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Cucurbit yellow stunting disorder virus and *Watermelon chlorotic* stunt virus induced gene silencing in tobacco plants

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***Cucurbit yellow stunting disorder virus* (CYSDV) and *Watermelon chlorotic stunt virus* (WmCSV) are the most widespread and damaging viruses to cucurbits in the Middle East. CYSDV and WmCSV are cucurbit-infecting bipartite whitefly-transmitted viruses. Post-transcriptional gene silencing (PTGS) is a universal mechanism by which plants are able to systemically switch off the expression of targeted genes via the reduction of steady-state levels of specific RNAs. PTGS was used in this study to control the two viruses. In this study, the efficiency of the dsRNA for the ability to trigger resistance against the CYSDV and WmCSV was investigated. Three regions of three genes of CYSDV genome were selected; the coat protein gene (CP), heat shock gene (Hsp70) and ORF3, while the two regions of two genes of WmCSV genome were selected; CP gene and rep gene. Bioassay, dot-blot hybridization and polymerase chain reaction (PCR) methods were capable to evaluate the resistance against viruses. Clear symptoms on tobacco plants took two to three weeks to appear and all non-infiltrating tobacco plants (positive control) showed viral symptoms after inoculation. Most of the agro-infiltrating sense/antisense constructs did not yield symptoms of the viruses. Dot-blot hybridization, showed that negative hybridization was obtained with infiltrating tobacco plants with prepared constructs compared to those non-infiltrating tobacco plants used as the control. Only one out of five gave positive signals with the construct pasCYSDV-Hsp70. Using PCR, positive reactions of the expected size of 500 bp fragment with WmCSV and 800 bp with CYSDV were obtained with the infiltrating tobacco plants with sense constructs, which pointed out the existence of viral genome in challenging tobacco plants. Infiltrating tobacco plants with sense/antisense constructs gave negative PCR pointed out the lack of the viral genome.**

Key words: Cucurbit yellow stunting disorder virus (CYSDV), watermelon chlorotic stunt virus (WmCSV), Post-transcriptional gene silencing (PTGS), coat protein (CP), Hsp70, ORF3, Rep, dot-blot, hybridization.

INTRODUCTION

Cucurbit crops are widely grown in the Middle East and consumed in large quantities in the traditional diet. The warm climate permits the nearly year-round development of insect vectors such as the whiteflies, which are the main vector for the viruses that infect cucurbits. The most widespread and damaging viruses are members of the

genus Begomovirus (*Geminiviridae*) and Crinivirus (*Closteroviridae*). The most important time of the year for whitefly infestation of cucurbit crops in Saudi Arabia is the autumn (Raja Al-Zahrani, 2010). During 2001, two new viral diseases were detected in cucurbits: watermelon (*Citrullus lanatus*), squash (*Cucurbita pepo*) and cucumber (*Cucumis sativus* L.*)* in the Middle East region. Sequence analysis revealed that the diseases were caused by the native *Watermelon chlorotic stunt virus* and *Squash leaf curl virus* (Abdel-Salam et al., 2006; Abudy et al., 2010; Ali-Shtayeh et al., 2014; Idris et al., 2006). WmCSV was first identified in Yemen (Jones et al., 1988) and after that, in the following years, it was reported through the Middle East and also in North Africa as the following: Sudan (Kheyr-Pour et al., 2000), Jordan (Al-Musa et al., 2011), Oman (Khan et al., 2012) and Palestine (Ali-Shtayeh et al., 2014). The symptoms caused by WmCSV are chlorotic mottling, vein yellowing, stunting and severe decrease of yield, generally in watermelon (*Citrullus* species) crops. WmCSV host range covers the most cucurbits including squash, cucumber, melons, and pumpkin.

The first detection of CYSDV in the Middle East was reported in the United Arab Emirates in 1982 (Hassan and Duffus, 1991). In subsequent years, it was identified throughout the Mediterranean region, in Europe, and in North America (Abou-Jawdah et al., 2000; Brown et al., 2007; Kuo et al., 2007; Papayiannis et al., 2009; Sweiss et al., 2007). CYSDV is a bunchy crinivirus, which has had a main negative impact on the production of cucumbers in several regions of the world, a phenomenon increased by the absence of effective preventive arrangements against the whitefly (Owen et al., 2016). WmCSV and CYSDV infect all cultivated cucurbits, some legumes (alfalfa, bean), and malvaceous species. CYSDV causes severe symptoms in cucurbit plants, green spots on the oldest leaves, severe interveinal chlorosis, and spot appears between 14 and 22 days' post-inculcation and severe symptoms including leaf rolling, complete leaf lamina yellowing, brittleness, severe stunting of young leaves and a drastic reduction of yield (Célix et al., 1996).

