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Identification of the experimental herbaceous host range of the *Apscaviroids* infecting citrus species

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Summary. – Citrus viroid V (CVd-V), citrus dwarfing viroid (CDVd) and citrus bent leaf viroid (CBLVd) (the genus *Apscaviroid*, the family *Pospiviroidae*) have been reported to be restricted to citrus species naturally. The herbaceous host range of these viroids was identified using the viroids infectious clones. Several herbaceous plants from the *Cucurbitaceae, Solanaceae, Fabaceae* and *Asteraceae* families were found to be susceptible to CVd-V, CDVd and CBLVd. Also, the viroids could be transferred to these hosts through rubbing of monomeric DNA plasmids and through mechanical inoculation of infected sap.

Keywords: citrus; viroid; host range; CVd-V; CBLVd; CDVd

Introduction

Viroids are small, circular, single-stranded non-coding RNAs. They are replicating 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 are containing 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 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 (CVd-V) and citrus viroid VI (CVdVI) (*Apscaviroid*) (Duran-Vila *et al*., 1988; Hadidi *et al*., 2017; Ito *et al*., 2002; Serra *et al*., 2008). These viroids are distributed worldwide (Hadidi *et al*., 2017). CVd-V has been reported from the USA, Spain, Iran, China, Japan and Pakistan (Sera *et al*., 2008; Bani-Hashemian *et al*., 2010; Ito and Ohta, 2010; Cao *et al.,* 2010). CVd-VI seems to be restricted to Japan (Ito *et al.,* 2002). CEVd and HSVd have broad host ranges in woody and herbaceous plants and they develop exocortis and cachexia symptoms in sensitive citrus species, respectively.

Apscaviroids infecting citrus plants induce mild symptoms on commercial citrus species with complex interaction in mixed infection. CBLVd, CDVd and CVd-V produce mild leaf bending and petiole necrosis symptoms on *Etrog citron* (Barbosa *et al.,* 2002). They are restricted to citrus species naturally, making the study of the biological properties of these viroids due to lack of suitable herbaceous host plants difficult.

Earlier attempts failed to transfer CVd-V or its artificial chimers to herbaceous plants (Serra *et al.,* 2009). However, 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.,*

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Abbreviations: AGVd = Australian grapevine viroid; ASSVd = apple scar skin viroid; CBCVd = citrus bark cracking viroid; CBLVd = citrus bent leaf viroid; CDVd = citrus dwarfing viroid; CVd-V = citrus viroid V; GYSVd1 = grapevine yellow speckle viroid 1; wpi = weeks post inoculation

2014). Moreover, natural infections of GYSVd1 in *Ixeridium dentatum* plants was previously reported (Lee *et al.,* 2015). These data hypothesize that apscaviroid members of the citrus viroids may also have herbaceous host plants.

Here, we provide evidence for replication of the apscaviroids infecting citrus plants in herbaceous host and compare their experimental host range. For this purpose, the infectious clones of CVd-V, CBLVd and CDVd have been constructed, and then inoculated into various herbaceous plants by agroinoculation, direct rubbing of DNA plasmids containing the viroid sequence and mechanical inoculation of infected sap. Eleven different plant species were identified in which CVd-V, CBLVd and CDVd can replicate, but only two of them were able to produce visual symptoms.

Materials and Methods

Source of viroids. The full length genome of CDVd (GenBank KY654681) and CBLVd (GenBank KY654680) were previously isolated from an infected Moro blood orange plant in citriculture of Ramsar in the north of Iran and cloned into pTZ57R vector (Ebrahimi-Moghadam, unpublished data). cDNA clone of CVd-V was kindly provided by Ricardo Flores and Pedro Serra (IBMCP, UPV, Spain).

Construction of infectious clones of viroids. The cDNA clones of CVd-V, CBLVd and CDVd were used as template for construction of viroid infectious clones. In order to make infectious clone of the viroid (Rezaian, 1999), the full-length genome was amplified using pUC-m13 universal primers from the cDNA clone. The PCR products were digested by *Xba*I/*Bam*HI endonucleases and then ligated into corresponding sites into 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 into corresponding site of pBin19 (Life science market, Hong Kong). The constructs were transferred to the competent cells of *Escherichia 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 (Thermo Scientific, USA). Finally, the constructs were transformed into *Agrobacterium tumefaciens* strain C5850 (Holsters *et al.,* 1978).

