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Research Article

Screening of *Cucurbitaceae* Family to Select Efficient Host Species for Transient Expression

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ABSTRACT

The use of plant viral vectors for the transient expression of heterologous proteins offer a useful tool for the large-scale production of importance proteins, such as antibodies and vaccines. Virus vectors are beneficial because of the autonomous replication and high levels of gene expression in a short time. Selecting the most productive host, and expression system are the most important effective factors in production of recombinant proteins. The ZYMV virus specifically infects Cucurbitacea family. Therefore, in order to find the best host among different spices from Cucurbitacea family were studied.

In this study, the hypothesis was the choice of plant host affects the foreign gene retention level by a *Zuchiini yellow mosaic virus*(ZYMV) vector expressing the green fluorescent protein (GFP).To accomplish this, a novel virus vector integrity bioassay was developed based on an old concept, whereby full-length cDNA of the ZYMV vector were rub-inoculated onto leaves of four species of *Cucurbitacea* family including Cucurbit, Cucumber, Melon and Cantaloupe and control plants (infected by ZYMV wild type). Survey expression levels of GFP in all infected plants were measured by several molecular and biochemical-based approaches. For investigates the GFP expression using fluorescence microscopy, RT-PCR, SDS-PAGE, Immunobloting and ELISA assays confirmed *gfp* gene was correctly transcribed and translated in the infected plants.

This host dependent effect, suggests some plants may present a more suitable environment than the others to support or maintain optimal levels of virus vector-mediated foreign gene expression. Our results demonstrated, that the cucurbit spices had the highest expression level of GFP and it is possible to use ZYMV-based viral vectors for molecular farming and producing recombinant proteins in *Cucurbitacea* family.

Keywords: Molecular farming; Recombinant protein; Transient expression. ZYMV-GFP vector

INTRODUCTION

Plants can be used as bioreactors for production of biomedical materials. Plants have many advantages over other expression systems such as the low cost of protein production and high safe[1].In the last decade, the use of plant viruses as gene vectors for the transient expression of heterologous proteins offer a useful tool for the large-scale production of

special proteins with pharmaceutical values in plant cells [2].Transient expression of foreign genes in plants through viral-vectors is considered to be an alternative or supplemental approach to stable transformation of plants because of the difficulty to transform some plant species. Several plant viruses were developed as transient expression vectors[3], heterologous genes and green fluorescent protein (GFP) as marker protein[3]. Zuchiini Yellow Mosaic Virus (ZYMV) causes one of the most devastating diseases in cucurbits including melon (*Cucumismelo* L.), cucumber (*Cucumissativus* L.), watermelon (*Citrulluslanatus* Schad.), squash (*Cucurbita pepo* L.) and pumpkin (*Cucurbita maxima* L., *C. moschata*L.)[4].The infected plants develop a range of foliage symptoms including plant stunting, bearing deformed leaves, severe mosaic and blistering. ZYMV is a member of the genus *potyvirus* which the largest group of plant-infecting viruses and its genome consists ofa positive-sense ssRNA which incoded a polyprotein[5].

In recent years, ZYMV has been engineered as aforeign proteins expression vector in cucurbits plants. The target protein is synthesized as a part of the viral polyprotein and produced in an equal molar ratio as with all viral proteins. Recently, a ZYMV vector containing a *cauliflower mosaic virus* (CaMV) 35S promoter and the full-length cDNA of ZYMV TW-TN3, an isolate collected from southern Taiwan,was constructed [6].

Still the expression of the foreign gene is oftentimes not maintained stably in the viral genome due to recombination events that may be related to host environment and size of the insert [7]. Therefore, availability of a stable and efficient ZYMV-GFP vector(s) will greatly enhance our abilities to examine virus–vector interactions, virus movement, trafficking, and virus–host interactions in di-cotyledons. We further examined the stability of ZYMV-GFP vectors and the distribution of ZYMV in melon, cucumber, cucurbit and cantaloupe using a GFP-tagged virus.

MATERIAL AND METHODS

Virus source

A previously constructed infectious plant virus clone, p35SZYMVGFPhis3, that contains the full-length cDNA of an Taiwan isolate of ZYMV (TW-TN3), driven by the *Cauliflower Mosaic Virus*35S promoter and a reporter GFP gene between the P1 and the HC-Pro genes [8], was engineered as an *in vivo* viral vector (Fig. 1).

