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Identification of Experimental Herbaceous Host Range of *Citrus viroid V*

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ABSTRACT

***Citrus Viroid V* (CVdV) is a member of the genus *Apscaviroid*, in the *Pospiviroidae* family. It is restricted to citrus species naturally. The herbaceous host range of CVdV was determined using the viroid infectious clone. Several herbaceous plants from the Cucurbitaceae, Solanaceae, Fabaceae, and Asteraceae families were found to be susceptible to CVdV. Also, CVdV could be transmitted to these hosts through rubbing of monomeric DNA plasmids and through mechanical inoculation of infected sap. The accumulation of CVdV in the tomato was monitored up to 28 days after inoculation and a further 56-fold increase of viroid titer was observed. Analysis of sequences of the viroid progenies from herbaceous plants revealed several nucleotide substitutions, which mostly concentrated in the pathogenicity domain on the secondary structure of the viroids.**

Keywords: Agro-inoculation, CVdV, Mechanical inoculation, Pathogenicity domain, Viroid species.

INTRODUCTION

Viroids are small, circular, single-stranded non-coding RNAs. They are replicated by rolling circle replication, lacking protein encoding capacity and recognized as the smallest known plant pathogens (Flores *et al.*, 2005). More than thirty viroid species are classified based on their molecular and biological properties into the *Pospiviroidae* and *Avsunviroidae* families; which contain a Central Conserved Region (CCR) and hammerhead ribozyme, respectively (King *et al.*, 2012). In the *Pospiviroidae* family, viroids with a broad host range fall into the *Pospiviroid* and *Hostuviroid* genera, howbeit the members of *Apscaviroid*, *Cocadviroid* and *Coleviroid* have restricted natural host range.

Citrus species are natural hosts of seven viroid species belonging to the *Pospiviroidae* family. They are *Citrus Exocortis Viroid* (CEVd) (*Pospiviroid*), *Hop Stunt Viroid* (HSVd) (*Hostuviroid*), *Citrus Bark Cracking Viroid* (CBCVd) (*Cocadviroid*) and *Citrus Bent Leaf Viroid* (CBLVd), *Citrus Dwarfing Viroid* (CDVd), *Citrus Viroid V* (CVdV) and *Citrus Viroid* VI (CVdVI) (*Apscaviroid*) (Duran-Vila *et al.*, 1988; Hadidi *et al.*, 2003; Ito *et al.*, 2002; Serra *et al.*, 2008). CEVd, HSVd, CBLVd, and CDVd are distributed worldwide (Hadidi *et al.*, 2003), whereas CBCVd has limited distribution in citrus growing areas (Cao *et al.*, 2010). CVdV has been reported from the USA, Spain, Iran, China, Japan and Pakistan (Serra *et al.*, 2008; Bani-Hashemian *et al.*, 2010; Ito and Ohta 2010; Cao *et al.*, 2010). CVdVI seems to be restricted to Japan (Ito *et al.*, 2002). CEVd and HSVd have broad host ranges in woody and herbaceous plants; they develop exocortis and cachexia symptoms in sensitive citrus species, respectively.

Apscaviroids infecting citrus plants induce mild symptom on commercial citrus species with complex interaction in mixed infection.

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CVdV produces mild leaf bending and petiole necrosis symptoms on citrus species (Barbosa *et al.*, 2002). It is restricted to citrus species naturally. Limited studies were carried out to determine the biological properties of this viroid due to lack of suitable herbaceous host plants.

Earlier attempts failed to transmit CVdV or its artificial chimers to herbaceous plants (Barbosa *et al.*, 2005); But, evidence of *de novo* replication of *Australian grapevine viroid* and *Apple scar skin viroid* (type member of the *Apscaviroid*) in several herbaceous plants have been reported (Rezaian 1990; Zakiaghl and Izadpanah 2010; Walia *et al.*, 2014). Moreover, natural infection of *Grapevine yellow speckle viroid1* (GYSVd1) in *Ixeridium dentatum* plants was recently reported (Lee *et al.*, 2015). These data raise the possibilities that CVdV may also have herbaceous host plants.

In this research, we aimed to provide evidence for replication of CVdV in herbaceous host plants by fulfilling Koch's postulates and compare their experimental host range. For this purpose, the infectious clones of CVdV has been constructed, which was inoculated to various herbaceous plants by agroinoculation, direct rubbing of DNA plasmids containing the viroid sequence and mechanical inoculation of infected sap.

MATERIALS AND METHODS

Construction of Infectious Clone of CVdV

cDNA clone of CVdV was kindly provided by Ricardo Flores and Pedro Serra (IBMCP, UPV, Spain). The cDNA clone was used as template for construction of viroid infectious clone.

