Transient expression of green fluorescent protein in radish (*Raphanus sativus*) using a turnip mosaic virus based vector

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Received: 17 Aug 2018; Accepted: 30 Jan 2019.
DOI: 10.30479/ijgpb.2019.9206.1205

Abstract

It is possible to use transgenic plants, as bioreactors, for the production of recombinant inexpensive chemicals and drug components. Transient gene expression is an appropriate alternative to stable transformation because it allows an inexpensive and rapid method for expression of recombinant proteins in plant tissues. In recent years, plant viral vectors have been increasingly developed as successful biotechnological tools for the expression of a wide range of foreign proteins in plants. Plant viruses-based vectors are useful because of the autonomous replication and the high level of gene expression in a short time. Here, we have used a vector derived from an infectious turnip mosaic virus (TuMV) for transient expression of the jellyfish green fluorescent protein (GFP), as a model heterologous protein, in radish plant. The GFP ORF was inserted between NIb and CP sites under control of CAMV35S promoter. The leaves were inoculated using surface scratch by carborundum and harvested 14 days after inoculation for analysis. The visualization of GFP fluorescence in leaf disks from inoculated plants using fluorescence microscopy demonstrated gene transformation and systemic infection. Expression of the desired protein were confirmed by RT-PCR, SDS-PAGE and Dot blotting analysis. The quantitative values of GFP in different inoculated leaves were compared by ELISA assay using an anti-GFP antibody. The results showed high level of expression of GFP protein in leaves of inoculated plants compared with wild type. The results demonstrated that the TuMV-based vector has high efficiency for the expression of the foreign protein in the radish plant. This is the first examination of TuMV-based vector in radish.

Key words: GFP, Radish, Transient expression, Viral vector.

ABBREVIATIONS

GFP (Green florescent protein), TuMV (Turnip mosaic virus), *Taq* (*Thermus aquaticus*), dNTPs (Deoxynucleotides), OD (Optical density), SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis), ELISA (Enzyme-linked immunosorbent assay).

INTRODUCTION

In recent years, transgenic plants have been developed as safe and cheap bio-factories for the production of the pharmaceutical and industrial recombinant proteins (Yamamoto *et al*., 2018). However, the establishment of transgenic plants is more time consuming (Sha *et al*., 2013) and the yield of the recombinant protein is relatively low (Habibi-Pirkoohi *et al*., 2014). Furthermore, the foreign genes might be affected by the site effects and gene silencing (Leuzinger *et al*., 2013). Viruses are known as important vehicle for foreign gene expression in plants (Tourino *et al.,* 2008). Transient expression through plant viral vectors is an efficient alternative for extensive, fast and inexpensive recombinant proteins production in plant systems (Wagner *et al.,* 2004). The heterologous protein can be

