 13).

The P19 protein blocks the spread of RNA silencing defenses beyond the site of local infection which enables the virus to infect the entire plant (14). This protein is originated from TBSV, a member of the Tombusvirus genus in the *Tombusviridae* (15). The genome of Tombusviruses is a linear, single stranded, positive-sense RNA with ~4.8 kb nucleotides long and contains five open reading frames coding for proteins with molecular masses of about 33, 92, 22, 19 and 41 kDa. Both P33 and P92 are required for viral replication, while P22 is required for cell-to-cell movement and P19 participates in virus spread in a host-specific manner (16).

The prime aim of this study was to improve the expression level of foreign gene in plant cell as an immune and safe eukaryotic host. In this content, the co- expression of human nerve growth factor with P19 silencing suppressor was reported for the first time.

In order to investigate the effect of a silencing suppressor on foreign protein yield, the transient expression of recombinant human nerve growth factor in *N. benthamiana* was described using a vacuum-agroinfiltration system. To enhance expression level, the co-agroinfiltration of *rhNGF* in combination with P19 viral suppressor of gene silencing was evaluated. Results demonstrated that co-agroinfiltration of *rhNGF* with *P19* suppressor gave higher rhNGF yields with an almost forty-fold increase compared to rhNGF transient expression alone.

Materials and Methods

Synthesis of P19 Gene

The P19 silencing-suppressor gene from TBSV (Gen Bank accession number: M21958) sequence was extracted from NCBI (http://www.ncbi.nlm.nih.gov) and synthesized by Biomatik (USA).

Construction of the P19- pCAMBIA 1304 Vector and Agrobacterium Transformation

The synthesized fragment which was delivered in pBMH, was digested with NcoI and BstEII restriction enzymes (Fermentase) to release the insert, then *P19* gene was gel-purified, and cloned directly into pCAMBIA (CAMBIA Co., Canberra, Australia).

The *P19* coding region was inserted in the downstream of the CaMV 35S promoter, and upstream of the Nos terminator in the pCAMBIA using NcoI and BstEII recognition sites.

After the ligation reaction between the *p19* gene and the pCAMBIA vector, the product was transformed into *E. coli* and grown in media containing 50 µg/ml of kanamycin. Cloning was confirmed by NcoI and BstEII double digestion.

The orientation and sequences of this fragment was confirmed by sequencing using the primers shown in Table 1.

Table 1. Primers used for colony PCR.

Primer Name	Oligo sequences 5'→3'	No. of Bases
P19-F	5' CCATGGAACGAGCTA TAC AA 3′	20
P19-R	5' AGA AAG CGA GTA AGG TGA CC 3'	20

P19-pCAMBIA1304 recombinant vector was transformed to Agrobacterium tumefaciens strain GV3101 competent cells via the standard freeze-thaw protocol (17). The transformed bacteria were selected on plates containing 50 µg/ml Kanamycin and it was verified by colony PCR using the primers shown in Table 1.

Construction of the rhNGF- PVX Vector

The PVX-GW vector (Lacorte et al., 2010), which was kindly gifted by Dr. Cristiano Lacorte (EMBRAPA Recursos Gene´ticos e Biotecnologia, Brası´lia, Brazil), was used as a viral vector. The cloned synthesized rhNGF fragment in pUC57 vector (Genscript, USA) was digested with ClaI and SalI restriction enzymes (Fermentas) to release the inserts, gel-purified, and cloned directly into PVX vector (unpublished data).

Co-agroinfiltration of N. benthamiana Leaves by P19 Silencing Suppressor Protein

To inhibit the silencing of foreign gene expression in *N. benthamiana*, agrobacterium containing the PVXrhNGF expression vector was co-infiltrated with Agrobacterium strain GV3101, harboring the P19 silencing-suppressor gene from TBSV.

In brief, the Agrobacteria cultures were first adjusted to an optical density at 600 nm>2 with TSB medium supplemented with 100 µg/ml Rifampicin, 25 µg/ml Gentamycin, and 50 µg/ml Kanamycin. The pellets of agrobacterium cultures harboring binary plasmids for the expression of rhNGF and P19 were mixed equally and resuspended in an induction medium containing 0.5x Murashige basal medium salts, 1x Murashige & Stoog vitamins, 5% sucrose, 1x benzylaminopurine, 200µM acetosyringone; pH 5.5 for 3 h at 28 °C and 150 rpm. The leaves of *N. benthamiana* were then immersed in the above suspension while a vacuum of maximal pressure (~25 in. Hg) was applied for 30-60 s. The infiltrated leaves were incubated at 22 °C with a 16-h photoperiod for seven days incubation until harvest. All culture media, chemicals, and antibiotics were purchased from Merck (Darmstadt, Germany) Sigma (St. Louis, MO) and companies.