Plant inoculation

Four species of *Cucurbitacea* family including cucurbit (three accessions), cucumber (five accessions and one landrace), melon (three landrace) and cantaloupe (four landrace) plants and three control plants (infected plant by ZYMV wild type) were used for whole plant expression analysis (Table 1). Plant seeds were cultured at 25°C in the greenhouse (25°C, 17-h photoperiod) for approximately 2 months. For viral infections, 10µl (1µg/µl) of either p35SZYMVGFPhis3 or wild-type ZYMV (as control) mechanically were rubbed with a cotton stick on leaves of local lesion host plants previously dusted with 600 mesh carborundum. Leaves were harvested 4, 8, 12, 14, 16, 20, 24 and 34 days post inoculation (dpi) and used for the GFP expression analysis.

Fluorescence microscopy

Leaves of ZYMV infected plants were observed under an Olympus fluorescent microscope (version IX71, Tokyo, Japan). Digital micrographs were taken using a mounted high-resolution Olympus DP70 DP71 digital camera at 4, 8, 12, 14, 16, 20 and 24 days post inoculation (dpi).

RT-PCR

Amount 0.2 g of young leaves of p35SZYMVGFPhis3 or wild-type ZYMV inoculated plants were used for the extraction of RNA. The leaves were ground to a fine powder with a mortar and pestle and thentotal RNA was extracted using Hybrid-RTM kit (GeneAll Biotechnology, Korea) following the manufacturer's instructions. In the next step, cDNA was synthesized by a First Strand cDNA Synthesis Kit (Fermentas). RT-PCR was done on cDNA by specific primers located in the

GFP sequence (5'-ACGACGGC AACTACAAGACC-3' as Forward primer and 5'-TTGTACTCCAGCTTGTGCCC-3' reverse primers).

Protein extraction and SDS-PAGE

Total proteins were isolated from 14 dpi Cucurbit, cucumber, melon and cantaloupe leaves by freezing 0.2 g leaf tissue in liquid nitrogen and macerating into a fine powder, and adding 1 ml of protein extraction buffer [50 mMTris-HCl, pH 7.5, 0.4% (v/v) 2-mercaptoethanol, 2 mM ethylene diamine tetraacetic acid (EDTA) pH 7.5 and 100 mM sucrose].

Extracts were centrifuged at 15,000g for 20 min at 4 °C and the supernatant was taken to remove excess plant material. Then, the total soluble protein (TSP) concentration was quantified using the Bradford assay. SDS-PAGE of proteins was performed using 12 % acryl amide gel followed by staining with Coomassie brilliant blue.

Western immunoblot analysis

For detection of the GFP protein expression patterns between infected plant (Cucurbit) with control plant, total soluble proteins (100 µg) were separated by 12 % SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane in a Bio-Rad semidry blotting system. The membrane was blocked by phosphate-buffered saline (PBS) 0.05% Tween 20 and 5% skim milk for overnight, and then incubation was performed with the mouse anti-GFP primary antibody (Biolegend, USA; diluted 1:2000 in PBST)for 1.5 h at 25°C. After washing three times, the blot was probed with the goat anti-mouse IgG antibody conjugated to peroxidase (Sigma; 1:3000 in PBST) for 1 h at 37°Cand was visualized by the DAB-HRP system. Color development reagent DAB (diaminobenzidine tetrahydrochloride) and H_2O_2 were used for detection.

Dot Blot Enzyme Immunoassay

To use dot blot technique for detection of GFP protein production in infected plant extracts, first, 20 µg of absolute extracted proteins from leaves of infected plants were placed on nitrocellulose paper. The membrane was blocked and then probed by the same method and antibodies previously mentioned for the western blot assay.

Enzyme-Linked Immunosorbent Assay (ELISA)

For this assay, TSPs were diluted with a carbonate/bicarbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6), and loaded into a 96-well micro plate in three replicates. After incubation overnight at 4 °C, the plate was cleaned three times using washing buffer (PBS with 0.05 % Tween 20). Consecutively, the remaining absorption sites were blocked by adding 100 µl of blocking buffer (1 % BSA in PBS/0.05 % Tween 20) to each well and incubated for 1 h at 37 °C.