produced in high yield within several steps by virus infection and replication (Jin *et al*., 2015; Mardanova *et al*., 2017) and that is comparable to bacteria and mammalian cell expression systems (Shah *et al*., 2013). Since the foreign gene does not incorporate into the plant genome, it does not inherit and affected by chromosomal effects (Voinnet *et al*., 2003). In compare to stably transgenic plant, this approach is more efficient for production of recombinant phytotoxic proteins that would hamper the development of transgenic plant. Because of inoculation of mature plant, the problems of phytotoxic proteins can be eliminated in transient expression (Wagner *et al*., 2004). Plant viral vectors have been successfully developed for the industrial-scale expression of recombinant proteins (Jin *et al*., 2015). So, several researchers have developed a range of virus-derived vectors for the expression of heterologous gene products (Pogue *et al*., 2002; Grill *et al*., 2005; Mardanova *et al.,* 2017). Potyviruses have a broad geographical distribution, including several species that can infect various host plants and are recognized as the second largest plant virus family after Geminiviridae (Ivanov *et al*., 2014). Some advantages of utilization of potyviruses for constructing viral vectors result from their rod shape that accommodate large inserts (Tourino *et al*., 2008). Turnip mosaic virus (TuMV) belongs to the genus *Potyvirus* was ranked second most important virus, infecting field-grown vegetables in many countries and regions (Walsh and Jenner, 2002). More than 300 plant species have been known as TuMV hosts (Tourino *et al*., 2008). The TuMV genome contains a single-stranded RNA molecule of about 10 kb in length (Zhu *et al*., 2016) and translated into a polyprotein of 358 kDa which binds to three proteases and leads to different proteins (Cotton *et al*., 2009). It was shown that the 6K2-VPg-Pro polyprotein is responsible for the formation of cytoplasmic vesicles derived from the ER through its hydrophobic 6K2 domain (Beauchemin *et al*., 2007). To date, the transcribed infectious RNAs derived frm full-length cDNA clones have been reported from more than 20 potyviruses including TuMV (Tou *et al*., 2015). It was demonstrated that the TuMV viral system has been extensively engineered to achieve high levels of recombinant proteins accumulation especially in Brassicaceae family (Tourino *et al*., 2008). Genetically encoded reporter genes such as green fluorescent protein (GFP) when inserted into viral vectors, allow evaluation of viral infection and movement through the plant (Bedoya *et al*., 2012). Radish (*Raphanus sativus* L.) belongs to the family Brassicaceae (Cho *et al*., 2008), is the most important root crop cultivated in S/E Asia (Park *et al*., 2005) and has been known as TuMV host (Li *et al*., 2017). In this study we inoculated the radish plant using an infectious TuMV-based vactor which carries a green fluorescent protein (GFP) gene for virus tracking and evaluating its systemic infection. The estimation of GFP accumulation in the plant demonstrated the efficiency of the viral vectors for transient expression of recombinant proteins in radish.

MATERIALS AND METHODS

Viral vector

The infectious plant viral vector, p35STuMVGFPHis (Kindly provided by Prof. Shyi-Dong Yeh, Department of Plant Pathology, National Cheng Hsing University, Taichnug, Taiwan) was used for leaf inoculation. This vector harbors the viral polyprotein (P1, HC-Pro, CIP, NIa-Vpg, NIa-Pro, NIa Protease and CP), GFP reporter, polyhistidine tag and ampicillin resistant gene under control of the constitutive CAMV (Cauliflower Mosaic Virus) 35S promoter (Figure 1). The Presence of full length GFP gene in p35STuMVGFPHis vector was confirmed using polymerase chain reaction.

Plant growth and inoculation

The seeds of Radish cherry Belle variety were planted in the pots containing soil and kept in the growth chamber (16 h light/8 h dark/25 ºC) for germination and growth. Three week old plants at the four-five true-

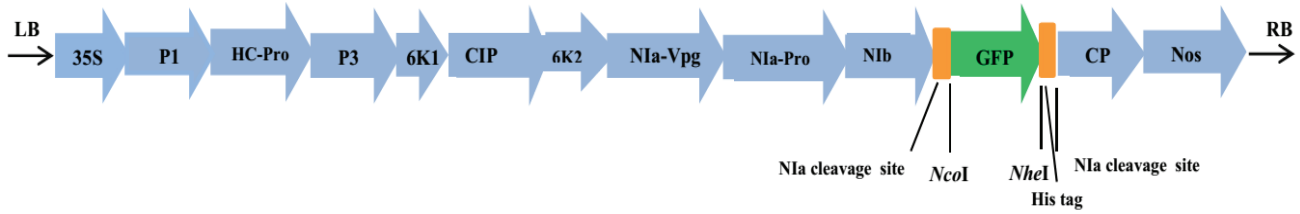

Figure 1. Schematic view of p35STuMVGFPHis vector for transient expression of the GFP in the radish. The construct contains *P1* (Protein 1), *HC-Pro* (Helper component-proteinase), *P3* (Protein 3), *6K1* and *6K2* (alphaviral *6k* gene), *CIP* (Cylindrical or Cytoplasmic inclusion protein), *NIa-Vpg* (Nuclear inclusion body component a- Viral9 protein genome-linked), *NIa-Pro* (NIa Protease), *CP* (Coat protein). This gene is under the control of the CaMV35S promoter and Nos terminator.

leaf stage were selected for inoculation (Adhab *et al*., 2013). Two or three expanded leaves were dusted with carborundum (600-mesh) and gently rubbed with 10 µl (1 µg/µl) of the either TuMV-GFP or wild-type TuMV (as negative control) vector in three independent experiments. Fourteen days post inoculation; the leaves were harvested for GFP expression analysis.

