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Research Article

Rapid Recombinant Protein Production using a TuMV-Based Vector in Lettuce (*Lactuca sativa* L.)

Malihe Mirzaee[1], Mokhtar Jalali-Javaran[1,*], Ahmad Moieni[1], Sirous Zeinali[2], Mahdi Behdani[3], Masoud Shams-Bakhsh[4] and Mostafa Modarresi[1]

[1] Department of Plant Breeding & Biotechnology,
Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran
[2] Department of Molecular Medicine, Pasteur Institute of Iran, Tehran, Iran
[3] Biotechnology Research Center, Biotechnology Department,
Venom & Biotherapeutics Molecules Lab., Pasteur Institute of Iran, Tehran, Iran
[4] Department of Plant Pathology, Faculty of Agriculture,
Tarbiat Modares University, Tehran, Iran
[*]Corresponding author: E-mail: m_jalali@modares.ac.ir, Tel: +98 (0)21 82882014; Fax: +98 (0)21 82884180

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ABSTRACT:
In recent years, plant viral vectors have been increasingly developed as a successful biotechnological tool to express a wide range of foreign proteins in plants. Plant viral vectors are beneficial because of the autonomous replication and high levels of gene expression in a short time. Here, we examined the utilization of an infectious turnip mosaic virus (TuMV)-derived vector for the transient expression of the jellyfish green florescent protein (GFP) in lettuce plants. After transfection by carborandum on lettuce leaves, GFP expression was detected using florescence microscopy at 14 days post inoculation. Further analysis of GFP expression using RT-PCR and ELISA assays confirmed that *gfp* gene was correctly transcribed and translated in lettuce plants. Our results demonstrated, for the first time, that it is possible to use TuMV-based viral vectors to produce recombinant proteins in lettuce.

Keywords: *Transient expression, viral vector, TuMV, GFP, lettuce*

[I] INTRODUCTION

Technical, biochemical and economic limitations of current prokaryotic and eukaryotic expression systems, and also the growing demand for diagnostic and therapeutic proteins have led to a wide interest in developing new recombinant protein expression platforms [1]. Typically, biopharmaceutical proteins are manufactured using microbes (bacteria and yeast) and mammalian cells such as the Chinese hamster ovary system, which are complex to manipulate and susceptible to contamination with human pathogens [2, 3]. During the past decade, plants have emerged as a promising competitive force in the large scale production of heterologous proteins [4]. This is described as 'molecular farming' and refers to the production of valuable recombinant pharmaceuticals proteins, industrial enzymes and secondary metabolites, in plants [5]. Plants offer a unique combination of benefits over mammalian and microbial production systems, since they provide a safe and inexpensive tool that might be easily scaled up to agricultural levels. In addition, plants can properly fold and assemble eukaryotic multimeric proteins and are able to perform many of the post-translational modifications essential for optimal biological function. Plants are unlikely to be contaminated by human-tropic pathogens or bacterial toxins. Unlike fermenter-based systems, there is no need to require large upfront investments, because plants can be grown in soil or artificial materials by only light, water and fertilizers [6].

Two strategies have been developed for the expression of recombinant proteins in plants, which resulted in stably or transiently transformed plants. Mainly, plant viral vectors or agroinfiltration method are used for transient protein expression. Compared to stable plant transformation, transient gene expression is a fast and easy approach and gives results in few days. However, transient expression could be used to verify the function of new constructs before proceeding to stable transformation, but it also provides a reliable alternative to the time and material consuming production of stably transformed plants [7, 8].