In order to make infectious construct, the full length genome was amplified using pUC-m13 universal primers from the cDNA clone. The PCR products were digested by *Pst*I and *Hind*III endonucleases; then, ligated into corresponding sites in pBin62sk binary vector under control of the 35S promoter. pBin62sk was derived from replacement of T-DNA fragment of pGreen62sk binary vector (Hellens *et al.*, 2005) into pBin19 plasmid (Bevan, 1984). To do this, *Bgl*II fragment of pGreen62sk was replaced in corresponding site of pBin19.

The construct was transferred to the competent cells of *E. coli* strain DH5α and the recombinant plasmids were recovered from bacterial cells using Plasmid DNA Isolation Kit (Denazist, Iran). Integrity of the construct was authenticated by sequencing using pUC-M13 universal primers. Finally, the construct was transformed into *Agrobacterium tumefaciens* strain C5850 (Holsters *et al.*, 1978).

Infectivity Test of Infectious Construction of CVdV

Agrobacterium tumefaciens C5850 cells containing the monomeric construct of CVdV were grown on an optical density (OD) of 0.8 in LB broth medium, pelleted down and suspended in agroinoculation buffer (10 mM Tris-HCl pH 6.5, 10 mM $MgCl_2$, 150 uM acetosyringone), stored for 1 hour at room temperature, then agroinoculaed into the stem of *Poncirus trifoliate* plants. The plants were maintained in an insect-proof cage at a controlled growth chamber. Five weeks after inoculation, total RNA was extracted and RT-PCR carried out to check the integrity of the viroid.

Determination of Host Range of CVdV

To identify herbaceous host range of CVdV, three species of Cucurbitaceae, i.e. cucumber (*Cucumis sativus*), Persian melon (*Cucumis melo* var. inodorus), watermelon (*Citrullus lanatus* var. lanatus), and six species of Solanaceae, including tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum* var. Turkish and *Nicotiana glutinosa*), potato (*Solanum tuberosum*), pepper (*Capsicum annuum*), Petunia (*Petunia hybrida*); a species of Fabaceae, i.e. a bean (*Phaseolus vulgaris*); and one species of Asteraceae (*Gynura aurantiaca*) were infiltrated with Agrobacterium cells harboring the infectious construct of CVdV. At least two young plants from each species were inoculated. Back-inoculation was performed on the same plants by mechanical inoculation of infected sap.

Three weeks after inoculation, nucleic acids were extracted from noninoculated newly grown leaves and the presence of viroids was checked by RT-PCR. Amplified products were cloned and sequenced to confirm their identity.

Mechanical Inoculation of Infected Plants Sap and Monomeric Plasmid

Leaf extracts of newly grown leaves of agro-infiltrated cucumber were prepared in 0.07 M Tris-HCl buffer pH 8.0, and then mechanically inoculated onto carborundum-dusted leaves of cucumber and tomato plants.

For mechanical inoculation, we used also cDNA inoculum of CVdV. For preparation of cDNAs inoculum, about 100 ng of plasmids containing CVdV sequence was linearized by *Hind*III, diluted in water and mechanically rubbed on carborundum-dusted leaves of cucumber and tomato plants.

RNA Extraction, RT-PCR, Cloning and Sequence Analysis

Total RNA was extracted by crashing of 500 mg of leaf tissue in 10 volumes of extraction buffer (100 mM Tris-HCl, pH8.0; 50 mM EDTA; 50 mM NaCl; 10 mM 2-mercaptoethanol). To the homogenate, 250 µL of 20% of SDS and 400 µL of 5M potassium acetate was added and placed at 65°C for 20 minutes, then chilled on ice. The tube was centrifuged at 12,000 rpm for 15 minutes and supernatant was transferred to a new tube. Nucleic acids were precipitated by addition of 2.5 volume of absolute ethanol followed by 15 minutes centrifugation at 14,000 rpm (Bernard and Duran Vila, 2006).

RT-PCR was performed using a specific primer pair (Table 1). The RT reaction mixture of 20 µL contained 5 µL of total RNA, 2 µL of MMuLV reverse transcription buffer, 1 µL of reverse primer (10 pmol), 2 µL of dNTP mix (40 mM), 0.5 µL of MMuLV reverse transcriptase (200 U μL^{-1}; Parstous, Iran). The RT reaction was incubated at 46°C for one hour, followed by 10 minutes at 70°C for enzyme inactivation. PCR reaction was carried out using 4 µL of the cDNA, 1 µL of each specific primer pair (10 pmol) and 12.5 µL of ready to use PCR Master Mix (Ampliqon) in a total volume of 25 µL.