Afterwards, the plate was washed and 100 µl of the mouse anti-GFP primary antibody was diluted with blocking buffer (0.1 % BSA in PBS-T) for 2 h at 37 ºC. The plate was washed as described above, and secondary goat anti-mouse IgG antibody diluted to 1:3000 with blocking buffer (0.1 % BSA in PBS-T) was added for 1 h at 37 ºC. Plates were then incubated for 30 min at room temperature with substrate solution [1 % TMB (tetramethylbenzidine), 200 mM citrate buffer, 0.01 % H2O2, pH 5.5] and the reaction was stopped with 1 M H_2SO_4. Absorbance was determined at 450 nm by a microplate reader (BioTek, USA).

Results

ZYMV-GFP inoculation on different hosts

Chlorotic local lesions similar to those induced by wild type ZYMV were developed on inoculated leaves of cucurbit, cucumber, melon and cantaloupe 7–9 days after inoculation with the chimeric constructs (Fig. 2). Systemic symptoms of yellow mosaic were noticed on all plants, as readily detected by the GFP under fluorescence microscopy (Fig. 3). Cucurbit and cantaloupe were sensitive to ZYMV-GFP vector rather than cucumber and melon. Cucumber and melon were somewhat more recalcitrant to inoculation to achieve acceptable levels of infection (Fig. 3a and 3b).The high-

density green fluorescence was observed on all plants at 14 days post inoculation (dpi) (data not shown) and disappeared after 35 days post inoculation.

RT-PCR

After RNA extraction and quality check on 1% Agarose gel, the RT-PCR results showed a 127bp product that confirmed GFP gene expression at the RNA level in the infected plants with ZYMV-GFP vector, whereas no product was seen for negative controls (Fig. 4).

SDS-PAGE

SDS-PAGE analysis of the extracted proteins showed a 26 kDa band in all plants (Fig. 5). It was observed a difference in the thickness of the 26 kDa protein band between infected and control plants. Further analysis was done for accurate detection of GFP protein.

Western immunoblotting

Western blot analysis was performed on infected plants for detection of GFP protein production. Western blot analysis confirmed protein expression and a very faint band in the expected size (26 kDa) detected in infected plants (Fig. 6). This showed that GFP transcripts were translated to GFP polypeptide successfully. However, no band was detected in the infected plant with wild-type ZYMV (Fig. 6).

Dot Blot Enzyme Immunoassay

In this experiment, the presence or absence of GFP protein among all the extracted protein in infected plant leaves was investigated with its specific antibody using dot blot analysis. After adding the substrate, the color variation was observed from colorless to brown only in extracts containing the GFP protein. No change was seen in control plants (Fig.7).

ELISA

The absorbance of extracted protein from inoculated and control plants (three replications) was measured at wavelength 450 nm, and the absorbance values of GFP in different inoculated plants were compared with the control plant. The results showed that GFP protein was produced in all infected plants. Accordingly, the results showed a significant difference between the absorbance means of the infected and the control plants (p=o.o5). The rate of absorbance in infected plants was 4.6 times more than in control plants (Fig. 8).

DISCUSSION

The investigation was designed to produce GFP protein, subsequently introduce it into Cucurbitaceae plants through p35SZYMVGFPhis3 vector-mediated transformation system, and finally survey expression levels of GFP in all infected plants by means of several molecular and biochemical-based approaches. In this context, following RT-PCR was also utilized and could successfully verify the presence of the GFP-specific transcription. Hence, as complementary assays, ELISA was employed, which, overall, confirmed the accurate expression function of the GFP.

The results of this study indicated that *Cucurbitaceae* plants can be used as a competent system for producing pharmaceutical recombinant proteins. This strategy can solve some plant expression system drawbacks like tissue culture problems.

Based on our results, GFP protein can be produced using ZYMV vector within 2 months without problems commonly raised by working on infected plants. Therefore, the use of plants can reduce the cost of production compared to other production systems like microbial fermentation systems.

In comparison to bacterial expression system, using viral vector for the raputics protein expression in plant is an efficient system which minimize the risk for contamination with endotoxins and maximizes potential for large-scale manufacturing of biopharmaceuticals to meet the global demand [9]. Also, this system does not have prokaryotic limitation including plasmid instability and the lack of capability for post-translational modifications such as the proper formation of disulfide bonds and glycosylation of the designated sites, often leading to misfolded proteins [10].