Turnip mosaic virus (TuMV) is a member of the genus *Potyvirus* in the family *Potyviridae* and causes diseases in 318 species in 156 genera of 43 families of vegetable plants. TuMV monopartite genome consists of a 10 kb positive-strand RNA genome whose translational product is a large polyprotein which is proteolytically processed into 10 mature proteins via three virus-encoded proteinases including Protein 1 (P1), Helper component-proteinase (HC-Pro) and Nuclear inclusion body component a (NIa). Potyviruses are very effective in expressing foreign proteins in plants, because of the existence of amenable sites for inserting genes, numerous replication of heterologous protein as a part of the polyprotein and subsequent release of recombinant protein by viral proteases activity [9-12]. In this study, to investigate the utility of TuMV as a viral vector in lettuce (*Lactuca sativa* L.), we applied an infectious TuMV vector carrying the reporter gene *gfp*. Lettuce is a robust and fast growing plant that is widely grown worldwide. The availability of agricultural infrastructure for large-scale lettuce cultivation provides advantage of rapid adaptation for therapeutic protein production [13].

Previously, several infectious clones of potyviruses have been used as useful biotechnolical tools for expression of heterologous genes in plants, such as TEV [14], PPV [15], ZYMV [16] and PVA [17, 18]. However, TuMV-based vectors have been recently applied to express foreign proteins in its indicator hosts, means *Brassica* spp., *Chenopodium quinoa* Willd., and *N. benthamiana* [9, 10], or in *Arabidopsis thaliana* as a model plant [19]. Their results and ours strongly showed that TuMV-derived vectors could effectively express heterologous proteins in a variety of plants.

[II] MATERIALS AND METHODS

2.1. Inoculation of plants

Romaine type lettuce plants were grown in a growth room with 16 h light cycles and 23 ºC temperature. For viral infections, two or three fully expanded leaves of 3-week old plants were dusted with carborundum and inoculated by gently rubbing with 10 µl (1 µg/µl) of either p35STuMVGFPHis or wild-type TuMV as control (Kindly provided by Prof. Shyi-Dong Yeh, Plant Pathology Department, National Cheng Hsing University, Taichnug, Taiwan). Leaves were harvested 14 days post inoculation (dpi) and used for the GFP expression analyses.

2.2. Detection of GFP fluorescence

In order to visualize the GFP expression in lettuce plant, leaf disks were examined using fluorescence microscopy (Olympus IX71, Tokyo, Japan), and images were captured using a mounted high-resolution Olympus DP71 digital camera.

2.3. Reverse transcription- Polymerase chain reaction (RT-PCR)

Total RNA was extracted from the leaves of p35STuMVGFPHis or wild-type TuMV inoculated plants by Hybrid-RTM kit (GeneAll Biotechnology, Korea) following the manufacturer's instructions. Then, 5 µg aliquot of total RNA was used for cDNA synthesis using First Strand cDNA Synthesis Kit (Fermentas). The resulting cDNA was amplified by PCR using primers located in the GFP sequence, forward primer (FG), 5'-ACGACGGCAACTACAAGACC-3', and reverse primer (RG), 5'-TTGTACTCCAGCTTGTGCCC-3'. Moreover, total RNA extracted from p35STuMVGFPHis infected plants was used as a control to consider the probability of genomic DNA contamination. PCR cycles was performed by an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C and 58 °C for 30 s and 72 °C for 20 s, and one final cycle of 5 min at 72 °C. Finally, the PCR fragments were analyzed by electrophoresis on a 1% agarose gel.

2.3. Protein extraction

For protein extraction, 0.2 g of leaf tissue was ground in liquid nitrogen with to a fine powder. Total soluble protein (TSP) was extracted by 1 ml of extraction buffer (50 mM Tris- HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.04% (v/v) 2-mercaptoethanol). After a brief vortex, the homogenate was centrifuged (15,000 × g for 20 min at 4 ºC) to ground and the clarified supernatant was used for dot blot and ELISA analysis. The concentration of TSP was determined by Bradford assay [20].

2.4. Dot blot immunoassay

For dot blot, 7 µl of plant extracts (20 ng) were spotted onto a nitrocellulose membrane and let dry at room temperature. The membrane was then blocked for 2 h with 5 % nonfat milk in PBST (PBS/0.1 % Tween 20) and probed with a mouse anti-GFP primary antibody (Biolegend, USA; diluted 1:2,000 in PBST) for 90 min at room temperature, followed by washing with PBST. After incubation with goat anti-mouse IgG antibody conjugated to peroxidase (Sigma; 1:3,000 in PBST) for 1 h, the membrane was washed with PBST and treated with DAB-H_2O_2 substrate solution for color demonstration.