PCR parameters consisted of initial denaturation at 94ºC for 5 minutes, followed by 35 cycles of 94ºC for 30 seconds, annealing temperature (Table 1) for 30 seconds and 72ºC for 1 minute and a final extension step at 72ºC for 5 minutes. PCR products were visualized on an agarose gel containing 0.2 µg of DNA green viewer™ (Parstous, Iran).

The PCR products were ligated into pTZ57R/T cloning vector according to

Table 1. List of oligonucleotide primers used in RT-PCR and qRT-PCR.

Name	Sequence (5´-3´)	Target	Annealing Temp. (°C)	Amplicon (bp)	Reference
CVdV-R CVdV-F	*TCGACGAAGGCCGGTGAGCA* *CGACGACAGGTGAGTACTCTCTAC*	CVdV	60	294	Serra *et al*., 2008
CVdV-R CVdV-L	*ACAGGGAGAGGGAGACCAC* *TCCTCTGGAGCTCTGCTCTAA*	CVdV	59	102	Design by author
GADPH-R GADPH-F	*ATCAACGGTCTTCTGAGTGGCTGT* *ACCACAAATTGCCTTGCTCCCTTG*	GADPH	59	110	Mascia *et al*., 2010

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manufacturer protocol (Thermo Scientific) and transformed into competent cells of *Escherichia coli* strain DH5α. Recombinant plasmids were purified from bacterial cells using Plasmid DNA Isolation Kit (Denazist-Iran). Finally, the purified recombinant plasmids were subjected to bidirectional sequencing using pUC-M13 universal primers using an ABI PRISM 377 apparatus by Macrogen Inc. (Seoul, South Korea).

Sequence comparisons against GenBank databases were performed using BLAST (http://blast.ncbi. nlm.nih.gov/Blast.cgi). Possible secondary structure of CVdV was predicted in mfold program (http://mfold.rna.albany. edu/?q=mfold). Multiple sequence alignment was carried out by ClustalW program implemented in MEGA6 software (Tamura *et al.,* 2013). Sequences were compared with the viroids type members (GenBank Acc. No. NC010165) and mismatches were plotted on the viroid secondary structure.

Dot Blot Hybridization

DIG-labeled DNA probe was synthesized by PCR amplification of the cloned viroid in 50 µL reaction volume containing 0.5 µM of each primer (Table 1), 1.5 mM $MgCl_2$, 120 µM each of the four dNTPs (containing DIG-labeled dUTP) and 1 unit of *Taq* DNA polymerase.

For dot blot hybridization, total nucleic acids were extracted at 3 wpi. The purified nucleic acid was treated with *Dnase* I (Sinaclon, Iran) followed by 10 minutes at 70°C for enzyme inactivation.

One microgram of total RNA was diluted with one volume of 1.2X standard saline citrate (SSC) containing 6% formamide and vacuum-blotted on nitrocellulose membrane, which were treated with l0X SSC (10 minutes) before use. Membrane was then air dried and baked at 80°C for 2 hours.

Processing of the blots for pre-hybridization (4 hours), hybridization (20 hours), and washing were carried out as described by Green and Sambrook (2012). The DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate and visualized with the substrate solution (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate *p*-toluidine salt, NBT/BCIP) in the dark.

Semi-Quantitative Real-Time PCR (qRT-PCR)

Accumulation of CVdV in inoculated tomato plants was monitored up to 28 days at seven days intervals. Total RNA were extracted (Bernard and Duran Vila 2006) and treated with *Rnase*-Free *DNase* I (Sinaclon, Iran) following the manufacturer instructions.

Specific primers for CVdV (CVdV-R/L) were designed by using the GenScript Real-time PCR Primer Design software (Table 1). The amounts of CVdV in the RNA preparations were estimated by reverse transcription followed by SYBR Green I based semi-quantitative PCR assay. Real-time PCR assay was performed in the CFX96™ Touch System (BioRad) using thermostable MMuLV Reverse Transcriptase (Parstous,Iran) and SYBR® Green qPCR Master Mix (Parstous,Iran) according to the manufacturer's instructions.

The real-time PCR program parameters consisted of an initial denaturing step at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 59°C for 30 seconds and 72°C for 30 seconds. Immediately after the final PCR cycle, specificity of the reaction was verified by melting curve analysis by a thermal denaturing cycle of 60–95°C at 1°C increments with 5 seconds between each step. All reactions were performed in triplicate and included no-template control and no reverse transcriptase as negative controls.