One of the primary aspects of virus-plant host interactions is derived from plant natural defenses through RNA silencing (Ding and Voinnet, 2007; Ruiz-Ferrer and Voinnet, 2009). Through infection, plants process viral double-stranded RNAs (dsRNAs) into small interfering RNAs (siRNAs) and use the siRNAs to guide specific antiviral silencing activities (Li et al., 2015). siRNAs guide members of the Argonaute protein family to specific mRNAs for cleavage, a process referred to as post-transcriptional gene silencing (PTGS), which is a universal defense mechanism against RNA viruses and transcripts produced by DNA viruses such as geminiviruses (Li et al., 2015). The lack of natural sources of resistance to WmCSV and CYSDV and the need to reduce pesticide application motivates us to develop multiple virus resistance through the development of transgenic cucurbit plants. This study was conducted to improve the socioeconomic status of the Saudi Arabia farmers and the local economy by the accurate diagnosis of cucurbit virus diseases and development of transgenic plant resistant to those viruses' adapted to local conditions. In the present study, infiltrating tobacco plants with sense/antisense constructs gave negative PCR which pointed out the lack of the viral genome while the infiltrating tobacco plants with sense constructs pointed out the existence of viral genome in challenging tobacco plants.

MATERIALS AND METHODS

The present work was conducted at Pest and Plant Diseases Unit (PPDU), College of Agriculture and Food Sciences, King Faisal University, Saudi Arabia.

Extraction of total nucleic acids from cucurbit tissues

The total RNA (for CYSDV) was extracted from infected cucurbit plants using the RNeasy® Plant Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer w`hile the total DNA (for WmCSV) was extracted from infected cucurbit plants using the DNeasy® Plant Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer.

Design and synthesis of oligonucleotide primers

Specific primers were designed for parts (selected fragments) of the coat protein gene (CP) of CYSDV (176 bp), heat shock gene (Hsp70) of CYSDV (232 bp), ORF3 of CYSDV (198 bp), CP gene of WmCSV (160 bp) and rep gene of WmCSV (230 bp) (Table 1) according to the sequences obtained from GenBank. The sense primers are CYcpf, Hsp702f, CYorf3f, Wmcp1 and Wmrep1 with *Asc*I and *Xba*I sites (underlined), respectively at the 5` end (Table 1). The antisense primers are CYcpr, Hsp702r, CYorf3r, Wmcp2 and Wmrep2 with *Swa*I and *Bam*HI sites (underlined) respectively, at the 5` end (Table 1). The restriction sites were inserted at the 5` end of the prepared primers to ease the cloning of the selected fragments into the binary vector.

One-step reverse transcriptase-polymerase chain reaction (RT-PCR) for the selected fragments of CYSDV

Platinum Quantitative RT-PCR Thermo Script One Step System experiment. The RT reaction started with incubation for 30 min at 50°C, then denaturation for 5 min at 95°C. Thirty-five cycles of PCR started with denaturation for 1 min at 95°C, primer annealing for 1 min at 55°C, and extension for 1 min at 72°C with a final extension

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for 7 min at 72°C.

PCR for the selected fragments of WmCSV

PCR cycle parameters were as follows: 2 min at 94°C, after that, 35 cycles of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C, then the final extension for 10 min at 72°C.

Cloning and sequencing of the amplified fragments of CYSDV and WmCSV

PCR products of CYSDV and WmCSV pGEM®T-Easy vector (Promega, USA) were ligated together. The insertion of recombinant plasmids in strain DH5α of *E. coli* occurred according to the instructions of the manufacturer. The Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) was applied for the isolation of DNA from different white colonies. Then the digestion of DNA was done using *Eco*RI and electrophoresed on agarose gels. The orientation of these inserts in the plasmid was determined using two different primers for sequencing, the first one was T7 forward primer in the promoter region (5' TAATACGACTCACTATAGGG '3) and the second was SP6 reverse in promoter region (5'ATTTAGGTGACACTATAG '3). The clones having inserts of accurate sizes were selected for sequencing using an automated instrument for DNA sequencing (ABI 377XL). Sequencing Analysis Software, ABI™ version 3.0 was used to analyze the data. All sequencing was carried out in Macrogen Company, Seoul, South Korea.

Sub-cloning of the selected fragments into a binary vector

The selected fragments for gene silencing (CYSDV-CP, CYSDV-Hsp70, CYSDV-ORF3, WmCSV-CP and WmCSV-rep*)* were re-amplified from the clones into pGEM-Teasy and sub-cloned into the binary vector pFGC5941 (kindly provided from the University of Arizona, USA) in both orientations once to give the sense orientation by restriction enzymes *Asc*I and *Swa*I and another one to give the antisense orientation with *Xba*I and *Bam*HI departing the intron *Chalcon Synthase* (*ChaS*) in the middle (Rezk et al., 2006; Soliman et al., 2008).