2.5. Enzyme-linked immunosorbent assay (ELISA)

To perform ELISA, 96-well plate was coated by 50 ng of TSPs in carbonate/bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and kept overnight at 4 °C. Then and after each of the following steps, the plates were washed three times using PBST buffer. Consecutively, the plates were treated with a blocking buffer (1 % BSA in PBST) for 1 h at 37 ºC. Afterwards, GFP was detected using mouse anti-GFP primary antibody for 2 h at 37 ºC. After washing, the plates were incubated with 1:3,000 PBS dilution of secondary goat anti-mouse IgG antibody for 1 h at 37 ºC. Finally for color development, substrate solution [1 % TMB (tetramethylbenzidine), 200 mM citrate buffer, 0.01 % H_2O_2, pH 5.5] was reacted for 30 min at room temperature and the reaction was stopped with 1 M H_2SO_4. Absorbance was determined at 450 nm by a microplate reader (BioTek, USA).

[III] RESULTS

3.1. Infectivity of TuMV vector expressing GFP in lettuce

Lettuce plants were transiently transformed with the construct p35STuMVGFPHis carrying the coding sequence of *gfp* as a reporter gene (Figure 1).

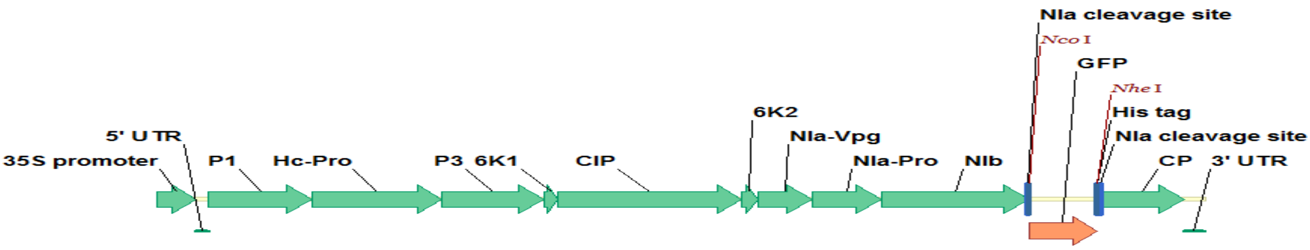
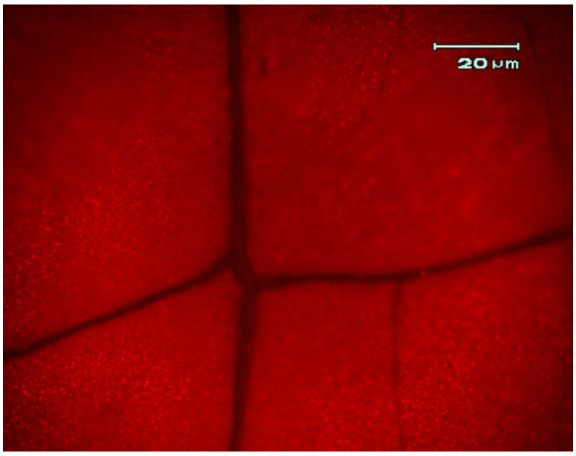


Fig. 1. Construction of the p35STuMVGFPHis vector. Schematic represents the viral polyprotein

under the 35S promoter which contains GFP sequence. The foreign gene insertion site in genomic TuMV map is between NIb and CP provided by *Nco*I and *Nhe*I restriction endonuclease enzymes. The recombinant protein is followed by a Histidine-tag and released with NIa protease activity. *35S promotor* Cauliflower mosaic virus 35S promoter, *P1* Protein 1, *HC-Pro* Helper component-proteinase, *CIP* Cylindrical (or Cytoplasmic) inclusion protein, *NIa-Vpg* Nuclear inclusion body component a- Viral protein genome-linked, *NIa-Pro* NIa- Protease, *CP* Coat protein

Inoculation of lettuce leaves with the TuMV viral vector caused systemic infection, as readily detected by the GFP under fluorescence microscopy (Figure 2).