For quantification of CVdV in tomato plants, the tomato *GAPDH* gene (GenBank Accession No. ES437736) was used as an internal control (Mascia *et al.,* 2010) for normalization of host RNA. Primer validation experiments were performed with fourfold serial dilutions of the plasmids containing DNAs of GAPDH and CVdV. Relative quantification was measured using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). In this method, change in amount of CVdV progeny was normalized to the expression of *GAPDH* gene. The $2^{-\Delta\Delta Ct}$ data analysis is where $\Delta\Delta Ct$= *(Ct of target-Ct of GAPDH* $)_{TimeX}$*-(Ct of target- Ct of GAPDH)*$_{Time1}$ which gives mean fold change in expression of target genes at each time point. In our experiments, time 1 was amplification of target gene at two weeks after inoculation.

Moreover, standard curve was constructed using serial dilutions of the plasmid containing sequence of CVdV. Quantification of CVdV in the tomato plants was performed by plotting the *Ct* value of each sample on the standard curve. The amount of starting template in a PCR reaction, expressed as the copy number of the target CVdV cDNA, was determined by this method.

RESULTS

Infectivity of CVdV Infectious Clone

The binary vector containing full length of CVdV was agro-inoculated to five *Poncirus trifoliata* plants. The plants were checked for the presence of the viroid by RT-PCR at 5 wpi. Amplification of 294 bp product from uninoculated leaves of inoculated *Poncirus trifoliata* plants revealed replication of CVdV in these plants (Figure 1). No amplification was observed in mock-inoculated plants. Sequencing of the RT-PCR products from three randomly selected plants confirmed the amplified fragments were identical to CVdV genome (data not shown). No visible symptom was observed in the inoculated trifoliate orange until 9 wpi.

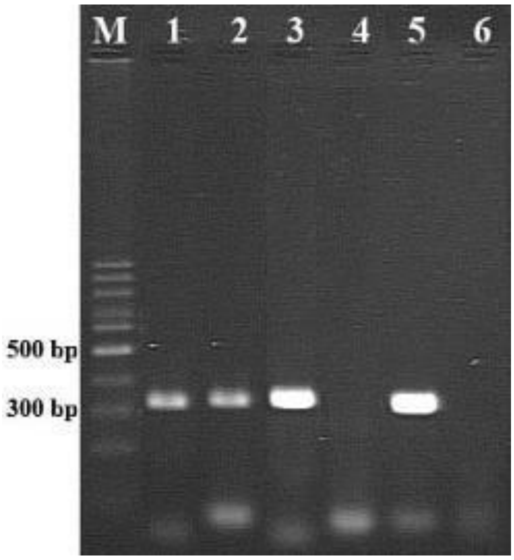

Figure 1. Electrophoresis of RT-PCR products from newly grown leaves of agro-inoculated trifoliate orange plants at five weeks post inoculation. (Lane1-5): Trifoliate oranges inoculated with infectious constructs of CVdV, (Lane6): Healthy control inoculated by empty plasmid vector. M: 100bp DNA ladder (Parstous, Iran).

Identification of the Experimental Host Range

Experimental host range of CVdV was determined by inoculation of several herbaceous plants from various families with the viroid infectious clones.

RT-PCR, dot blot hybridization and mechanical inoculation indicated that some of these plants were susceptible to CVdV (Figure 3, Table 2).

As shown in Table 2, eleven species of herbaceous plants were susceptible to CVdV. It was replicated in *Cucumis sativus*, *Cucumis melo*, *Citrullus lanatus*, *Solanum lycopersicum*, *Nicotiana tabacum*, *Nicotiana glutinosa*, *Solanum tuberosum*, *Capsicum annuum*, *Petunia hybrid*, *Phaseolus vulgaris*, and *Gynura aurantiaca* plants.

Most of the infected plants were symptomless, except for tomato and bean plants. In tomato, CVdV generated mottling, epinasty, bushy growth, leaf deformation and leaf curl (Figure 2, Table 2) within 2 months after inoculation. *Phaseolus vulgaris* plants infected with CVdV showed leaf crinkle, crazy top, and leaf deformation 2 months after inoculation (Figure 2, Table 2). Cucumber plants only showed stunting (data not shown).

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Table 2. Identification of herbaceous host rang of CVdV.

	Hosts	No. plants	Symptoms
Cucurbitaceae	*Cucumis sativus*	5/5[a] (4/5[b])	Leaf deformation
	Cucumis melo var indorus	3/3 (3/3)	Symptomless
	Citrullus lanatus	2/3 (2/3)	Symptomless
Solanaceae	*Solanum lycopersicum*	4/5 (3/3)	Leaf curl, leaf deformation, epinasty, mottling, bushy growth
	Solanum tuberosum	4/4 (2/2)	Leaflet joining
	Capsicum annum	4/4 (3/3)	Symptomless
	Petonia hybrida	3/3 (2/3)	Symptomless
	Nicotiana glutinosa	3/3 (2/2)	Increased in leaf thickness
	Nicotiana tabacum Var. turkish	3/3 (2/2)	Symptomless
Leguminosae	*Phaseolus vulgaris*	4/4 (3/3)	Leaf crinkle, crazy top, leaf deformation
Asteraceae	*Gynura sarmentosa*	3/3 (1/3)	Symptomless
Rutaceae	*Poncirus trifoliata*	4/5	Symptomless

[a] No. positive plants/No. of agro-inoculated plant. [b] No. positive plants/No. of plants inoculated by infected sap of the same species.