The most successful expression systems today for high accumulation are using viral vectors [11] which our results confirmed it. Moreover,

in comparison to transgenic plants, the major advantages of a transient expression approach are time efficiency, high levels of target protein expression, uniformity and consistency of target accumulation, fewer environmental concerns due to contained facility production [12].

In summary, in the present work, we have demonstrated the feasibility of using attenuated ZYMV as an expression vector in cucurbits. We now have a tool to produce recombinant proteins at high levels making the future very promising for the role of plant viral vectors as a vehicle for industrial and pharmaceutical protein production.

This vector may also be useful as a tool for in planta genomic studies, as it can express a gene with effective protein expression.

REFERENCE

1. COMMANDEUR, C, THORIMBERT, S, AND MALACRIA, M, 2003.CHEMO-AND STEREOSELECTIVE PALLADIUM-CATALYZED ALLYLIC ALKYLATIONS CONTROLLED BY SILICON. THE JOURNAL OF ORGANIC CHEMISTRY. 68(14): P. 5588-5592.
2. FISCHER, R, ET AL., 2012.GMP ISSUES FOR RECOMBINANT PLANT-DERIVED PHARMACEUTICAL PROTEINS. BIOTECHNOLOGY ADVANCES. 30(2): P. 434-439.
3. FOLIMONOV, AS, ET AL., 2007.A STABLE RNA VIRUS-BASED VECTOR FOR CITRUS TREES. VIROLOGY. 368(1): P. 205-216.
4. DESBIEZ, C, ET AL., 1997.CHARACTERIZATION OF EPITOPES ON ZUCCHINI YELLOW MOSAIC POTYVIRUS COAT PROTEIN PERMITS STUDIES ON THE INTERACTIONS BETWEEN STRAINS. JOURNAL OF GENERAL VIROLOGY. 78(8): P. 2073-2076.
5. REVERS, F, ET AL., 1999.NUCLEOTIDE SEQUENCE OF THE 3′ TERMINAL REGION OF THE GENOME OF FOUR LETTUCE MOSAIC VIRUS ISOLATES FROM GREECE AND YEMEN. ARCHIVES OF VIROLOGY. 144(8): P. 1619-1626.
6. LIN, S-S, HOU, RF, AND YEH, S-D, 2001.COMPLETE GENOME SEQUENCE AND GENETIC ORGANIZATION OF A TAIWAN ISOLATE OF ZUCCHINI YELLOW MOSAIC VIRUS. BOTANICAL BULLETIN OF ACADEMIA SINICA. 42.
7. KELLONIEMI, J, MÄKINEN, K, AND VALKONEN, JP, 2008.THREE HETEROLOGOUS PROTEINS SIMULTANEOUSLY EXPRESSED FROM A CHIMERIC POTYVIRUS: INFECTIVITY, STABILITY AND THE CORRELATION OF GENOME AND VIRION LENGTHS. VIRUS RESEARCH. 135(2): P. 282-291.
8. HSU, C-H, ET AL., 2004.ORAL ADMINISTRATION OF A MITE ALLERGEN EXPRESSED BY ZUCCHINI YELLOW MOSAIC VIRUS IN CUCURBIT SPECIES DOWNREGULATES ALLERGEN-INDUCED AIRWAY INFLAMMATION AND IGE SYNTHESIS. JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY. 113(6): P. 1079-1085.
9. YEVTUSHENKO, D AND MISRA, S. *TRANSGENIC EXPRESSION OF ANTIMICROBIAL PEPTIDES IN PLANTS: STRATEGIES FOR ENHANCED DISEASE RESISTANCE, IMPROVED PRODUCTIVITY, AND PRODUCTION OF THERAPEUTICS.* IN *ACS SYMPOSIUM SERIES*. 2012.
10. BAGHERI, K, ET AL., 2013.EXPRESSION OF HUMAN INTERFERON GAMMA IN BRASSICA NAPUS SEEDS. AFRICAN JOURNAL OF BIOTECHNOLOGY. 9(32): P. 5066-5072.
11. EGELKROUT, E, RAJAN, V, AND HOWARD, JA, 2012.OVERPRODUCTION OF RECOMBINANT PROTEINS IN PLANTS. PLANT SCIENCE. 184: P. 83-101.
12. METT, V, ET AL., 2008.PLANTS AS BIOFACTORIES. BIOLOGICALS. 36(6): P. 354-358.