GFP detection using fluorescent

The GFP fluorescence in leaf disks from inoculated plants was visualized using fluorescence microscopy (ZEISS, Germany) and photographed using high-resolution digital camera.

RNA extraction and RT-PCR analysis of *GFP* gene transcripts

Total RNA was isolated from 500 mg inoculated radish leaves and ground in liquid nitrogen using RNX™-plus solution according to a standard protocol (http://www.cinnagen.com/Catalogue.pdf). Extracted RNA was treated with DNase enzyme (Fermentas) and cDNA synthesis was performed according to the manufacturer's instructions using a Reverse Transcription System (Fermentas). The first-strand cDNA was used as a template in the following PCR conditions: pre heating at 94 ºC /5 min and 35 cycles denaturation at 94 ºC /1 min; annealing at 60 ºC /40 s; extension at 72 ºC /40 s and finally the reaction mixture were placed for 10 min at 72 ºC to amplify 127 bp located in the middle of *GFP* using specific primers (forward: 5' ACGACGGCAACTACAAGACC 3' and reverse: 5'TTGTACTCCAGCTTGTGCCC3'). The RT-PCR products (127 bp) were analyzed by electrophoresis on a 2% agarose gel.

Protein extraction and SDS-PAGE

Total soluble protein (TSP) was extracted using 200 mg of inoculated leaves and ground into a powder in liquid nitrogen and resuspended in 1 mL of protein extraction buffer (0.2 M Tris–HCl pH 8, 5 mM ethylene diamine tetraacetic acid (EDTA), 100 mM sucrose and 0.1 mM 2-mercapthoethanol). After stirring, the suspension was centrifuged at 12000 g in a micro centrifuge for 20 min at 4°C (Abdolinasab *et al*., 2013). The supernatant was collected for determination of the total soluble protein concentration using the Bradford assay (Bradford, 1976). Protein samples were boiled for 4-5 min and SDS-PAGE was performed using 12% acrylamide gel followed by staining with Coomassie brilliant blue (Laemmli, 1970).

Dot blotting assay

The presence of GFP protein in the inoculated leaves was evaluated by standard protein dot blotting assay. Briefly, 20 µg of the extracted protein samples from inoculated and non-inoculated plants was dotted on the PVDF membrane and allowed to dry. The membrane was incubated with BSA 1% as blocking solution for 1 h. After incubation, the membrane was cleaned in PBST (PBS with 0.05% Tween 20) washing buffer (3*5 min), and incubated (shaking) with 200 µg/mL Rabbit polyclonal GFP antibody (Cat No: 338042) for 1 h in 37 ºC. The membrane was washed three times with PBST buffer and incubated with Goat anti Rabbit IgGHRP conjugate secondary antibody (Cat No: 405405). The membrane was washed three times with PBST and incubated in TMB stabilized substrate for horseradish peroxidase (Promega, USA) until spots were visible.

Validation of the infectivity of TuMV-GFP on radish plant

Expression of the GFP protein in inoculated plants was evaluated using enzyme-linked immunosorbent assay. A 96-well micro plate was coated with 50 µg of total soluble proteins from the inoculated plants in three replications and incubated overnight at 4 ºC. The plate was washed using the buffer (PBS with 0.05% Tween 20) and followed by incubation with 1% bovine serum albumin (BSA) in PBS for 1 h at 37 ºC to prevent non-specific binding. After washing, the plate was incubated with Rabbit polyclonal GFP primary antibody for 1 h at 37 ºC. The plate was washed and 100 µL of the Goat anti Rabbit IgGHRP conjugate secondary antibody was added per well and incubated in room temperature for 30 min. The unbound conjugate was released by washing the plate and it was then incubated at room temperature with 100 µL of TMB substrate Reagent (Biolegend, USA) for 15 min. The reaction was stopped with 50 µL of 2N H_2SO_4 and the plate was read at 450 nm using a micro plate reader (ML-2571N, Korea). The analysis of variance (ANOVA) was performed at first to test the levels of absorbance and it was followed by the pairwise comparison between leaves using Tukey's honest significant difference (HSD) test.