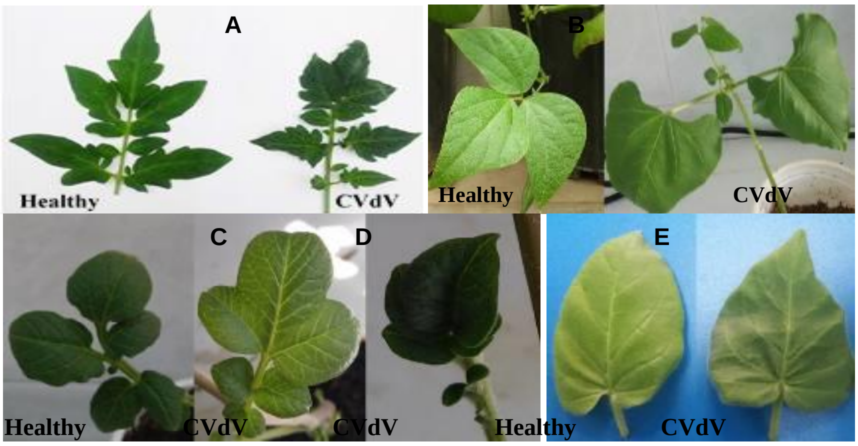

Figure 2. Symptoms of CVdV in herbaceous hosts. Mottling and bushy growth of CVdV (A) in tomato; Open petiolar sinus and leaf crinkle (B) in bean; leaflet joining (C) and bushy growth of leaf (D) in potato and mottling and crinkle in *Nicotiana glutinosa* (E).

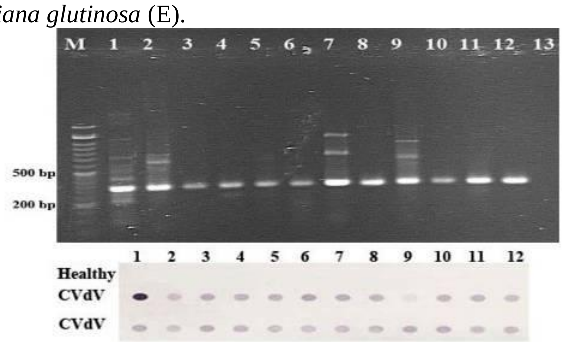

Figure 3. Identification of *de novo* population of CVdV in non-inoculated leaves of herbaceous plants using RT-PCR (Top) and dot blot hybridization with the full-length DIG-labelled probe of the viroids (Bottom), confirming the infectivity of the viroids in various inoculated plants.

Positive DNA control (1), *Solanum lycopersicum* (2), *Cucumis sativus* (3), *Nicotiana tabacum* var. *Turkish* (4), *Nicotiana glutinosa* (5), *Phaseolus vulgaris* (6), *Petunia hybrid* (7), *Gynura aurantiaca* (8), *Capsicum annuum* (9), *Citrullus lanatus* var. *lanatus* (10), *Cucumis melo* (11), *Solanum tuberosum* (12), negative control (13). M: 100bp DNA ladder (Partous, Iran).

Cucumis melo, *Citrullus lanatus*, *Nicotiana glutinosa*, *Nicotiana tabacum* and *Petunia hybrid*; either, mock-inoculated controls did not show any symptoms.

Accumulation of CVdV Progenies in Inoculated Tomato Plants

To ascertain replication of CVdV in tomato plants, a time-course experiment assay was carried out to monitor accumulation of the viroid progenies over 28 days post-inoculation with 7 days interval using semi quantitative real time RT-PCR.

Normalization of host RNA using an internal control gene GADPH was performed before the quantification of CVdV in tomato plants. The normalized templates were then used for quantitative assay of CVdV. Utilizing the comparative Ct ($2^{-\Delta\Delta Ct}$) method for relative quantification of viroid needs validation of efficiency of the cv5F/R primers with respect to the endogenous control primers, GAPDH. Comparison of the standard curves generated from amplification of four fold dilutions of GAPDH and CVdV demonstrated that the efficiencies of viroid and GAPDH amplification were similar (Figure 4).

