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TuMV as an efficient transient vector for expressing heterologous proteins in *Nicotiana tabacum* and *N. benthamiana*

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All relevant data are within the paper and its Supporting Information files.

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Abstract: **Nowadays the production of recombinant proteins such as drugs and commercial protein compounds in plants is called molecular farming. It has some benefits such as fast and large quantity production of recombinant proteins with low cost. In this research, the green fluorescent protein (GFP) was transiently expressed in two tobacco species via turnip mosaic virus (*TuMV*) derived vector, a virus which can infect a wide range of plant species. Florescence microscopy results indicated that *TuMV* could infect tobacco plants and accumulate GFP protein in plant leaves. In addition, RT-PCR, Dot-Blot and ELISA assays demonstrated the recombinant gene transcription, translation and stability. This is the first report of using *TuMV*-based viral vectors for producing recombinant proteins in tobacco. Optimized *TuMV*-based viral vectors could be used for producing recombinant proteins in tobacco.**

1. Introduction

Various expression systems, such as bacteria, yeast, plants, insects and mammalian cell cultures can produce recombinant proteins. The benefits of expressing recombinant proteins in plants include economic, agricultural scale, safe and authentic production (Sijmons *et al.*, 1990; Ma *et al.*, 2003; Mardanova *et al.*, 2015;). Molecular farming (also known as molecular pharming or biopharming) uses genetically engineered plants for the production of biopharmaceutical products, vaccine subunits, industrial enzymes therapy peptides and other compounds of interest (Boothe *et al.*, 1997; Wang and Ma, 2011; Yarbakht *et al.*, 2015).

Recombinant proteins in plants may be gained by stable genetic transformation (nuclear or plastid) or through transient expression. Transient expression is usually used for fast and flexible expression of genes of

interest (GOI), evaluation of expression system performance and components such as promoter and enhancers (Chiera *et al.*, 2008). In plants, a number of virus-based vectors are utilized for the transient expression of foreign genes, such as tobacco yellow dwarf virus (*TYDV*) for transient expression of chalcone synthase in *Petunia hybrida* (Atkinson *et al.*, 1998), tobacco mosaic virus (*TMV*) for transient expression of *GFP* (jellyfish, *Aequorea victoria* green-fluorescent protein) in tobacco (Shivprasad *et al.*, 1999), bean pod mottle virus (*BPMV*) for expression the *GFP* in the soybean (Zhang *et al.*, 2010), wheat streak mosaic virus (*WSMV*) for expression the *GFP* in cereals (Tatineni *et al.*, 2011) etc.

Turnip mosaic virus (*TuMV*) belongs to *Potyviridae* family and infects a wide range of plant species especially cruciferous (*Brassicaceae* family). It is a positive-sense single stranded RNA virus with a linear and monopartite genome and average length of 720 nm (Brunt *et al.*, 1996). Previously Beauchemin *et al.* (2005) strongly expressed *GFP* and *GUS* (bacterial β-glucuronidase) reporters genes in *Brassica perviridis* plants via *TuMV* virus. Furthermore, Chen *et al.* (2007) introduced *GFP* in some *Brassica* hosts such as *B. campestris* and *B. juncea* and high levels of the recombinant protein expression were observed. Therefore, in this study, to investigate the performance of recombinant protein production, the *GFP* reporter gene was introduced into the tobacco (*Nicotiana tabacum* and *N. benthamiana*) plants by using *TuMV* vector.

2. Materials and Methods

Plant material and growth conditions

Nicotiana tabacum cv. Xanthi and cv. Samsun and *N. benthamiana* seeds were grown in pots containing autoclaved soil, including 40% farm soil, 30% peat moss and 30% perlite. They were kept at 25°C in a phytotron under a 16-hour photoperiod (16:8 h L: D).