Protein Extraction

The infiltrated *N. benthamiana* leaves were frozen with liquid nitrogen and were ground into a fine powder using a mortar and pestle. Subsequently, 200 µl extraction buffer containing 100 mM NaCl, 10 mM EDTA (pH 8), 200 mM Tris-HCl (pH 8), 0.1% SDS, 0.05% Tween-20, 14 mM β-mercaptoethanol, 200 mM sucrose, and 2 mM phenyl methyl sulfonyl fluoride was added per 100 mg of leave (18). The TSP was separated using centrifugation for 20 min at 4 °C (14000 rpm). The concentration of TSP wasdetermined using Bradford assay (Bradford, 1976) by bovine serum albumin (BSA) as standard.

Dot-blot and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Analysis

Dot-blot analysis was performed on protein extracts from transformed plants containing rhNGF either in the presence or in the absence of P19 silencing suppressor. The procedure was done using anti-NGF HRP-conjugated Rabbit monoclonal antibody (ab195536; Abcam, USA). In brief, 5 µg and 25µg of total soluble extracts of control and recombinant plants were dotted on nitrocellulose membrane. After that, blocking solution 1X was added and incubated for 90 min at RT with shaking. The solution was washed out and then substituted with anti-NGF antibody. It was incubated for 2 hours, and after the solution was poured out; washing with TBST1x was repeated three times for 10 min each time. Subsequently, the recombinant proteins on the membrane were identified by anti-NGF antibody (1:2000 dilution) and were detected by DAB-Peroxidase substrate solution.

For SDS-PAGE analysis, the protein extracts from transformed plants containing rhNGF either in the presence or in the absence of P19 silencing suppressor was loaded onto a 12% SDS-polyacrylamide gel. For both Dot-blot and SDS-PAGE analysis extracted protein from transformed plants by *A. tumefaciens* alone was used as negative control; at the end of electrophoresis, the protein bands were visualized with coomassie brilliant blue. Also, the amount of expressed rhNGF protein was calculated by AlphaEaseFC software.

Results

Synthesis of P19 Gene

The synthesized P19 sequence was delivered in pBMH (Biomatik, USA). Figure 1 shows the P19 nucleotide and amino acid sequences.

atggaacgagctatacaaggaaacgacgctagggaacaagctaacagtgaacgttgggatggaggatcaggaggtaccacttctcccttcaaacttcctgacgaaagtccgagttggactg
agtggcggctacataacgatgagacgaattcgaatcaagataatccccttggtttcaaggaaagctggggtttcgggaaagttgtatttaagagatatctcagatacgacaggacggaagcttc
actgcacagagtccttggatcttggacgggagattcggttaactatgcagcatctcgatttttcggtttcgaccagatcggatgtacctatagtattcggtttcgaggagttagtatcaccgtttctg
gagggtctcgaactcttcagcatctctgtgagatggcaattcggtctaagcaagaactgctacagcttgccccaatcgaagtggaaagtaatgtatcaagaggatgccctgaaggtactgaga
ccttcgaaaaagaaagcgagtaa

Figure 1. A) TBSV P19 nucleotide sequence (GenBank accession number: M21958).

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          10         20         30         40         50         60         70
MERAIQGNDA REQANSERWD GGSGGTTSPF KLPDESPSWT EWRLHNDETN SNQDNPLGFK ESWGFGKVVF
          80         90        100        110        120        130        140
KRYLRYDRTE ASLHRVLGSW TGDSVNYAAS RFFGFDQIGC TYSIRFRGVS ITVSGGSRTL QHLCEMAIRS
         150        160        170
KQELLQLAPI EVESNVSRGC PEGTETFEKE SE
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B) TBSV P19 amino acid sequence (Uniprot ID: P11690-1).

P19 Gene Silencing Suppressor Coding Plant Vector

Figure 2 represents the schematic diagram of the constructed pCAMBIA vector harboring the P19, located under the control of the CaMV35S promoter, upstream of the NOS-Ter transcriptional terminator.