As shown in Figure 4, a significant correlation between levels of accumulation of the viroid progenies and sampling time was observed. A 7.1 fold increase in accumulation of the CVdV progenies at 21 dpi was observed compared to 14 dpi (Figure 4). Then, level of CVdV RNA leaped at 28 dpi to 56.5 fold. Four weeks after inoculation, the number of viroid

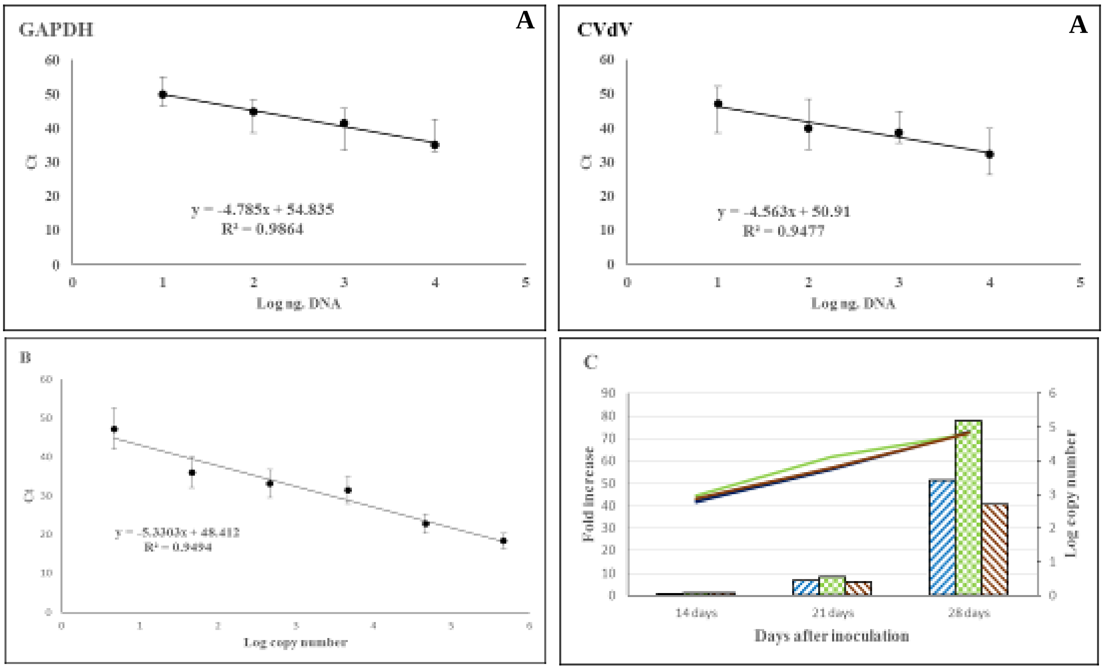

Figure 4. (A) Primer efficiency validation determined using fourfold serial dilutions of cDNA amplified by RT_PCR using CVdV and GAPDH specific primers. (B) Standard curve obtained by plotting Ct values of amplification of 6-fold serial dilutions of the pTZ57R-CVdV plasmid vs. starting CVdV copy number. (C) Fold changes in accumulation levels and titer of CVdV in tomato plants in a time-course experiment assay over 28 days post-inoculation with 7 days interval. The relative quantity of CVdV was calculated using the comparative cycle threshold method. The CVdV level at 14 dpi was chosen as the calibrator and all other samples were quantified relative to it. GAPDH RNA was used as an internal control to normalize the data. The *x*-axis indicates the days after inoculation and the primary *y*-axis reports fold increase and secondary *y*-axis shows logarithmic values of CVdV copy number/nanogram of total RNA. The Ct values for each dilution are the means of three replicates.

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copies ng^{-1} of total RNA was in the range of 6.57×10^{1} to 7.06×10^{1} in the tomato plants, without substantial differences between them.

The symptoms induced by CVdV, such as rugosity, leaf epinasty, and stunting in tomato could be observed in 8 weeks post inoculations (Figure 2).

Increasing levels of CVdV in time-course experiments in the tomato plants indicated replication of the viroid in this plant.

Sequence Analysis of the Viroids Progeny

To determine whether the replicated RNA preserves its primary sequence or suffers nucleotide alteration, viroid progenies were sequenced. The sequencing data, provided from 22 clones, revealed the presence of several mutations in the progenies of CVdV.

The sequencing of the CVdV clones revealed the progenies were 96% identical to the wild type, but differing at 11 nucleotide positions: C43A, A48U, A53G, G55C, C56A, A60G, T163G, C222T, C224T, +243A, T257C. These mutations lie in the Pathogenicity (P), Central Conserved Region (CCR) and Terminal Right (TR) domains of the viroid secondary structure (Figure 5).