Plasmid and viral constructs

The *TuMV-GFP* construct (Fig. 1) was kindly provided by Dr. Shyi-Dong Yeh, Plant Pathology Department, National Cheng Hsing University, Taichnug, Taiwan. The plasmid contains a cauliflower mosaic virus 35S promoter (CaMV 35S) and *GFP* coding sequence between the NIb (nuclear inclusion protein b) and CP (coat protein) positions. Recombinant viral construct, p*TuMV-GFP*, was transferred into bacterial (*Escherichia coli* DH5α) competent cells (Sambrook and Russell, 2001). Bacteria were grown in 200 ml Luria-Bertani medium and then, p*TuMV-GFP* was extracted (Engebrecht *et al.*, 1991).

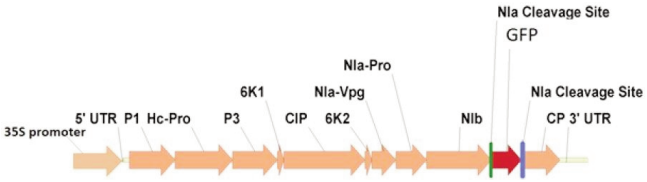

Fig. 1 - Schematic representation of the viral construct containing *GFP* under the *35S* promoter that was used in this expression analysis. The foreign gene insertion site is between NIb (nuclear inclusion protein b) and CP (the virus coat protein gene) provided by *NcoI* and *NheI* restriction endonuclease enzymes.

Plant Rub-inoculation with TuMV-derived vector

Wild-type TuMV (for control plants) and p*TuMV-GFP* was mechanically inoculated on upper surface of two top leaves (10 µg in 10 µl per leaf), using a cotton stick and carborandum powder according to Hosseini *et al.* (2013). Systemically infected (non-inoculated leaves) were used for further analysis.

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

The presence of the *GFP* gene in inoculated leaves was determined by RT-PCR. Total RNA was extracted from inoculated and control plant leaves (five independent samples) by Qiagene kit (South Korea) twelve days after incubation according to the manufacturer's instruction. RNA was extracted from non-inoculated leaves for confirming replication and movement of the virus. After treating with DNase I (Thermo Fisher Scientific, USA), cDNA was synthesized using the RevertAid Reverse Transcriptase (Thermo Fisher Scientific, USA) and *GFP* reverse primer (5' –TTG TAC TCC AGC TTG TGC CC-3') according to the producer's instructions. RT-PCR was conducted using the cDNA and the following forward and reverse primers under the following cycling conditions: forward (5'- ACG ACG GCA ACT ACA AGA CC -3') and reverse (5'- TTG TAC TCC AGC TTG TGC CC -3'). PCR cycling conditions were as follows: 94°C for 3 min for initial denaturation; 35 cycles of 94°C for 30 s, 51°C for 30s, and 72°C for 30 s; and 72°C for 10 min for a final extension. Then PCR products were analyzed by 1% TAE agarose gel.

Fluorescence microscopy

Leaves from TuMV-based vector inoculated and control plants were observed under an Olympus fluorescent microscope 6 (version IX71, Tokyo, Japan).

The fluorescence photographs were taken using a mounted high-resolutionm7 Olympus DP70 DP71 digital camera at 12 days post-inoculation (dpi).

Protein extraction and GFP analysis

Proteins were extracted from 0.5g tissues of control and inoculated tobacco leaves (five independent samples) with extraction buffer, including 0.2M Tris-HCl (pH 8.0), 5mM ethylenediaminetetraacetic acid (EDTA), 100 mM sucrose, and 0.1 mM 2-mercapthoethanol (Abdoli-Nasab *et al.*, 2013) and the concentration was assessed by Bradford's assays (Bradford, 1976). Dot-Blot (Stott, 1989) and indirect enzyme-linked immunosorbent assay (ELISA) (Wang and Gonsalves, 1990) were carried out to quantitative detection of the GFP protein in the inoculated tobacco plants.

Statistical analysis

All experiments were done according to a completely randomized design at five independent samples. Data analyses were performed using *Microsoft Excel* program software and *SPSS version 22*. When significant differences were found least significant difference (LSD) test at $P<0.05$ was applied to separate means.