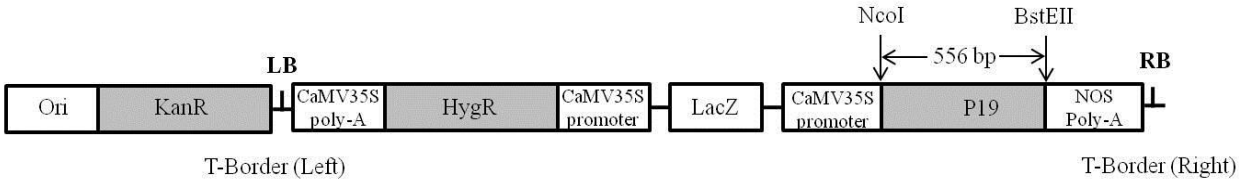


Figure 2. pCAMBIA-CaMV35S:P19. The P19 coding region is inserted in the downstream of the CaMV 35S promoter, and the upstream of the Nos terminator in the pCAMBIA using NcoI and BstEII recognition sites.

The sequencing results confirmed the presence of the cloned P19 gene in pCAMBIA vector with no alterations in the nucleotides. The resulted plasmid was transformed into *A. tumefaciens* GV3101. The transformed Agrobacteria were screened using specific primers (P19-F and P19-R) and the colony PCR assay indicated the expected 528 bp fragment. One positive colony was subsequently used for Co-agroinfiltration of *N. benthamiana* leaves with agrobacterium harboring PVX-rhNGF recombinant vector.

Analysis of Human Nerve Growth Factor Co-expression in N. benthamiana Leaves by Dot-blot and SDS-PAGE

To detect and analyze rhNGF, the protein extracts of the transformed *N. benthamiana* were evaluated by Dot-blot assay using anti-NGF antibody (Figure 3).

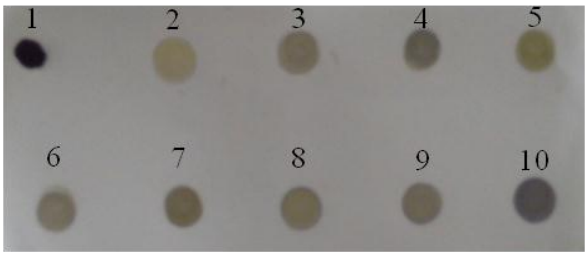
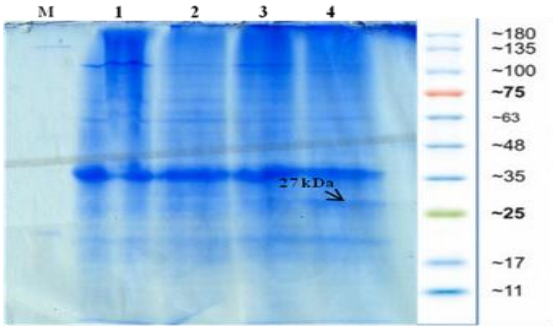


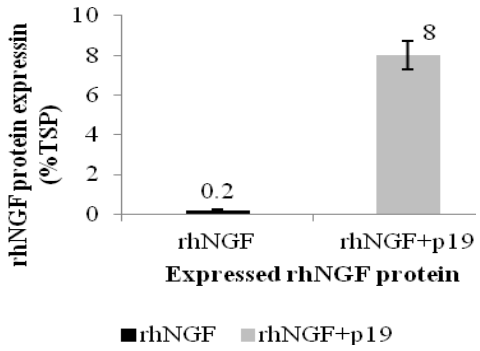
Figure 3. Dot-blot analyses, 1) positive control (purified human NGF protein extracted from E. coli); 2) Negative control (TSP of plants transformed by A. tumefaciens alone) in dpi 3; 3) TSP of plants transformed by PVX+rhNGF construct in dpi 3; 4) TSP of plants transformed by PVX+rhNGF construct in the presence of P19 silencing suppressor dpi 3; 5) Negative control (TSP of plants transformed by A. tumefaciens alone) in dpi 5; 6) TSP of plants transformed by PVX+rhNGF construct in dpi 5; 7) TSP of plants transformed by PVX+rhNGF construct in the presence of P19 silencing suppressor in dpi 5; 8) Negative control (TSP of plants transformed by A. tumefaciens alone) in dpi 7; 9) TSP of plants transformed by PVX+rhNGF construct in dpi 7; 10) TSP of plants transformed by PVX+rhNGF construct in the presence of P19 silencing suppressor dpi 7.

In addition, coomassie-stained SDS-PAGE showed aa27 kDa band (Figure 4a).

As shown in Figure 3, the results of Dot-blot analysis on the protein extracted from *N. benthamaina* leaves using anti-NGF antibody indicated that the expression of human NGF in the presence of P19 silencing suppressor in *N. benthamaina* leaves is several times higher than in the absence of P19.