Preparation of sense, antisense and sense/antisense constructs

The sense constructs were prepared through the digestion of the DNA plasmids (pCYSDV-CP, pCYSDV-Hsp70, pCYSDV-ORF3, pWmCSV-CP and pWmCSV-rep) with *Asc*I (Thermo Fisher Scientific, USA) and *Swa*I (Thermo Fisher Scientific, USA) as the following: 50 U of *Swa*I and 10 µl of 10X buffer O were used to digest 50 µl of each DNA plasmid and dH_2O were added to reach 100 µl. The tubes were incubated at 30°C for 4 h and 50 U of *Asc*I and 10 µl of 10X buffer Tango™ were added, and incubated at 37°C for 4 h. The antisense constructs were prepared through the digestion of the plasmids with *Xba*I (Promega, USA) and *Bam*HI (Promega, USA) as the following: 50 U of each of *Xba*I and *Bam*HI, 5 µl of BSA (100 µg/µL), 10 µl of multicore buffer (PROMEGA) were used to digest 50 µl of DNA plasmids and dH_2O was added to reach 100 µl. The tubes were incubated at 37°C for 4 h. The sense/antisense constructs were prepared through the digestion of the plasmids with *Xba*I and *Bam*HI as described in antisense preparation and the tubes were incubated at 37°C in water bath for 4 h. The digested DNAs were electrophoresed in 2% agarose gel. A clean sharp scalpel was used to cut out the digested bands for gene cleaning. The restriction enzymes (the same enzymes) were used to digest the binary vector as described in each case of sense and antisense constructs. At the same time, the prepared sense constructs (psCYSDV-CP, psCYSDV-Hsp70, psCYSDV-ORF3, psWmCSV-CP and psWmCSV-rep) were digested in another reaction with *Xba*I and *BamH*I. The digested vector (100 ng) was added with 15 ng of the digested DNA of the selected fragment to a clean tube (both of them were digested with the same restriction enzymes). 1 U of T_4 DNA ligase, 1 µl of 10X ligase buffer and final volume of the 10 µl of the ligation mixture was obtained using nuclease-free water, and finally incubated overnight at 4°C.

Transformation of the constructs into *E. coli* competent cells

Competent cells *E. coli* (strain DH5α) were thawed on ice for 30 min and 100 µl was transferred to a pre-chilled 5 ml tube and 5 µl of ligated DNA was added and incubated on ice for another 30 min. Cells were heat-shocked for 2 min at 42°C by immersing the tube into a water bath for 2 min and then incubated on ice for 2 min. One milliliter of Luria-Bertani (LB) medium was added and cells grown for 1 h at 37°C in shaking water bath. Transformed cells were plated on the surface of prepared plates (LB solid medium with 50 µg/ml kanamycin) for overnight at 37°C and bacterial colonies containing recombinant plasmids were selected for further characterization. The enzyme digestion for the DNA minipreps of the selected colonies of constructs (sense, anti-sense, sense/anti-sense) were carried out using the restriction enzymes *Swa*I and *Asc*I (sense), *BamH*I and *Xba*I (anti-sense) and *Asc*I and *Xba*I (sense/anti-sense) as described earlier.

Nucleotide sequence analysis

Two primers were designed in the binary vector pFGC5941; one in the p35S promoter location (pFGC35SF, 5'AAGATGGACCCCCACCCACGAGG3') and the other in the OCS location (pFGC-OCSR, 5`AGGATCTGAGCTACACATGCTCAGG3`).

Transformation into *Agrobacterium tumefaciens* LBA4404

One microgram of plasmid DNA was added to the cells. Freezing the cells in liquid nitrogen was performed. The cells were thawed by incubating the tubes in a 37°C water bath for 5 min. 1 ml of YEP medium was added to the tube and incubated at 28°C for 2 to 4 h with gentle shaking. This period allowed the bacteria to express the antibiotic resistance genes. The tubes were centrifuged for 30 s in a table centrifuge. The supernatant solution was discarded and the cells were resuspended in 0.1 ml YEP medium. The cells were spread on a YEP agar plate containing 50 µg/ml kanamycin and 50 µg/ml streptomycin. The plate was incubated at 28°C. Transformed colonies should appear in 2 to 3 days.

Gene delivery using the syringe-spotting technique (SST)

After *A. tumefaciens* LBA4404 has been transformed with vectors harboring different constructs using chemical transformation, 5 ml culture was grown overnight at 28°C in the appropriate antibiotic selection medium. The next day, the culture was inoculated into a 50 ml LB medium containing the selective antibiotics. The culture was grown overnight in a 28°C shaker. *Agrobacterium* cells were harvested and resuspended in infiltration media [10 mM $MgCl_2$, 10 mM MES (2-N-morpholino ethane sulfonic acid), and 20 µM acetosyringone (4'-hydroxy-3,5-dimethoxyacetophenone)], adjusted

Table 1. Nucleotide sequences of the prepared primers to amplify the selected fragments of CYSDV and WmCSV.