Agroinoculation. Agrobacterium tumefaciens C5850 (Takara, Japan) containing the viroids monomeric constructs were grown to an O.D. 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 μM acetosyringone), incubated for 1 h at room temperature, then infiltrated into the cotyledonary leaves of *Cucumis sativus* or agroinoculated into the stem of *Solanum lycopersicum* and seedlings of *Poncirus trifoliata*. The negative control was inoculated by *A. tumefaciens* cells containing wild type pBin62sk plasmid. The plants were maintained in an insect-proof cage in a growth chamber. Three weeks after infiltration, total RNA was extracted and RT-PCR carried out to verify the viroids infectivity. Three species in *Cucurbitaceae*, such as cucumber (*Cucumis sativus*), Persian melon (*Cucumis melo* var. inodorus), watermelon (*Citrullus lanatus* var. lanatus), six species of *Solanaceae*, such as tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum* var. Turkish and *Nicotiana glutinosa*), potato (*Solanum tuberosum*), pepper (*Capsicum annuum*), petunia (*Petunia hybrida*); one species of *Fabaceae*, bean (*Phaseolus vulgaris*); one species of *Asteraceae*, purple passion (*Gynura aurantiaca*), were inoculated with *Agrobacterium* cells harboring the viroid constructs for the host range assay of CDVd, CBLVd and CVd-V (Table 2). At least two young plants from each species were inoculated. Back-inoculation was performed on the same plant species by mechanical inoculation of infected sap. Three weeks after inoculation, nucleic acids were extracted from non-inoculated newly grown leaves and the presence of viroids was proved by RT-PCR. Amplified products were cloned and sequenced.

Mechanical inoculation of infected plants sap and the monomeric plasmids. Sap of newly grown leaves of agroinfiltrated cucumber were prepared in 0.07 M Tris-HCl buffer pH 8.0 and then mechanically inoculated onto carborundum-dusted leaves of tomato plants. For mechanical inoculation, we used either cDNA inoculum (Podstolski *et al.,* 2005) of CVd-V, CBLVd and CDVd. For preparation of cDNAs inoculum, about 100 ng of plasmids containing CVd-V, CBLVd or CDVd sequences were linearized (Podstolski *et al.,* 2005) by *Bam*HI, diluted in water and mechanically rubbed on carborundum dusted leaves.

RNA extraction, RT-PCR, cloning and sequence analysis. Total RNA was extracted from 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 5 M potassium acetate was added and placed at 65°C for 20 min, then chilled on ice. The tube was centrifuged at 12000 rpm for 15 min and the supernatant was transferred to a new tube. Nucleic acids were precipitated by addition of 2.5 volume of absolute ethanol followed by 15 min centrifugation at 14,000 rpm (Bernard and Duran Vila, 2006). RT-PCR was performed using specific primer pairs for each of the viroids (Table 1). The RT reaction mixture of 20 μl contained 5 μl of total RNA, 2μl of MMuLV reverse transcription buffer, 1μl of reverse primer (10 pmol), 2μl of dNTP mix (40 mM), 0.5 μl of MMuLV reverse transcriptase (200 U/μl; Parstous, Iran). The RT reaction was incubated at 46°C for 1 h, followed by 10 min at 70°C for enzyme inactivation. PCR reaction was carried out using 4 μl of cDNA, 1μl of each specific primer pair (10 pmol) and 12.5 μl of ready to use PCR Master Mix (Ampliqon, Denmark) in a total volume of 25 μl. PCR parameters consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, specific annealing temperature (Table 1) for 30 s and 72°C for 1 min and a final extension step at 72°C for 5 min. PCR products were visualized on an agarose gel containing 0.2 μg of DNA green viewer™ (Parstous, Iran). The PCR products were

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

Name	Sequence (5'–3')	Target	Annealing temp. (°C)	Amplicon (bp)	Reference
CBLV-R CBLV-F	ACGACCAGTCAGCTCCTCTG CGTCGACGAAGGCTCGTCAGCT	CBLVd	60	319	designed by author
CDV-R CDV-F	CTCTGCGTTTTATTTTCGGCA AGGGAAAAGGGAACTTACCTGTC	CDVd	57	294	designed by author
CVdV-R CVdV-F	TCGACGAAGGCCGGTGAGCA CGACGACAGGTGAGTACTCTCTAC	CVd-V	60	294	Serra *et al*., 2008

ligated into pTZ57R/T cloning vector according to manufacturer's protocol (Thermo Scientific), transformed into competent cells of *E. coli* strain DH5α. Recombinant plasmids were purified from bacterial cells using Plasmid DNA isolation kit (Denazist). Finally, the purified recombinant plasmids were subjected to bidirectional sequencing using pUC-M13 universal primers using an ABI PRISM 377 apparatus by Macrogen Inc. (South Korea).