RESULTS

Initial infectivity assay

The infectivity of TuMV in radish plants was confirmed using appearance green fluorescent in leaves that were mechanically inoculated with TuMV-GFP, whereas no green fluorescent appeared in wild-type TuMV inoculated plants (Figure 2 A and C). The local spots were observed on the leaves of inoculated plant, which is a reason for systemic infection (Figure 2 B). The photographs were taken at 14 days after inoculation.

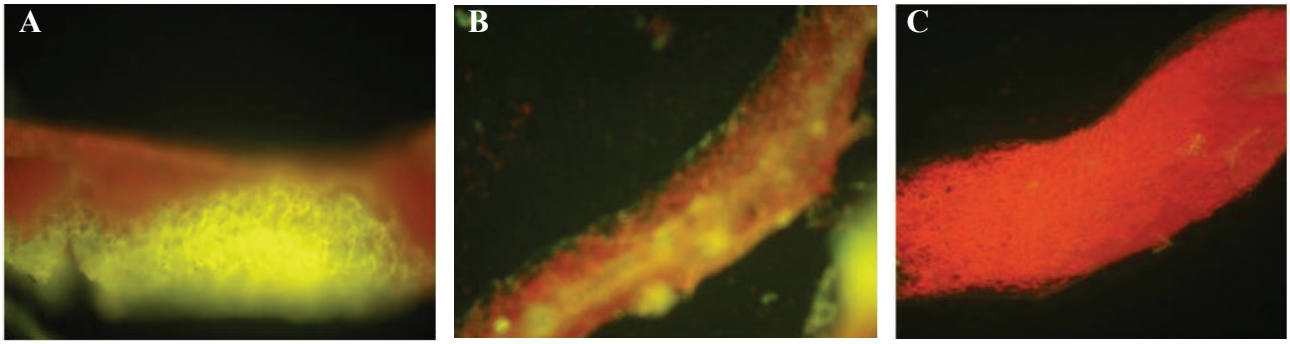

Figure 2. Detection of the expressed GFP in the radish leaves using fluoresce microscopy. **A:** the leaf inoculated with TuMV-GFP vector, **B:** Systemic infection of TuMV-GFP in non-inoculated leaf of inoculated plant, **C:** the leaf inoculated with wild type TuMV (negative control).

Expression of the *GFP* gene in inoculated radish plants

To analyze the presence of *GFP* transcripts in the inoculated plants, RT-PCR was performed. The specific primer pair designed from the middle of *GFP* gene yielded a specific DNA band of approximately 127 bp in size from the RNA extracts of inoculated plants with TuMV-GFP (Figure 3, Lane1). This DNA product was absent in RT-PCR reactions from the plants inoculated with the wild-type TuMV construct and also in PCR control reactions of total RNA extracted from inoculated radish plants with TuMV-GFP (Figure 3, Lane 2 and 3).

Figure 3. RT-PCR amplification of a 127 bp fragment from the middle of *GFP* gene with specific primers. M: 100 bp molecular weight marker (Fermentas), Lane 1: inoculated leaf, Lane 2: RNA treated with *DNase* as template (negative control), Lane 3: the plant inoculated with the wild-type TuMV (negative control), Lane 4: TuMV-GFP as template (Positive control).

Protein extraction and SDS-PAGE

To indicate the presence of GFP protein in inoculated plants, protein were extracted from leaves of inoculated radish plants with TuMV-GFP and wild-type TuMV (as negative control). SDS-PAGE analysis showed a distinctive band with approximately 27 kDa molecular weight in the inoculated plants with TuMV-GFP (Figure 4, Lane 2-4) whiles, it was not observed in the inoculated plants with wild-type TuMV (Figure 4, Lane 1).