Viruses'name	Primers' name	Nucleotide sequences (5`----------3`)	Restriction enzymes	Size (bp)
CYSDV	CYcpf	ATATTCTAGAGGCGCGCCACCAACACACTCATGCACGG	*Xba*I, *Asc*I	176
	CYcpr	ATATGGATCCATTTAAATATGTTCATGAGCCCTGGCG	*Bam*HI, *Swa*I	
	CYorf3f	ATATTCTAGA GGCGCGCCACTTGAAGTACAGGGGC	*Xba*I, *Asc*I	198
	CYorf3r	ATATGGATCCATTTAAATATGCCCTAGCCCTTAGAAGATCC	*Bam*HI, *Swa*I	
	Hsp702f	ATATTCTAGA GGCGCGCCAGGTGATTCGTTTTTGGGAGG	*Xba*I, *Asc*I	232
	Hsp702r	ATATGGATCCATTTAAATTCACATTATGAAGTTTGCCTG	*Bam*HI, *Swa*I	
WmCSV	Wmcp1	ATATTCTAGAGGCGCGCCAGATATTCTCATTTCCACTCCCG	*Xba*I, *Asc*I	160
	Wmcp2	ATATGGATCCATTTAAATCCTGTACATCCTCGGCTTCC	*Bam*HI, *Swa*I	
	Wmrep1	ATATTCTAGAGGCGCGCCAAGTGCTTTAGATAGTGCGG	*Xba*I, *Asc*I	230
	Wmrep2	ATATGGATCCATTTAAATACCGCCTGAGCTCGTTGATTGGG	*Bam*HI, *Swa*I	

to OD_{600} of 2.0 and left at room temperature for 3 h. The prepared constructs (pasCYSDV-CP, pasCYSDV-Hsp70, pasCYSDV-ORF3, pasWmCSV-CP, and pasWmCSV-rep) in sense and sense/antisense direction were used to inoculate all experimental plants in this study. Tobacco (*Nicotiana benthamiana*) plants were infiltrated at the stage of seedlings in 2 to 3 leaves (each treatment contains 10 plants) with *Agrobacterium* containing both prepared constructs and the empty binary vector without construct (as a control) using a 5 ml syringe with no needle (Johansen and Carrington, 2001; Abhary, 2003). Fifteen days after the infiltration of the constructs, the challenge with infection viruses was carried out using the syringe spotted technique with infectious clones of WmCSV and CYSDV.

Evaluation of the resistance triggered against viral infection

The agro-infiltrating plants of *N. benthamiana* with different constructs were inoculated with viral infectious clones and the development of the disease symptoms was observed. In addition, RNA for CYSDV and DNA for WmCSV were extracted as described earlier; after that, PCR and non-radioactive hybridization methods were applied to detect the two viruses in the challenged plants.

RESULTS

PCR and RT-PCR analysis of the selected fragments

The designed specific primers CYcpf/CYcpr, Hsp702f/Hsp702r and CYorf3f/CYorf3r (Table 1), were used successfully for the amplification of the selected fragments of the coat protein gene (CP), heat shock 70 (Hsp70) and ORF3, respectively. In addition, the designed specific primers Wmcp1/Wmcp2 and Wmrep1/Wmrep2 (Table 1) were used successfully for the amplification of the selected fragments of coat protein gene (CP) and rep gene. The selected fragments of CYSDV were 176 bp of CP, 232 bp of Hsp70 and 198 bp of ORF3. While the selected fragments of WmCSV were 160 bp of CP and 230 bp of rep gene (Figure 1).

Cloning and sequencing of the selected fragments

The recombinant plasmids were digested with *Eco*RI restriction endonuclease to test for inserts of 176, 232 and 198 bp of CYSDV and 230 and 160 bp of WmCSV. One recombinant plasmid with inserts pCYSDV-*CP*, pCYSDV-*Hsp70*, pCYSDV-*ORF3*, pWmCSV-*CP* and pWmCSV-*rep* were selected for sequencing and sub-cloning in the binary vector pFGC5941.

Nucleotide sequence analysis of the selected fragments

Sequences of the PCR amplified fragments in the recombinant (pCYSDV-*CP*, pCYSDV-*Hsp70*, pCYSDV-*ORF3*, pWmCSV-*CP* and pWmCSV-*rep)* were completed. The sequences obtained with T7 forward, SP6 reverse primers were aligned with CYSDV, and WmCSV sequences available in the GenBank.