Dot blot hybridization. For dot blot hybridization, total nucleic acids were extracted at 3 weeks post innoculation (wpi). The purified nucleic acid treated by *Dnase* I (Sinaclone, Iran) followed by 10 min at 70°C for enzyme inactivation. DIG-labeled DNA probes were synthesized by PCR amplification of the cloned viroids 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. One microgram of total RNA was diluted with one volume of 1.2 x standard saline citrate (SSC×20: 3 M sodium chloride, 300 mM trisodium citrate, pH 7.0) containing 6% formamide and vacuum-blotted on nylon membrane which was treated with l0x SSC (10 min) before use. Membrane was then airdried and baked at 80°C for 2 h. Processing of the blots for prehybridization (4 h), hybridization (20 h) 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 (Murcia *et al.,* 2009).

Results

Infectivity of the viroids infectious clones

Initially, the binary vector containing full length of the viroids were agroinoculated to 5 cucumber and 5 tomato plants. Newly grown leaves were checked for the presence of the viroids by RT-PCR at 3 wpi. Amplification of 319, 294 and 294 bp product from all uninoculated newly grown leaves of cucumber and tomato plants revealed replication of CBLVd, CDVd and CVd-V in these plants, respectively (Fig. 1). No amplification was observed in non-inoculated plants. Also, CVd-V, CBLVd and CDVd were detected in upper leaves of *Poncirus trifoliata* plants by RT-PCR 3 wpi (Fig. 1).

For each viroid, the RT-PCR products from three randomly selected plants were sequenced. These results confirmed that the amplified fragments were identical to CBLVd, CDVd and CVd-V genome (data not shown). The inoculated plants also generated positive signal in dot blot hybridization (Fig. 1).

The plants were kept 5–6 wpi in order to observe any symptoms. CVd-V, CBLVd and CDVd developed symptoms in tomato plants (Fig. 2) but there were no visible symptoms observed on cucumber plants, meaning that cucumber is symptomless host for these viroids. Moreover, no visible symptoms were observed in the inoculated trifoliate orange until 9 wpi.

Apart from agroinoculation, CVd-V, CBLVd and CDVd were mechanically inoculated into 5 tomato plants using linearized plasmid containing the viroids genome; and 5 other tomato plants were inoculated using sap from agroinoculated cucumber plants. 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 efficiency of infectivity for three inoculation methods revealed that agroinfiltration and mechanical inoculation of sap were the best inoculation methods with approximately 100% efficiency (data not shown).

Identification of the experimental host range

Experimental host range of CVd-V, CBLVd and CDVd 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 CVd-V, CBLVd and CDVd (Fig. 1, Table 2).

As shown in Table 2, eleven species of herbaceous plants were susceptible to CVd-V. It replicated in *Cucumis sativus*, *Cucumis melo*, *Citrullus lanatus*, *Solanum lycopersicum*, *Ni-*