Comparison of Different Inoculation Methods

Three different inoculation methods including agro-inoculation, mechanical inoculation of infected sap, and mechanical inoculation of linearized plasmid were compared. To do this, three groups of 5 cucumber plants were separately inoculated by bacterial cells harboring CVdV infectious construct, linearized plasmid containing the viroids genome, or sap of CVdV-infected cucumber. The plants were checked for the presence of the viroids at 3 wpi by RT-PCR. Amplification of a single expected band using viroids specific primers indicated successful transmission of the viroids.

Comparison of the percent of infectivity for each inoculation method revealed that agro-infiltration and mechanical inoculation of sap were the best inoculation methods, with approximately 100% efficiency. All plants (5 cucumbers) inoculated using agroinoculation or mechanical inoculation of the infected sap were infected by CVdV, but in the case of linearized plasmid as inoculum for mechanical inoculation, only 3 out of five inoculated cucumbers were infected by CVdV.

DISCUSSION

Without encoding protein, viroids are infectious in many plant species (Ding, 2009; Flores *et al.*, 2009). They are interesting biological entities, which may be used as models in biological research (Ding and Itaya, 2007). Therefore, many studies have been carried out to determine factors involved in replication, movement, and pathogenicity of the viroids, especially for the genus pospiviroids (Ding, 2009; Flores *et al.*, 2009; Gora-Sochacka *et al.*, 1997; Owens *et al.*, 1996; Owens and Hammond,

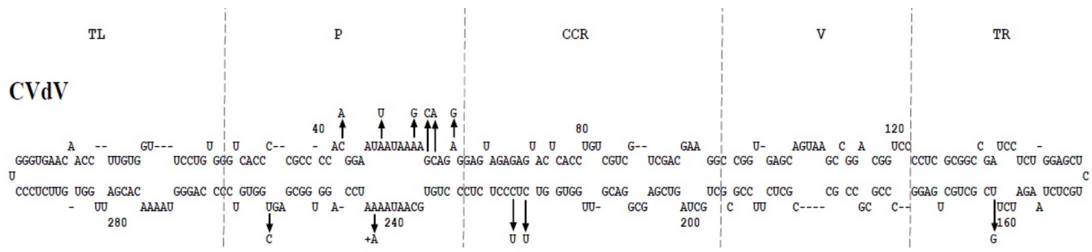

Figure 5. Locations and sequence variations found in 22 isolates of CVdV on the secondary structures of the viroid.

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2009; Tabler *et al.,* 1992; Takeda and Ding, 2009).

Citrus plants are harboring several viroids species belonging to the *Pospiviroid*, *Hostuviroid*, *Cocadviroid* and *Apscaviroid* genera. Naturally, members of the genus *Apscaviroid* are restricted to woody plants. To study the biological properties of the viroids, herbaceous plants are better hosts than woody plants, due to shorter time required to grow and display symptoms; so, the genetic information for apscaviroids is rather scant. To date, there have been no successful reports for CVdV, CBLVd and CDVd, apscaviroids infecting citrus, transmission to any herbaceous plant species. Transmission of AGVd and ASSVd to several herbaceous host plants (Rezaian, 1990; Zakiaghl and Izadpanah, 2010; Walia *et al.,* 2014) and identification of GYSVd1 in *Ixeridium dentatum* (Lee *et al.,* 2015) raised the probability that apscaviroid members of the citrus viroids may also have herbaceous hosts. Therefore, among the apscaviroids infecting citrus, we chose CVdV for studying its experimental host range. We constructed the infectious clones of CVdV to fulfill Koch's postulates and determine its host range.

Prevailing evidence indicates that longer than unit length of viroid or monomers of the viroid regulated by artificial promoter are infectious in plants (Daros and Flores, 2004; Gardner *et al.,* 1986; Gomez and Pallas, 2006; Gora-Sochacka *et al.,* 1997; Podstolski *et al.,* 2005; Rezaian, 1999; Tabler *et al.,* 1992). We made monomeric construct under control of the 35s promoter for CVdV. The construct was infectious in *Poncirus* plants as confirmed by RT-PCR, dot blot hybridization, and mechanical inoculation of infected plants sap.

Among viroids infecting citrus species, infectious clones for exocortis (Martin *et al.,* 2007; Visvader *et al.,* 1985), HSVd (Kofalvi *et al.,* 1997) and CBCVd (Jakse *et al.,* 2015) were previously made, but for the first time we developed monomeric infectious clone of CVdV.

Past attempts had failed to transmit CVdV to non-citrus species (Barbosa *et al.,* 2005; Serra *et al.,* 2008). In this study, eleven herbaceous plant species were identified as systemic hosts for CVdV. *Cucumis sativus*, *Cucumis melo*, *Citrullus lanatus*, *Solanum lycopersicum*, *Nicotiana tabacum*, *Nicotiana glutinosa*, *Solanum tuberosum*, *Capsicum annuum*, *Petunia hybrida*; *Phaseolus vulgaris* and *Gynura aurantiaca* are reported for the first time as experimental hosts for CVdV. However, only *Solanum lycopersicum* and *Phaseolus vulgaris* plants displayed visual symptoms (Table 2). These plant species were also symptomatic hosts for AGVd (Zakiaghl and Izadpanah, 2010) and ASSVd (Walia *et al.,* 2014).