Sub-cloning of the selected fragments into the binary vector

Sub-cloning of the selected fragments into the binary vector were done in the two orientations once by the *Asc*I and *Swa*I (sense orientation) and second time with *Xba*I and *Bam*HI (antisense orientation) departing the Chalcon Synthase (*ChaS*) Intron in the middle and finally the third

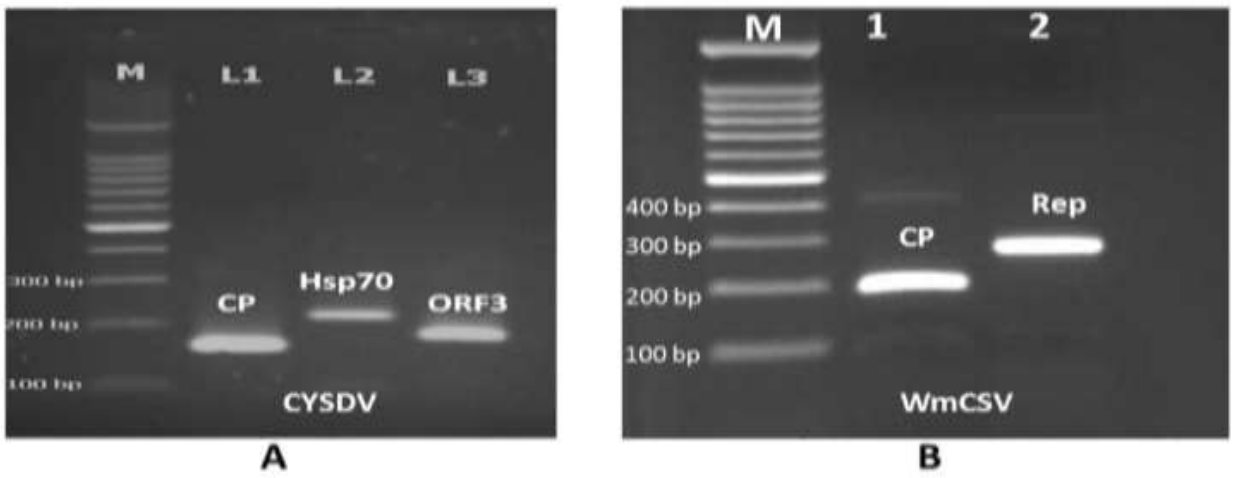

Figure 1. RT-PCR products of CYSDV and PCR products of WmCSV. **(A)** M: 100 bp DNA ladder; L1, 176 bp amplified fragment with primers CYcpf/CYcpr; L2, 232 bp amplified fragment with primers Hsp702f/Hsp702r; L3, 198 bp amplified fragment with primers CYorf3f/CYorf3r. **(B)** M, 100 bp DNA ladder; 1, 160 bp amplified fragment with primers Wmcp1/Wmcp2; 1, 230 bp amplified fragment with primers Wmrep1/Wmrep2.

construct of sense/antisense was prepared.

Nucleotide sequence analysis

The selected clones of the sense/antisense constructs were prepared for sequencing as described earlier. Nucleotide sequencing of the silencing fragments from the sense/anti-sense constructs were completed and the sequences were the same as the sequence obtained earlier.

PCR for the recombinant plasmids

PCR was done on the recombinant plasmids obtained from the Alkaline Lysis Miniprep method as described earlier. Using the previous profile of PCR, it was found that the transformation was carried out successfully and the bands appeared at their expected sizes.

Transformation into *Agrobacterium*

The constructs pasCYSDV-CP, pasCYSDV-Hsp70, pasCYSDV-ORF3, pasWmCSV-CP and pasWmCSV-rep in sense and anti-sense direction and binary vector pFGC5941 without construct as a control were transformed into *A. tumefaciens* LBA4404. Tobacco (*N. benthamiana)* plants were transformed using Syringe-Spotting Technique.

Evaluation of the transgenes

Screening for viral symptoms

The monitoring of symptoms appearance, after inoculation with viral infectious clones, was done daily to record the severity of symptoms. Clear symptoms on tobacco plants (*N. benthamiana*) took two to three weeks to appear (Table 2). All non-infiltrating tobacco plants (positive control) showed viral symptoms after inoculation. Most of the agro-infiltrating sense/antisense constructs did not yield symptoms of the viruses.

Screening with dot-blot hybridization

Tobacco plants inoculated with viral infectious clones were used as sources for DNA in dot-blot hybridization for the detection of viral genome in challenging plants. Total RNA and total DNA were extracted from new leaves using the methods mentioned earlier and blotted into nylon membranes. Hybridization was carried out using probes prepared to detect the viral genome in infiltrating plants with the different constructs. Results presented in Figure 2 showed that negative hybridization was obtained with infiltrating tobacco plants prepared constructs compared to those of non-infiltrating plants used as control. Only one out of five gave positive signals with the construct pasCYSDV-Hsp70.