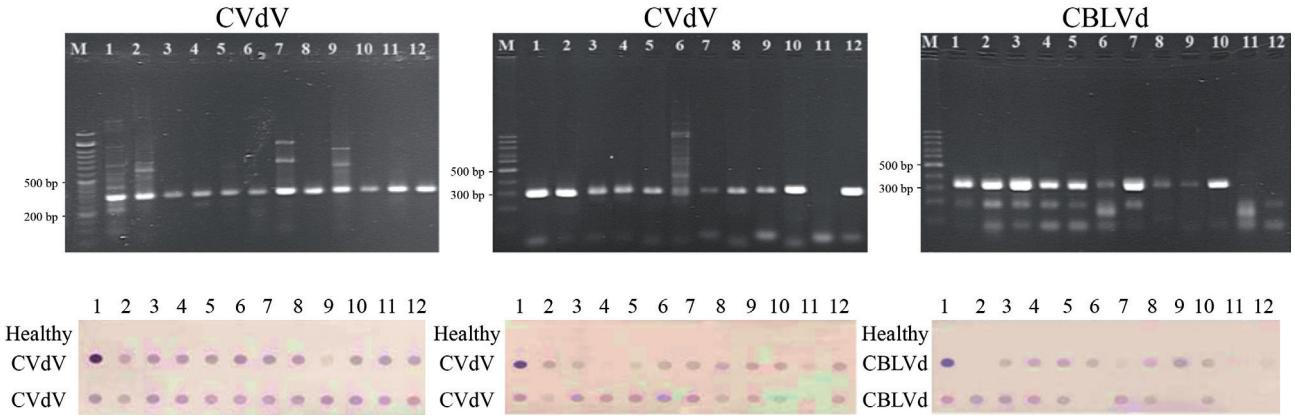

Fig. 1

Infectivity of the viroids in various inoculated plants

Identification of *de novo* population of CVd-V, CDVd and CBLVd in non-inoculated leaves of herbaceous plants using RT-PCR (top) and dot blot hybridization with the full-length DIG-labeled probe of the viroids (bottom), confirming the infectivity of the viroids in various inoculated plants. 1: Positive DNA control, 2: *Solanum lycopersicum*, 3: *Cucumis sativus*, 4: *Nicotiana tabacum* var. *Turkish,* 5: *Nicotiana glutinosa,* 6: *Phaseolus vulgaris*, 7: *Petunia hybrid*, 8: *Gynura aurantiaca*, 9: *Cucumis melo,* 10: *Citrullus lanatus* var. *lanatus*, 11: *Capsicum annuum,* 12: *Solanum tuberosum*. M: 100 bp DNA ladder.

Table 2. Transmission of apscaviroids infecting citrus to different herbaceous plants by agroinoculation of infectious clone and mechanical inoculation of infected sap

		CVd-V	CBLYd	CDVd
Cucurbitaceae	*Cucumis sativus*	5/5* (4/5**)	5/5 (5/5)	5/5 (4/5)
	Cucumis melo var. *indorus*	3/3 (3/3)	2/3 (3/3)	3/3 (2/3)
	Citrullus lanatus	2/3 (2/3)	3/3 (3/3)	3/3 (2/3)
Solanacea	*Solanum lycopersicum*	4/5 (3/3)	5/5 (3/3)	3/5 (2/3)
	Solanum tuberosum	4/4 (2/2)	0/4	3/4 (2/2)
	Capsicum annum	4/4 (3/3)	0/4	0/4
	Petonia hybrida	3/3 (2/3)	3/3 (3/3)	2/3 (3/3)
	Nicotiana glutinosa	3/3 (2/2)	3/3 (2/2)	3/3 (2/2)
	Nicotiana tabacum Var. turkish	3/3 (2/2)	2/3 (2/2)	2/3 (2/2)
Leguminosae	*Phasolis vulgaris*	4/4 (3/3)	3/4 (2/3)	3/4 (2/3)
Asteraceae	*Gynura aurantiaca*	3/3 (1/3)	3/3 (2/3)	2/3 (1/3)
Rutaceae	*Poncirus trifoliata*	2/2	2/2	2/2

*: No. positive plants/No. of agroinoculated plants; **: No. positive plants/No. of plants inoculated by infected sap of the same species.

cotiana tabacum, *Nicotiana glutinosa*, *Solanum tuberosum*, *Capsicum annuum*, *Petunia hybrida*; *Phaseolus vulgaris* and *Gynura aurantiaca* plants.

In the case of CBLVd, it replicated in nine herbaceous plant species, including cucumber, Persian melon, watermelon, tomato, *Nicotiana tabacum*, *Nicotiana glutinosa*, *Gynura aurantiaca*, petunia and common bean plants, but it did not infect pepper and potato plants (Table 2).

CDVd was infectious in ten out of eleven herbaceous species. It replicated in all inoculated plants, except pepper plants (Table 2).