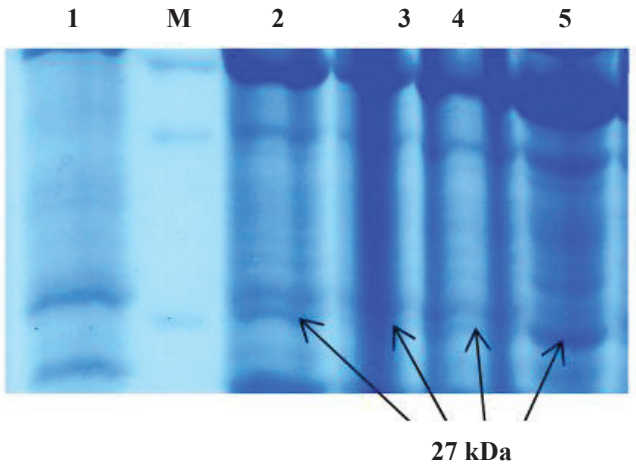

Figure 4. SDS-PAGE analysis of inoculated radish plants to detect GFP expression. Lane 1: inoculated leaf with wild-type TuMV (as negative control), Lanes 2-5: inoculated plants with TuMV-GFP, M: Molecular weight marker (Fermentas, SM0601).

Dot blotting assay

The production of recombinant GFP protein was evaluated by dot blot assay using specific anti-GFP antibody (Figure 5). It confirmed the expression of the *GFP* gene at translation level, whereas no signal was observed in the inoculated plants with the wild-type TuMV (Figure 5).

ELISA assay

Expression of the recombinant GFP protein was further quantitatively measured by ELISA. The absorbance of TuMV-GFP (OD 450) showed signals in the inoculated (OD 450 0.92±0.34) and systemic infected leaves (OD

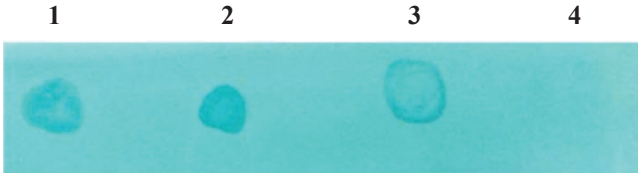

Figure 5. Dot-blot analysis of GFP expression in inoculated radish plants. 1-3: inoculated plants with TuMV-GFP, 4: inoculated plant with wild type TuMV (as negative control).

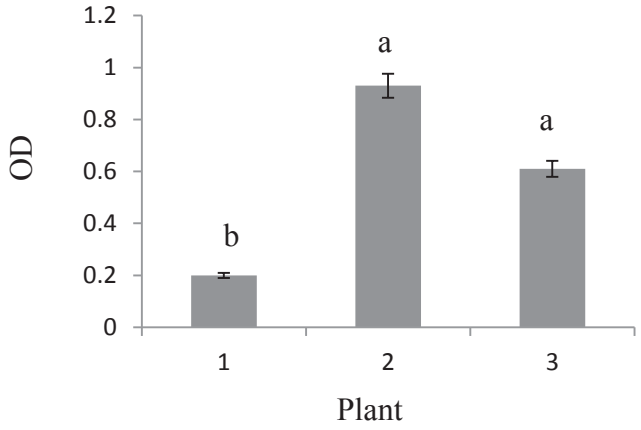

Figure 6. ELISA assay of inoculated plants using specific anti-GFP antibody. 1: inoculated plant with wild-type TuMV (as negative control), 2: inoculated leaf with TuMV-GFP, 3: systemic infected leaf with TuMV-GFP (Values correspond to means of three replicates along with standard deviations).

450 0.65±0.19) which was significantly higher than that of the inoculated leaves with wild-type TuMV (Figure 6). ELISA analysis of transiently expressed GFP from the total protein extraction of infiltrated leaves confirmed the expression of recombinant proteins that was specifically recognized by the conjugated anti-GFP antibody.