Screening with RT-PCR and PCR

RT-PCR and PCR were carried out on the extracted RNA and DNA, respectively for the detection of the viral genome in challenging plants, after 15 days of inoculation with infectious clones of CYSDV and WmCSV. RT-PCR was applied using CYSDV-CP1 and CYSDV-CP2 primers to detect CYSDV. While, PCR was done using WmF2 and WmR2 primers to detect WmCSV. Positive reaction of the expected size, 500 bp of WmCSV and 800 bp of CYSDV were obtained with the infiltrating plants in case

Table 2. Viral symptoms severity after challenging with viral infectious clones showed by the number of infected tobacco plants per the number of infiltrating tobacco plants.

Construct's name	No symptoms	Mild symptoms	Severe symptoms
pasCYSDV-CP	5/5	0/5	0/5
pasCYSDV-Hsp70	2/5	2/5	1/5
pasCYSDV-ORF3	4/5	1/5	0/5
pasWmCSV-CP	5/5	0/5	0/5
pasWmCSV-rep	5/5	0/5	0/5
pFGC5941	0/5	0/5	5/5
Untreated plants	0/5	0/5	5/5

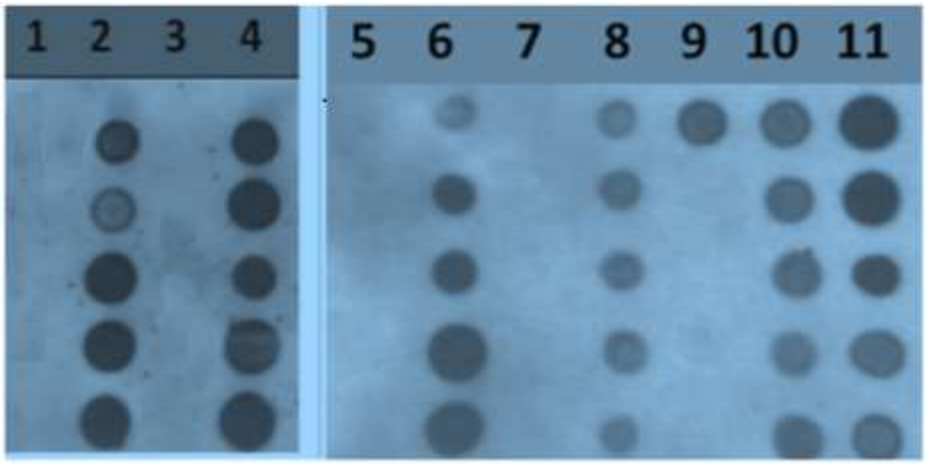

Figure 2. Non-radioactive labeling system dot-blot hybridization for virus detection in the treated plants. 1, pasWmCSV-CP; 2, psWmCSV-CP; 3, pasWmCSV-rep; 4, psWmCSV-rep; 5, pasCYSDV-CP; 6, psCYSDV-CP; 7, pasCYSDV-ORF3; 8, psCYSDV-ORF3; 9, pasCYSDV-Hsp70; 10, psCYSDV-Hsp70; 11, Untreated plants as control.

of sense constructs (ps) which proved the existence of the genome of both viruses in challenging plants (Figures 3 and 4). Infiltrating plants with sense/antisense (pas) constructs gave negative reactions indicating the lack of the viral genome. The obtained results indicate that the pasWmCSV-CP and pasCYSDV-CP constructs succeeded in preventing viral replication and infection in infiltrating plants through the siRNA-mediated resistance.

DISCUSSION

The application of new techniques is becoming a widely used strategy to control diseases caused by plant viruses. Post-transcriptional silencing (PTGS) of viral genes using co-suppression or anti-sense constructs performs a little ratio of silenced individuals (Wesley et al., 2001). There are two hallmarks in PTGS; first, silencing of target miRNAs occurs in the cytoplasm. Secondly, small interfering RNA (siRNA) molecules (21-25 nt) are generated from the silenced target mRNAs. Based on differences in their biogenesis, types of small RNAs (miRNA and siRNA) have been identified (Carthew and Sontheimer, 2009). The second type (siRNA) was used as a tool to have resistance cucurbit plants for CYSDV and WmCSV. The *A. tumefaciens*-mediated transient expression system is a multilateral system to quickly introduce genes into plant tissue. This system enables gene expression within a short period of time and without the demand for regenerating transgenic plants. The *Agrobacterium* mediated expression system has also been used effectively as a way to deliver RNA silencing suppressors and inducers into transgenic plants that express a silencing reporter gene (Johansen and Carrington, 2001).