Figure and Tables (Continue):

Table 1. ID number, Species, Genotype and Site of collection of Plants tested for ZYMV infection and observation of green fluorescent foci.

ID No	Species	Genotype	Site of collection
1	*Cucumissativus*	n.94.197	Iran, Karaj
2		n.94.201	Iran, Karaj
3		n.94.215	Iran, Karaj
4		n.94.226	Iran, Karaj
5		n.94.264	Iran, Karaj
6		Dastgerd	Iran, Esfehan
7	*Cucurbita pepo*	n.95.585	Iran, Karaj
8		n.95.594	Iran, Karaj
9		n.95.569	Iran, Karaj
10	*Cucumismelo var. inodorus*	Ivanaki	Iran, Garmsar
11		Sarakhs	Iran, Sarakhs
12		Soghan	Iran, Orzoye
13	*Cucumismelo var. cantalupensis*	Korki	Iran, Khorasan
14		Habib Abadi	Iran, Habib Abad
15		Shah Abadi	Iran, Shah Abad
16		Semsori	Iran, Karaj

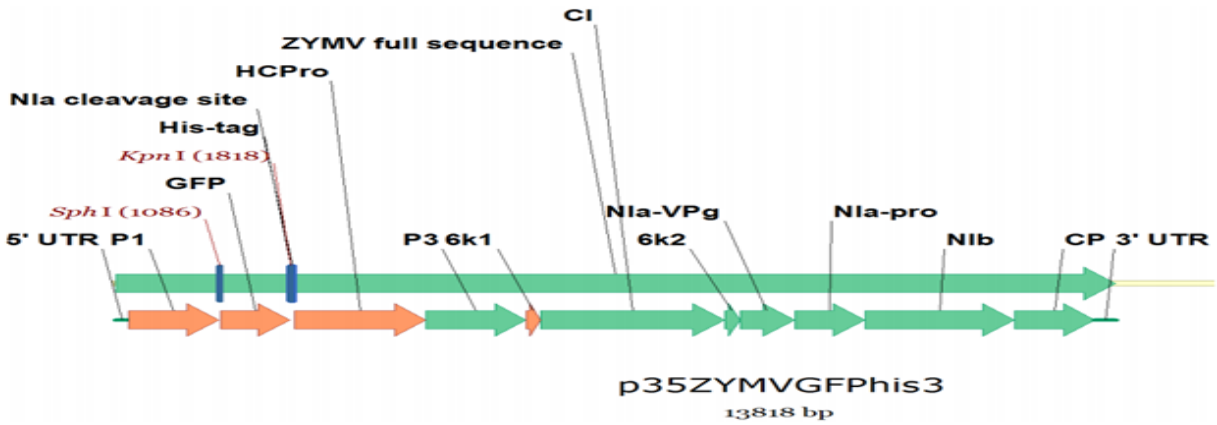

Fig1. Construction of the p35SZYMVGFPhis3 vector. Schematic represents the viral polyprotein under the 35S promoter which contains GFP sequence. The foreign gene insertion site in genomic ZYMV map is between P1 and HC-Pro provided by *Sph*I and *Kpn*I restriction endonuclease enzymes. The recombinant protein is followed by a Histidine-tag and released with NIa protease activity.

Fig 2a

Fig 2b

Fig 2. The local lesions of ZYMV-GFP on cucumber (a) and cucurbit (b).