CVdV showed mottling and leaf deformation in cucumber plants. Cucumber is known as symptomless assay host plant of three other apscaviroids, such as *Pear blister canker viroid* (Flores *et al.,* 1991), *Australian grapevine viroid* (Rezaian *et al.,* 1990, Zakiaghl and Izadpanah 2010) and *Apple scar skin viroid* (Walia *et al.,* 2014). It seems that cucumber serves as a relatively good host plant for biological indexing of apscaviroids.

In the cases of *Nicotiana tabacum*, *Nicotiana glutinosa* and *Petunia hybrida*, replication of CVdV induced no symptoms. Similar results were obtained for ASSVd (Walia *et al.,* 2014) and AGVd (Zakiaghl and Izadpanah, unpublished data).

Increase in accumulation of viroid RNA over the period of 28 days indicated the successful replication of CVdV in tomato plants. Previous data indicated that replication of *Potato spindle tuber viroid* (Qi and Ding, 2002), CEVd (Martin *et al.,* 2007) and CDVd (Rizza *et al.,* 2009) in their host plants are coupled with the accumulation of viroid transcripts in plant tissues.

In addition to identification of herbaceous host plants, different types of inoculation strategies were also examined. CVdV could be transmitted through agro-inoculation of monomeric constructs, mechanical inoculation of plasmid DNA containing viroids monomer, and through the sap inoculation. Agro-inoculation and mechanical inoculation of sap yielded approximately 100% efficacy. There is no report for successful transmission of CVdV to herbaceous host plants (Barbosa *et al*., 2005; Serra *et al*., 2008). However, not only this study was able to transmit CVdV to various herbaceous host plants but also it was readily transmissible via several inoculation methods.

Previous studies conducted with viroids have revealed that several variants can be generated *de novo* from a single sequence (Ambros *et al.,* 1999; Gandia and Duran-Vila, 2004; Gora-sochacka *et al.,* 1994; Owens *et al.,* 1996). Comparison of sequences of progenies of CVdV with the wild type, revealed the presence of several mutations in *de novo* populations of the viroids.

Most of the substitutions concentrated at P domain on the secondary structure of the viroids. Earlier reports suggested that variability of viroids in the family *Pospiviroidae* is generally found in the V and P domains (Keese and Symons, 1985). In CEVd and HSVd, most changes are located in the P and TL domains, without significant changes in the secondary structure (Fagoaga and Duran-Vila, 1996; Gandia and Duran-Vila, 2004). These substitutions may *de novo* occur in the herbaceous host plants to protect viroids against the host defense system or to induce fitness to the new host. It has been reported for *Peach latent mosaic viroid* and *Apple scar skin viroid* that the generation of new variants may undergo transitions in the host plants (Ambros *et al.,* 1999; Walia *et al.,* 2014).

In conclusion, we analyzed experimental host range of CVdV, an apscaviroid naturally infecting citrus species. We fulfilled the Koch's postulates to show that several herbaceous plants belonging to Solanaceae, Fabaceae, Cucurbitaceae, and Asteraceae families are systemic hosts for CVdV.

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شناسایی دامنه میزبانی آزمایشگاهی ویروئید پنج مرکبات در گیاهان علفی

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چکیده

ویروئید پنج مرکبات متعلق به جنس اپسکاویروئید از خانواده پوسپی ویروئیده است. این ویروئید در طبیعت محدود به گونه های مرکبات است. با استفاده از همسانه عفونت زا دامنه میزبانی این ویروئید در گیاهان علفی بررسی و مشخص شد که چندین گونه از خانواده های کدوئیان، سیب زمینیان، حبوبات و

Asteraceae به این ویروئید حساس هستند. همچنین با مایه زنی مکانیکی پلاسمید حاوی ژنوم ویروئید یا عصاره گیاه آلوده نیز ویروئید پنج مرکبات به میزبانهای علفی منتقل شد. بررسی تغییرات غلظت ویروئید در گوجه فرنگی تا ۵۶ برابر افزایش مقدار ویروئید را در ۲۸ روز پس از مایه زنی نشان داد. مقایسه توالی ژنوم نتاج ویروئید در گیاهان علفی بیانگر ایجاد چندین تغییر در ناحیه بیماری زایی در ساختار ثانویه ویروئید نسبت به تیپ وحشی آن بود.

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