Data from this study demonstrate clearly the effectiveness of the gene silencing application technique to trigger resistance against CYSDV and WmCSV. Three conserved, un-translated sequences of CYSDV genome were selected; the coat protein gene (CP), heat shock gene (Hsp70) and ORF3. While two conserved, un-translated sequences of WmCSV genome were selected;

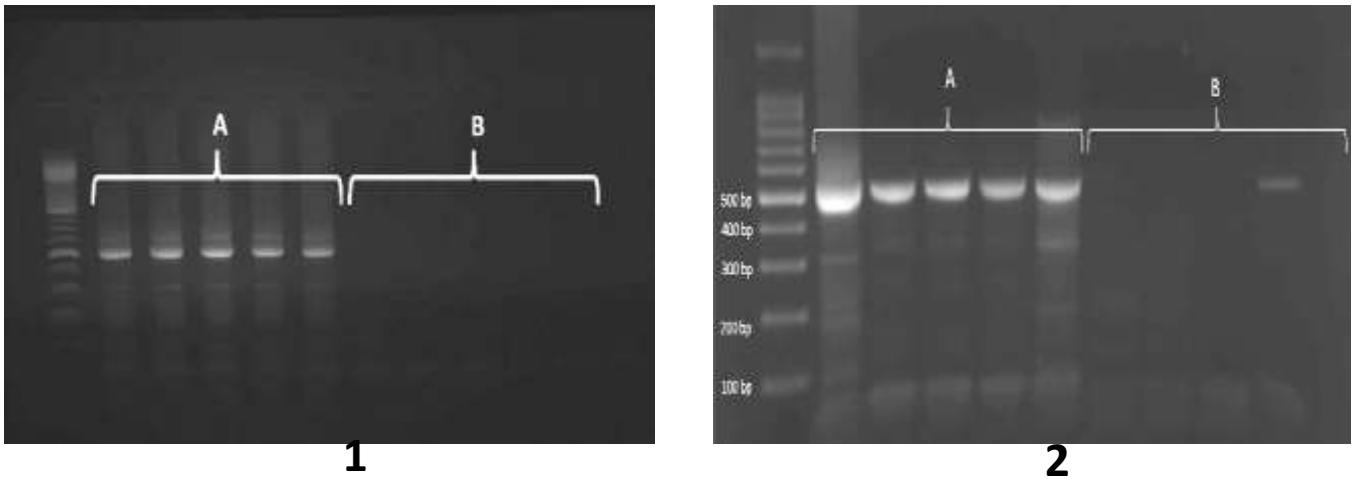

Figure 3. PCR for DNA extracted from different agro-infiltrating plants after 15 days of inoculation with infectious clone, using the primers WmF2 and WmR2. **(1)** A, plants treated with psWmCSV-CP; B, plants treated with pasWmCSV-CP. **(2)** A, plants treated with psWmCSV-rep; B, plants treated with pasWmCSV-rep.