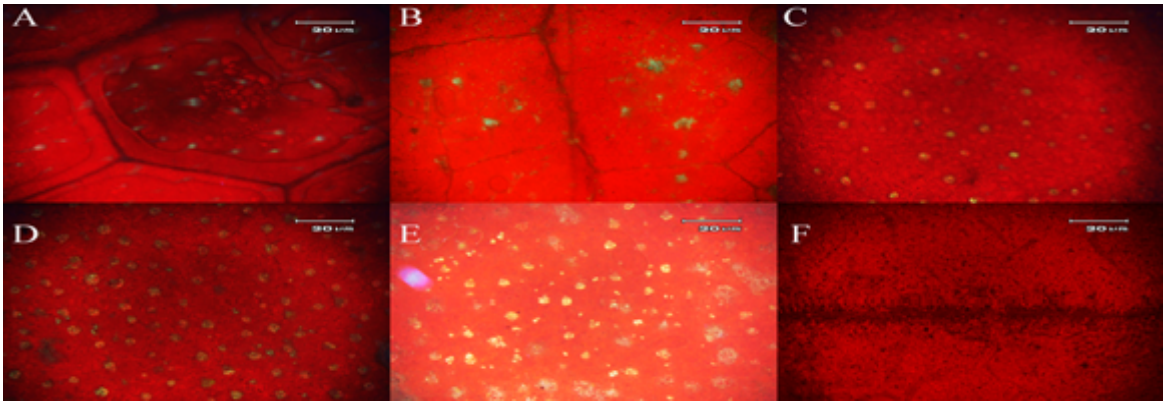

Fig 3. Fluorescence microscopy analysis of GFP expression in leaves that infected by a ZYMV-GFP vector. GFP was detected in (A) cucumber, (B) melon, (C and D) cucurbit, (E) cantaloupe leaves inoculated with p35SZYMVGFPhis3 vector. Control plant (F) was infected with wild-Type ZYMV which did not show any green fluorescence. The red color indicated autofluorescence of chlorophyll.

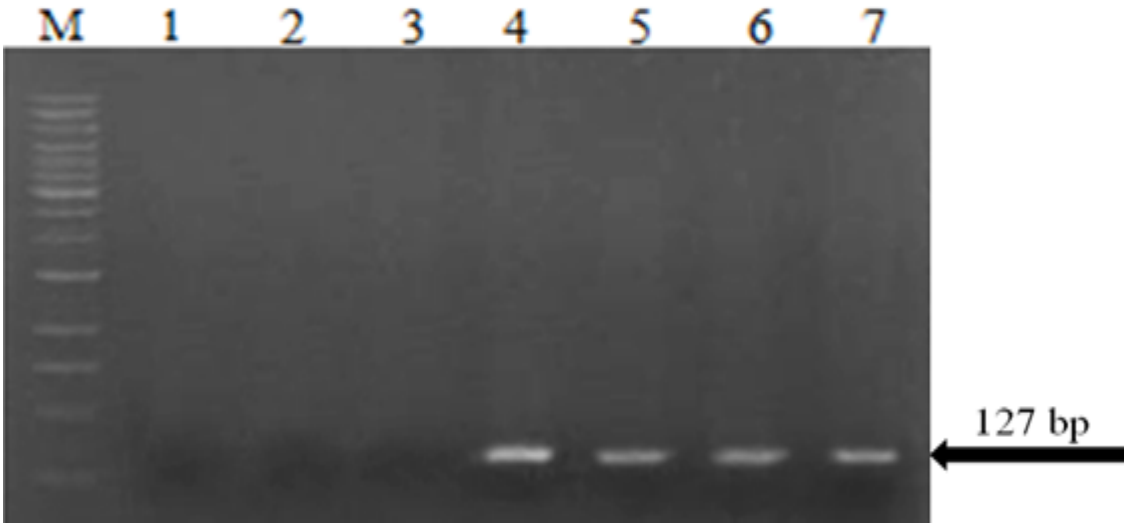

Fig 4. RT-PCR amplified a 127 bp fragment by GFP specific primers. M 100 bp molecular ladder (Fermentas), 1: water negative control, 2: RNA negative control, 3: wild-type TuMV-infected plant as negative control, 4: p35SZYMVGFPhis3 vector as positive control, 5-7 different p35SZYMVGFPhis3 inoculated plants

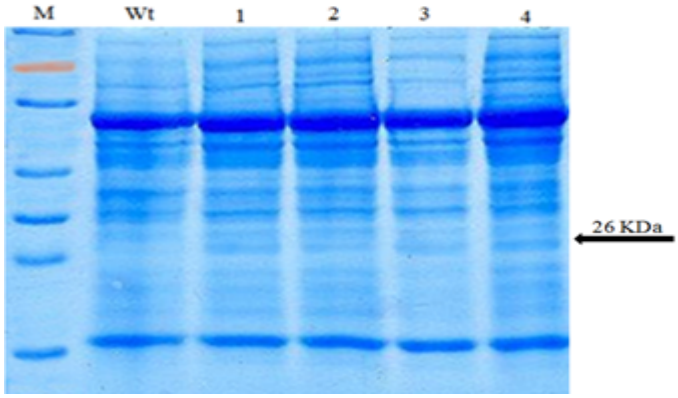

Figure 5. SDS-PAGE analysis of infected and control plants shows the 26 kDa band in all plants. M, molecular ladder (Fermentas); wt, control plant; 1–4 infected plants.