3. Results and Discussion

The main benefits of the plant made proteins (PMPs) are lower costs and potential to produce a very large scale of recombinant proteins. Viral vectors have the ability to express transgenes in hosts and they are suitable and rapid platform for production high-level of recombinant proteins.

In this research, we utilized a *TuMV* viral vector (Fig. 1) under the control of the CaMV 35S promoter for transient expression of the *GFP* in tobacco plants. Although systemic symptoms of *TuMV* were not observed on infected plants, *GFP* was detected by the fluorescence microscopy (Fig. 2) twelve days post-inoculation.

This research has displayed for the first time that recombinant protein (GFP) can accumulate in tobacco plants via *TuMV* based viral vector with CaMV 35S promoter. *TuMV* can infect tens different plant species (Chen *et al.*, 2007) (to compare common viral vectors which can affected specific plant species), therefore, *TuMV* based viral vector can be economical.

In this study, two different tobacco species, *N. benthamiana* and *N. tabacum* (two different cultivars Xanthi and Samsun) were investigated. Fluorescence

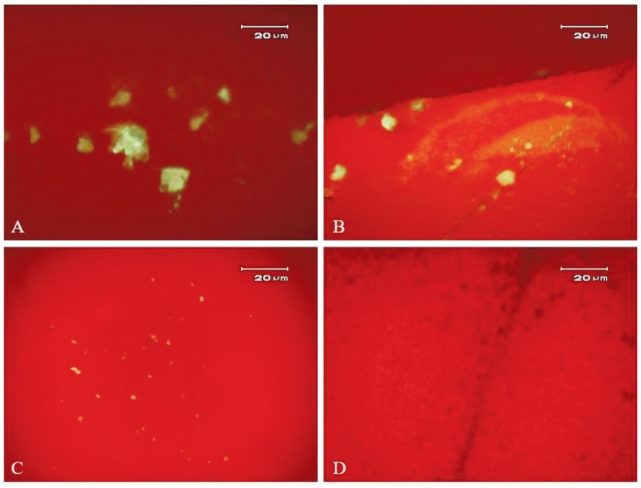

Fig. 2 - Fluorescence microscopy analysis of GFP expression in tobacco plant's leaves which infected by pTuMV-GFP. (A) *Nicotiana benthamiana*, (B) *N. tabacum* cv. Xanthi, (C) *N. tabacum* cv. Samsun (D) Negative control (tobacco plant infected with wild-type TuMV). Green color indicated GFP expression and the red indicated chlorophyll autofluorescence.

microscopy analysis of *GFP* (Fig. 2), RT-PCR (Fig. 3), Dot-Blot analyses (Fig. 4) and ELISA assay (Fig. 5) indicated that recombinant protein expression in tobacco plants leaves occrued. RT-PCR (Fig. 3) showed that, as expected, 160 bp bands were found in infected plants, while not observed in the negative control (wild type). It shows that the *TuMV* virus can infect the plant and replicate its genome. Viruses (like *TuMV* from Potyviridae family) have developed proteins such as Helper Component Proteinase (HCPro),