A



B

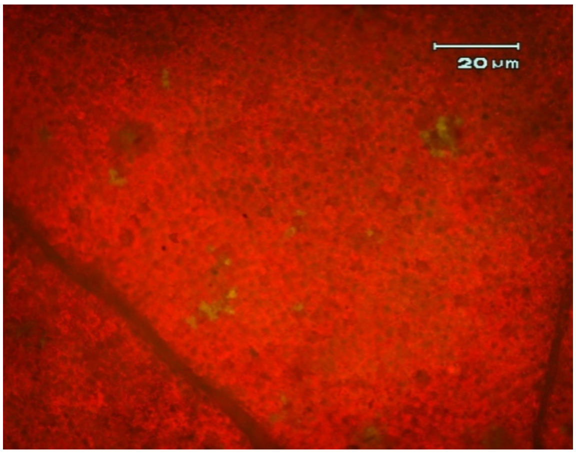


Fig. 2. Fluorescence microscopy analysis of GFP expression in lettuce leaves infected with a TuMV-based vector. (A) A leaf of lettuce with wild-type TuMV did not show any green fluorescence. The red color indicated autofluorescence of chlorophyll. (B) GFP was detected in lettuce leaves inoculated with p35STuMVGFPHis vector.

3.2. RT-PCR

RT-PCR assay utilizing *gfp*-specific primers amplified, as expected, a 127 bp fragment in lettuce plants infected with the TuMV recombinant virus, while this band was absent in wild-type TuMV inoculated plants (Figure 3).

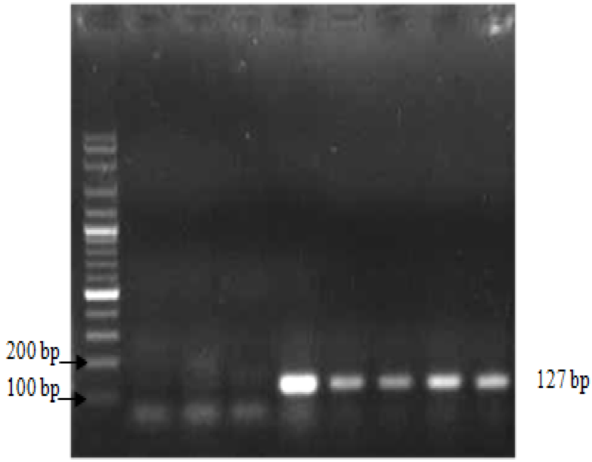


Fig. 3. RT-PCR amplified a 127 bp fragment by *gfp* specific primers. *M* 100 bp molecular ladder (Fermentas), C^-_1 water negative control, C^-_2 RNA negative control, C^-_3 wild-type TuMV-infected plant as negative control, C^+ p35STuMVGFPHis vector as positive control, *1-4* different p35STuMVGFPHis inoculated plants

3.3. Dot blot

The expression of GFP in infected plants was also screened by dot blot analysis. For p35STuMVGFPHis leaf extract, positive signal was clearly distinguished from the wild-type negative control ones (Figure 4).