DISCUSSION

In the recent years, several studies have been initiated to investigate the expression of recombinant proteins in vegetables as oral vaccines (Beihaghi *et al.,* 2017). Plant transient gene expression provides low cost, fast, the ease of manipulation and usually higher heterologous protein yield (Shah *et al.,* 2013). The radish plant is rich in antioxidant constituents and it has been reported that this plant has the highest per capita consumption within the Brassicaceae family (Papi *et al.,* 2008). Because of low regeneration frequency in radish, production of recombinant proteins in transgenic radish via tissue culture approach had been scarcely reported (Park *et al.,* 2005). Radish, being edible and easily grown, has been used for all transient expression experiments (Liu *et al.,* 2018). Because of autonomous replication and high levels of expression of positive-sense ssRNA viral vectors, in addition to their possession of amenable genomic contexts to insert expressible foreign ORFs, they have been employed for the expression of heterologous genes in host plants effectively (Chen *et al.,* 2007). Previously, potyviral-based vectors have been developed for transient expression of different recombinant proteins specially in Brassicaceae family (GermanRetana *et al.,* 2000; Fernández-Fernández *et al.,* 2001; Arazi *et al.,* 2001; Beauchemin *et al.,* 2005; Tourino *et al.,* 2008) because of their efficiency for production of high amounts of the heterologous proteins up to 1 g/kg of plant biomass (Gleba *et al.,* 2007; Egelkrout *et al.,* 2012; Thuenemann *et al.,* 2013). In the present study we exploited the TuMV virus-based vector as a platform for the expression of the foreign gene in radish. Until now, there have been no reports on successful use of infectious viral vectors for the production of heterologous proteins in radish. We describe the potential of a viral vector derived from an infectious TuMV clone for the systemic expression of the green fluorescent protein (as a model heterologous protein) in radish plant. The GFP gene was chosen because of its many advantages in plant transformation studies. The detection of this protein is easy and does not require the addition of exogenous substrate; also, the protein is stable and has relatively small size (Malabadi *et al.,* 2008). This protein is usually well-expressed in plant cells (Mardanova *et al.,* 2017) and is not toxic and does not affect the physiology of the living intact cells (Bellucci *et al.,* 2003). Also, it was reported that the *GFP* gene is more stable than other reporter genes such as the *uidA* in a TuMV based vector (Tourino *et al*., 2008). The results indicated the success rate for the TuMV initial infection and heterologous gene (GFP) expression in radish by fluorescence microscopy and Reverse Transcriptase-PCR analysis. Also, the heterologous protein translation was demonstrated by SDS-PAGE and Dot Blot assay. Our results showed that the TuMV-GFP vector could synthesize a protein with the expected band (27 kDa). Moreover, recognition of the GFP protein by the anti-GFP specific antibody in ELISA and Dot blot indicated that the GFP expressed is correctly folded and fully functional. Some factors including the insertion site, the nature of recombinant protein, the plant species that employed as host (Tourino *et al*., 2008) and the length of heterologous sequence are affected on successful expression of recombinant proteins in viral vector (Chen *et al*., 2007). Several potyvirus based vectors have been constructed to insert alien ORFs adjacent

to the junction between P1 and HC-Pro or NIb and CP by directional insertion (Chen *et al.,* 2007). We used the TuMV viral vector carrying GFP ORF in the insertion site between NIb and CP ORF since some previous reports claimed that this insertion site is less prone to recombination events than the insertion site between the P1 and HC-Pro (Arazi *et al*., 2001; Tourino *et al*., 2008), resulting in the high level expression of GFP protein in several hosts (Chen *et al*., 2007). Since the HC-Pro is one of the most potent RNA silencing pathway suppressors (Qu and Morris, 2005), it was reported that co-expression with HC-Pro boosts expression level of heterologous genes by protecting mRNA from degradation (Bucher *et al.,* 2003). The heterologous protein is flanked by two NIa-Pro recognition sites and will be processed out of the viral polyprotein upon translation (Tourino *et al*., 2008). Since the potyviruses are aphid-transmitted viruses, the major concern of using TuMV is spread of the foreign gene by aphids. With the induction of a mutation in the CP aphid transmissibility motif, its transmission by aphid was prevented (Tourino *et al*., 2008). The ELISA results showed a high level of expression of GFP protein in leaves of inoculated plants compared with the wild type and demonstrated the feasibility of using attenuated TuMV as an expression vector in radish. It is in accordance to previous findings reported by Mirzaee *et al*. (2016) in lettuce. Our results showed that the insertion of the foreign gene in the virus genome does not affect the ability of TuMV to infect radish plants. Thus, we propose that the TuMV based vector could be used for the production of various recombinant proteins in radish quickly and conveniently. This is the first report of the expression of a foreign gene in radish plant using virus- based vector.

ACKNOWLEDGEMENTS

I would like to thank Dr. Masoud Shams Bakhsh at the Tarbiat Modares University. The authors would like to acknowledge the financial support of Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kemran, Iran, under grant number of 96.42.

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