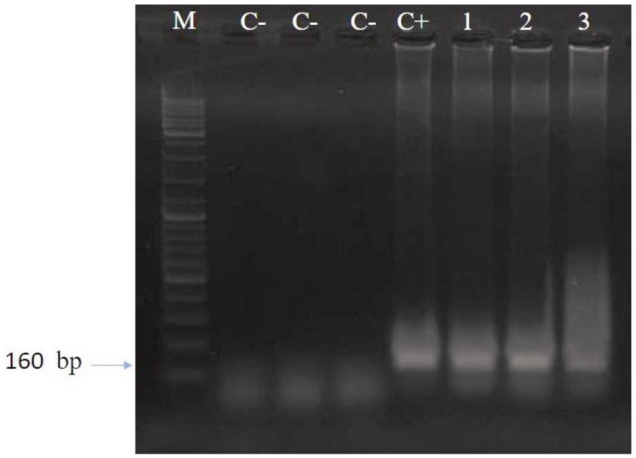

Fig. 3 - RT-PCR amplified a 160 bp fragment from the GFP with specific primers in 1% agarose gel. C- 1= negative control (water template), C- 2= negative control (RNA template), C- 3= negative control (Wild type (non-inoculated plant)), C+= positive control, Lane 1= *Nicotiana benthamiana* transformed plants, Lane 2= *N. tabacum* cv. Xanthi, Lane 3= *N. tabacum* cv. Samsun transformed plants, M= molecular weight marker (1 kb standard GeneRuler).

which suppress the plants silencing defense (Voinnet, 2001). Furthermore, HCPro has protease activity and it is necessary for virus genome replication and viral movement and transmission (Klein *et al.*, 1994; Chiera *et al.*, 2008).

Dot-Blot assay (Fig. 4) indicated that GFP protein was recognized by specific antibody and developed brown color in transformed plants and positive control. ELISA assay indicated that expression levels of GFP was estimated approximately ≤0.5% of total soluble protein (TSP) of fresh weight of tobacco leaves. These results show lower accumulation of recombinant proteins compared with a number of previous studies which expressed by viral vectors such as Artichoke mottled crinckle virus (Lombardi *et al.*, 2009), Beet curly top virus (Kim *et al.*, 2012) etc. Some strategies, such as codon-optimization (Love *et al.*, 2012) and the use of improved viral vector elements including strong viral promoters (Gleba *et al.*, 2007) can increase the expression of recombinant protein.

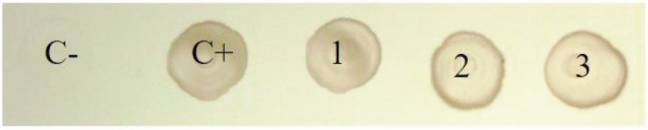

Fig. 4 - Dot-Blot analysis of GFP transient expression in *Nicotiana benthamiana* (spot 1), *N. tabacum* cv. Xanthi (spot 2) and *N. tabacum* cv. Samsun (spot 3), C-= negative control [Wild type (non-transformed plant)] and C+= Purified GFP protein.

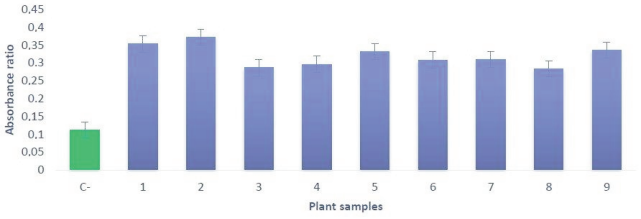

Fig. 5 - Analysis of GFP expression in tobacco plants by ELISA assay. C-= negative control (Wild type TuMV-infected plant), 1-3= *Nicotiana benthamiana* transformed plants, Lane 4-9= *N. tabacum* transformed plants. The data represent means ± SE from five independent infected samples.

It seems that, *N. benthamiana* lacks RNA-dependent RNA polymerases (RdRPs) which required for defense against viruses, therefore *N. benthamiana* infected plant displays more strong symptoms and its products more than do other tobacco species (Yang *et al.*, 2004).

In many previous studies (Kumar and Kirti, 2010; Sasaki *et al.*, 2015; Vojta *et al.*, 2015; Park *et al.*, 2016), *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) delivery systems has been used to express the transient expression of recombinant protein using a viral vector. However, in this study, a direct virus inoculation system via dusted with carborundum has been used. Our study indicated that this method is useful to accelerate the production of recombinant proteins in tobacco plants.

4. Conclusions

In conclusion, our results showed that *TuMV*, as a virus that could infect a wide range of plant species, could be used to produce recombinant proteins in tobacco. In this investigation, all inoculated plants expressed GFP protein. Results showed that incubated *N. benthamiana* has more accumulated recombinant protein compared to the two *N. tabacum* cultivars. Although the level of expression is low and should be optimized for future studies.

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