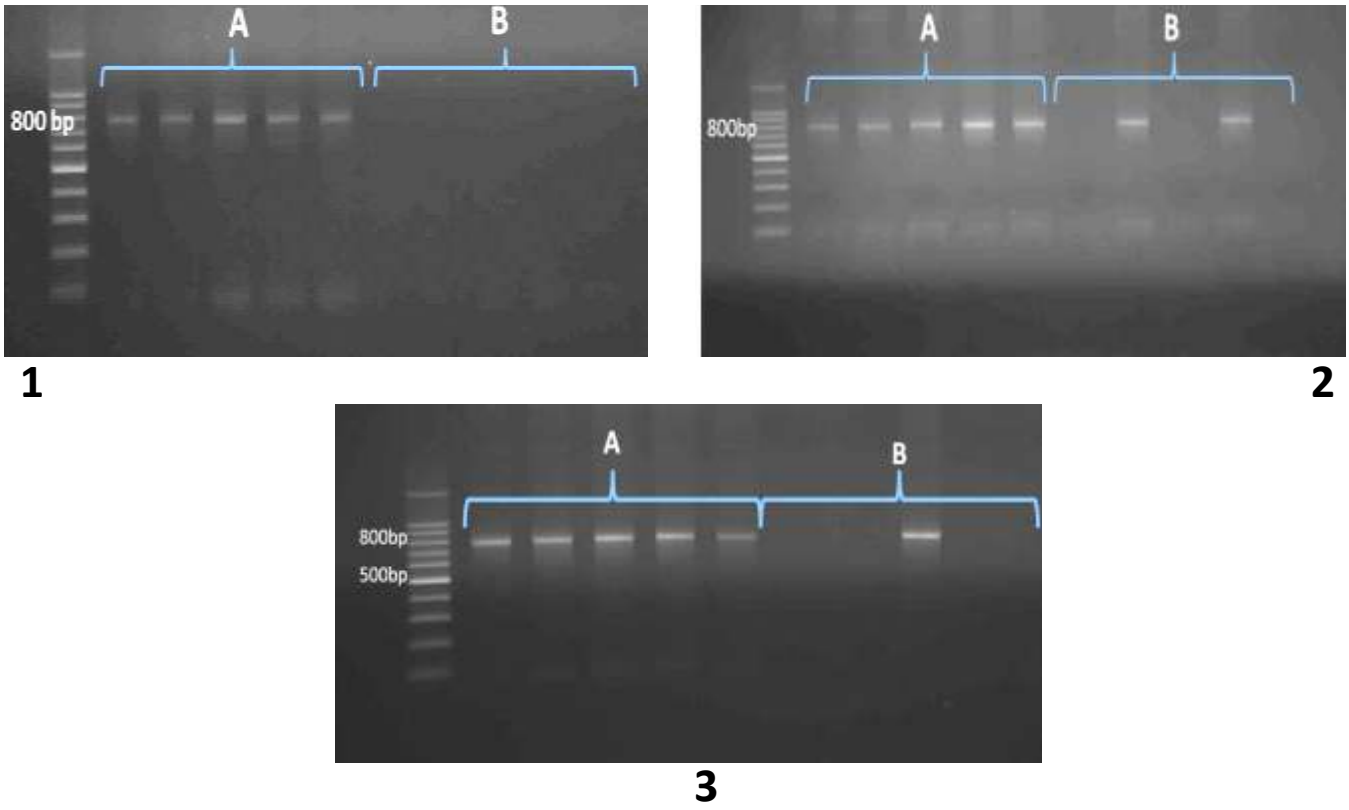

Figure 4. RT-PCR for RNA extracted from different agro-infiltrating plants after 15 days of inoculation with infectious clone, using the primers CYSDV-CP1 and CYSDV-CP1. (1) A, plants treated with psCYSDV-CP; B, plants treated with pasCYSDV-CP. **(2)** A, plants treated with psCYSDV-ORF3; B, pasCYSDV-ORF3. **(3)** A, psCYSDV-Hsp70; B, pasCYSDV-Hsp70.

CP gene and rep gene. In this study, the Syringe-Spotting Technique has been adapted to deliver different constructs into tobacco (*N. benthamiana*) cells. The high efficiency of this technique to convey the constructs may be attributed to the presence of large amount meristematic cells in small leaf tissues of newly evolved

seedlings, which supply the most suitable host-cells for the DNA to be expressed, and the bacterium to be linked (Soliman et al., 2008). Although the transformed plant cells are limited to the spotted region, RNA silencing signals could be systemically transmitted, as supposed by Eckardt (2002). Andrieu et al. (2012) mentioned that, the results of their work indicate that the method is efficient at inducing gene silencing in the agro-infected leaf area.

The transfer of low amounts of siRNA, probably occurring passively through the symplastic pathway from the agro-infected area, seemed sufficient to trigger degradation of target transcripts in the adjacent tissues. Data presented in this study shows that all transformed tobacco plants with empty vector and subsequent inoculated with CYSDV and WmCSV showed viral symptoms after 2 to 3 weeks of inoculation. These results suggest that the binary vector was single-handedly incapable to trigger resistance against CYSDV and WmCSV. In a similar way, all transformed tobacco plants with sense construct were oversensitive (susceptible) to the infection with CYSDV and WmCSV. These results suggest that, the transcription of the sense silencing trigger resulted in a mRNA that lacked the coding for any protein and did not recombine to the viral mRNA during the viral infection cycle.

Plants that had been transformed with antisense construct were all susceptible to the infection with CYSDV and WmCSV. However, these plants developed disease symptoms after two weeks of inoculation with CYSDV and WmCSV. This suggests that the progression rate of disease in these plants was lower than that with other treatments. High level of resistance could be induced in tobacco plants against CYSDV and WmCSV using sense/antisense construct in transformation. No disease symptoms could be observed in plants two and three weeks after inoculation and these plants remained symptomless until the experiment has been terminated (3 weeks after inoculation with CYSDV and WmCSV). These results clearly showed that, dsRNA folding could be produced by the sense/antisense orientation through the complementarities between the sense and the antisense orientations of the silencing triggers after the splicing of the ChaS intron post-transcriptionally. As previously described in the literature review, the dsRNA would be chopped into small temporal RNAs that can guide the host cell RISC protein to degrade the complementary sequence, which is the viral mRNA, in a sequence specific manner. This might explain the reason why plants transformed with sense/antisense construct did not show any disease symptoms during the experiment. The sequence homology of the silencing trigger might guide the RISC protein in tobacco cells to degrade the CYSDV and WmCSV messenger RNA before expressing the replicas and the coat protein, preventing the virus from initiating the replication cycle. Previous studies showed that the 21-23 nucleotides RNA known as siRNA is the main factor of gene silencing (Voinnet, 2002). The siRNA is derived from dsRNA by the action of an RNase III-like enzyme (Dicer). In another study, Llave (2002) showed that the small RNAs, extracted from inflorescent tissues, were accumulated in tissue-specific manner.

Conclusion

Data presented in this study provide evidence that the gene silencing (siRNA) technology could be used as significant virus-control measure. As indicated, high rate of plants was resistant to CYSDV and WmCSV when they were transformed with sense/antisense construct.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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