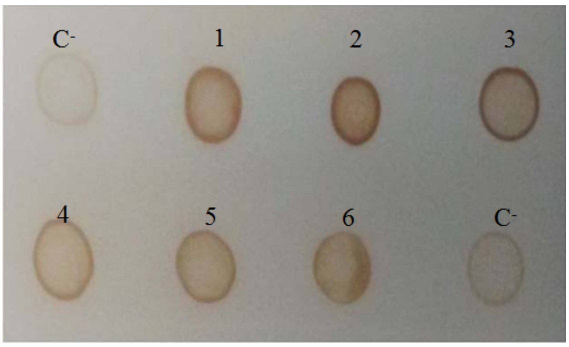


Fig. 4. Dot blot analysis of GFP transient expression in lettuce leaves. C^- wild-type TuMV-infected plant

as negative control, *1-6* different p35STuMVGFPHis inoculated plants

3.4. ELISA

The absorbance values of GFP in different inoculated lettuce plants were compared by direct ELISA using an anti-GFP antibody. The ELISA results demonstrated that plants infected with recombinant TuMV expressed different levels of GFP which are significantly greater than that of the wild-type TuMV inoculated plants showed (Figure 5).

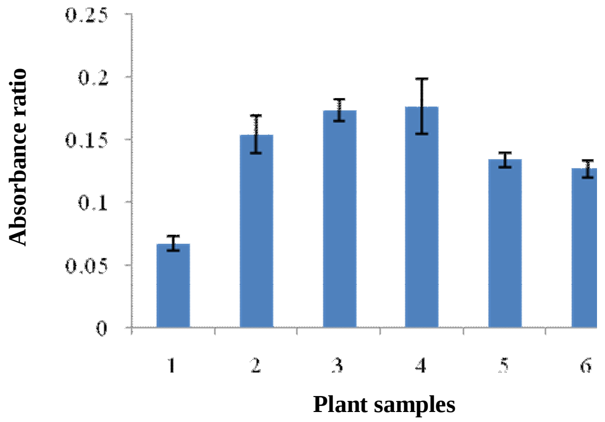


Fig. 5. ELISA assay for comparison of GFP expression in infected lettuce leaves. *1* wild-type TuMV-infected plant as negative control, *2-6* different p35STuMVGFPHis inoculated plants. Data represent means ± SE from three independent infected samples.

[IV] DISCUSSION

Plant viral vectors have been considered as a high-value alternative to stably transformed plants [21]. Transient gene expression using a modified plant viral vector provides the systemic spread of the virus throughout the plant. The foreign gene, as a part of viral genome, is transcribed by viral RNA replication and then translated into the protein of interest, therefore production takes about two weeks [22]. The obvious advantages of viral vectors are speed, low cost, ease of manipulation and the gene expression in different plant species using the same vector construct because of the wide plant host range of viruses [23, 24].

Among the various viral vectors, some of them such as tobacco mosaic virus (TMV), potato virus X (PVX), cowpea mosaic virus (CPMV), alfalfa mosaic virus (AMV) and bean yellow dwarf virus (BeYDV) –based vectors are the most commonly used in transient expression system [25]. However, these affect a few host species. TuMV, a *potyvirus* of economic importance, infects 43 plant species [10] and can be used as a truly versatile virus vector.

In the present study, first our results illustrated quite convincingly that the TuMV vector could fluorescently express *gfp* gene in lettuce leaves. Then, for further analysis of GFP expression in lettuce plant system, ELISA assay by the mouse anti-GFP monoclonal declared apparently significant accumulation and correct conformation of the GFP.

Actually, we exploited the TuMV-based viral vector as a new platform for the production of recombinant proteins in lettuce plants and in this way, we assessed whether this vector can express the *gfp* as a reporter gene. In many researches, *Agrobacterium* delivery systems have been used for the transient expression of a vast variety of valuable recombinant proteins in lettuce plants [13, 22, 26, 27, 28, 29]. However, to the best of our knowledge, there is no report that directly and successfully consider infectious viral vectors for the production of heterologous proteins in lettuce until now. While, they offer a reasonable alternative to *Agrobacterium* due to the high level expression and the ability of autonomous replication in host cells. These can potentially result in the much higher yield of recombinant proteins [30, 31].

[V] CONCLUSION

In summary, our results have provided evidence that recombinant TuMV vector can be used as an expression platform for GFP production and possibly for pharmaceutically important proteins in lettuce. This achievement will open up a new prospect in molecular farming and now we are examining the efficiency of this system in expressing a therapeutic protein.

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