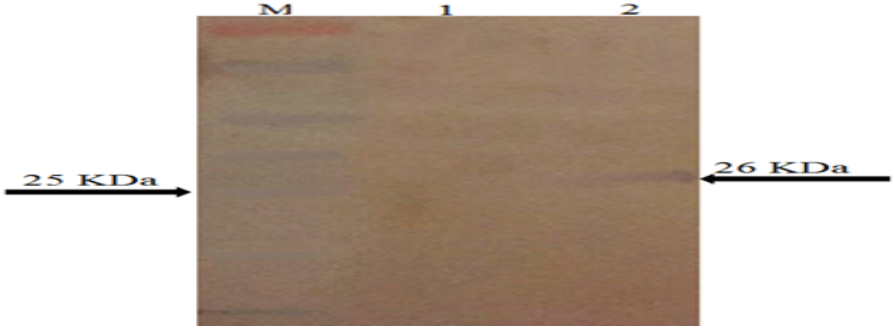

Figure 6. Western blot analysis on infected plants contain GFP gene. M: molecular ladder (Fermentas), 1: infected plant with wild-type ZYMV, 2: infected cucurbit plants with ZYMV-GFP vector.

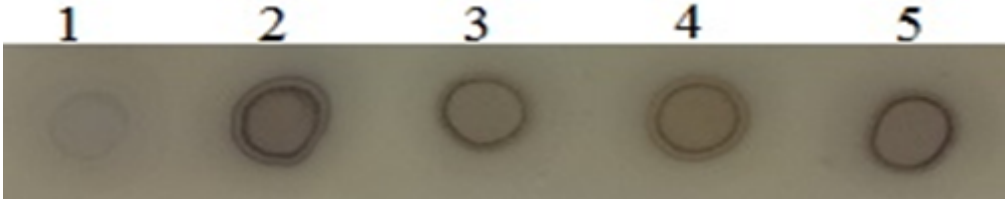

Figure 7. Dot-blot analysis of infected and control plants. 1: negative control, 2: infected cucurbit plant, 3: infected cucumber plant, 4: infected cantaloupe plant and 5: infected melon plant.

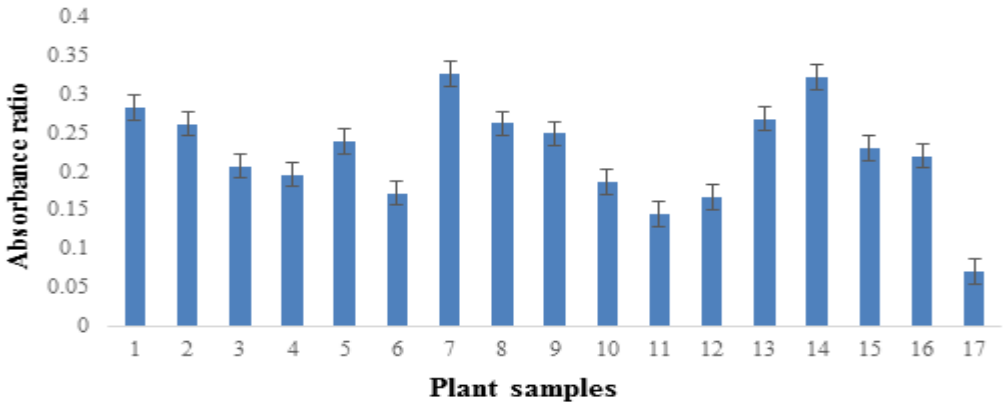

Figure 8. ELISA assay for infected plants using mouse anti-GFP primary antibody. Values correspond to means of three replicates along with standard deviations. 1-16: different p35SZYMVGFPhis3 inoculated plants, 17: wild-type ZYMV-infected plant as negative control.