A



B

Figure 4. a) SDS-PAGE analysis, 27 KDa band related to proNGF is indicated by the arrow. Lane M: Protein molecular weight marker (Thermo Scientific, Cat. No. 26610), Lane 1: Negative control (TSP of plants transformed by *A. tumefaciens* alone); Lane 2: Negative control (TSP of plants transformed by recombinant *A. tumefaciens* containing P19 gene); Lane 3: TSP of plants transformed by recombinant *A. tumefaciens* containing rhNGF gene; Lane 4: TSP of plants transformed by a mixture of recombinant *A. tumefaciens* containing rhNGF and P19 genes. **b)** The comparison of expressed rhNGF in the presence of P19 silencing suppressor and in the absence of P19.

Also SDS-PAGE result indicated one protein band about 27 kDa attributed to the recombinant pro-NGF form. The results demonstrated that co-agroinfiltration of human NGF with P19 suppressor gave higher rhNGF yields with an almost forty-fold (8%TSP) increase compared to rhNGF transient expression alone (0.2%TSP) (Figure 4b).

Discussion

In the present study, the co-agroinfiltration of rhNG

was evaluated using PVX viral vector in combination with P19 viral suppressor of gene silencing.

To our knowledge, no attempt on transiently co-expression of human NGF with P19 silencing suppressor has been reported in literature until now. Therefore, with the aim of increasing the expression level of plant-produced human NGF, we evaluated an efficient vacuum-agroinfiltration system in *N. benthamiana* boosted by P19 gene silencing suppressor. Previous studies reported at least three RNA silencing pathways in plants. In these pathways, silencing signals could be transmitted between cells, and even might be self-regulated by feedback mechanisms (19).

The first pathway is cytoplasmic short interfering RNAs (siRNA) silencing. This pathway is important in virus-infected plant cells in which the double stranded RNA (dsRNA) could be a replication intermediate of viral ssRNA. The second one is endogenous messenger RNAs silencing by miRNAs. These miRNAs bind to their target mRNA, resulting in either RNA cleavage or arrest of protein translation. The third pathway of RNA silencing in plants is related to DNA methylation and suppression of transcription (20).

All these three pathways of RNA silencing are sharing an ancient origin as there are examples of these pathways in animals, fungi and plants. Plants are remarkable in that they have retained the capacity for all three types of silencing, but other organisms may have lost one or more of these pathways (21).

Moreover, previous works showed that the use of TBSV-P19 enhanced expression levels of different proteins in *N. benthamiana* up to 50 folds (22). The results illustrated herein, demonstrated that the leaves co-agroinfiltrated with PVX-rhNGF and TBSV-P19 gave higher yield (mean value of 8% TSP). This prohNGF expression level is compared with plants infiltrated without TBSV-P19. In accordance with our results, previous studies also showed the effect of this silencing suppressor on the expression of foreign genes. Some of them are listed here: Lombardi et al (2009) showed a three-fold increase of Nef expression level compared to plants infiltrated without P19 (3). Also, Voinnet et al (2003) demonstrated that transient co-expression of viral suppressors of PTGS alleviates the host silencing response in *Nicotiana benthamiana*. The most effective suppressor was the P19 protein derived from TBSV. This protein significantly enhanced transient expression of a broad range of proteins, in several instances, the yields were in excess of 50 folds (23). In addition, in the study done by Boivin et al (2010), it had been shown that a dual transient Agrobacterium-mediated transformation process of suspension plant cells, using a viral suppressor such as P19 in combination with an expression vector enhances production of recombinant protein by 2 folds when compared with that for the use of the expression vector alone(24). Garabagi e t al (2012) reported P19-enhanced expression of the model therapeutic mAb trastuzumab that is targeted to the apoplast using binary vectors at about 2.3% of TSP (25).

Taken together, the results presented here suggest that all of the above mentioned reports confirm our data, and the use of viral suppressor of gene silencing, P19 derived from TBSV, could efficiently increase the transient expression of recombinant proteins in *N. benthamiana* manifold.

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Author contributions

HO and ZAB and PE designed the constructs and analyzed the data. MZ performed experiments and wrote the manuscript. HO supervised this work.

All authors have reviewed and approved the final manuscript.

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Conflict of interest

The authors have no competing interests.

Role of sponsor

The funding organizations played no role in the design of study, interpretation of data, or preparation or approval of manuscript.

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