Most of the infected plants were symptomless, with the exception of tomato and bean plants. In tomato, CDVd developed mottling and leaflet deformation, CBLVd induced epinasty and leaflet deformation and CVd-V generated mottling, epinasty, bushy growth, leaf deformation and leaf curl (Fig. 2, Table 3) within 2 months after inoculation. *Phaseolus vulgaris* plants infected with CVd-V, CBLVd and CDVd showed leaf crinkle, crazy top and leaf deformation, respectively at 2 months after inoculation (Fig. 2, Table 3). Cucumber plants only showed stunting 2 months after inoculation (data not shown). *Cucumis melo*, *Citrullus lanatus,*

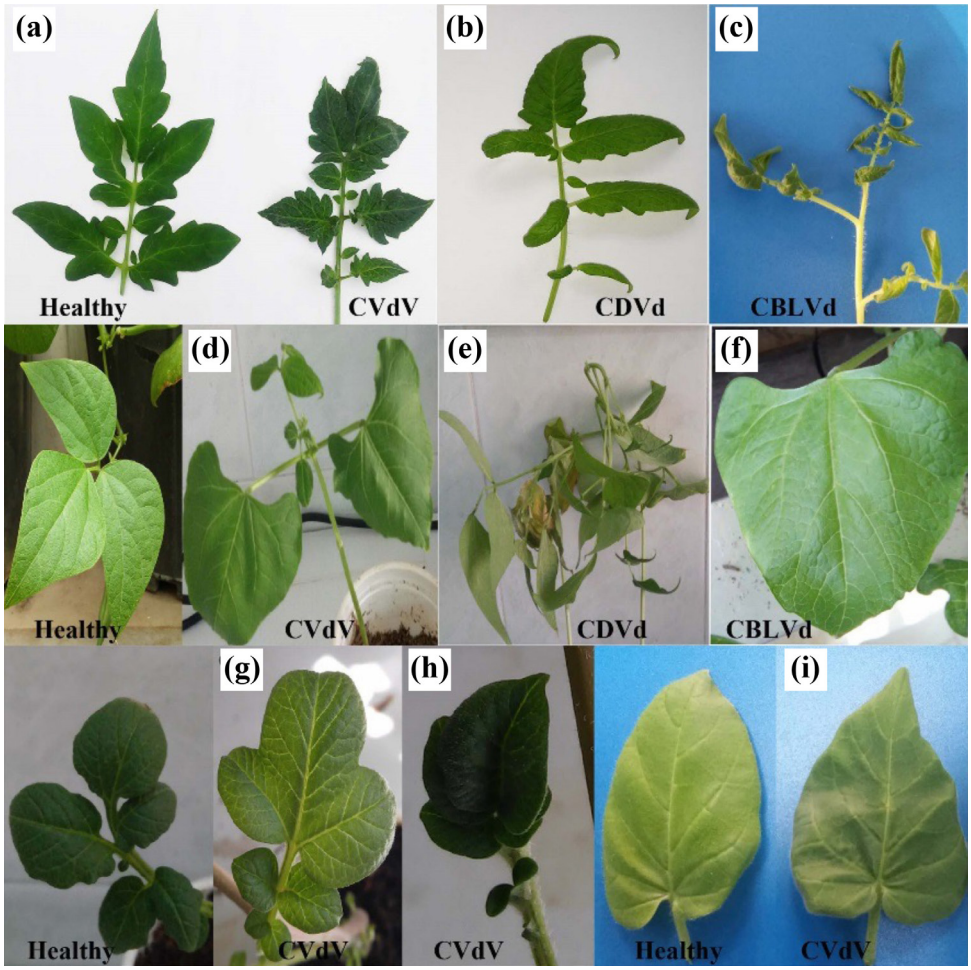

Fig. 2

Symptoms of CVd-V, CDVd and CBLVd in herbaceous hosts

As indicated by mottling and bushy growth of CVd-V **(a)**, mottling and leaf deformation and crinkle generated by CDVd **(b)**; leaf deformation and epinasty induced by CBLVd **(c)** in tomato; Open petiolar sinus and leaf crinkle induced by CVd-V **(d)** and CBLVd **(f)**, crazy top induced by CDVd **(e)** in bean; leaflet joining **(g)** and bushy growth of leaf **(h)** in potato and mottling and crinkle in *Nicotiana glutinosa* **(i)** induced by CVd-V.

Table 3. Symptoms of the citrus apscaviroids on experimental herbaceous hosts at 8 weeks after inoculation

		CVd-V	CBLVd	CDVd
Cucurbitaceae	*Cucumis sativus*	LD,M	SL	SL
	Cucumis melovar Indorus	SL	SL	SL
	Citrullus lanatus	SL	SL	SL
Solanaceae	*Solanum lycopersicum*	LC, LD, E, M, BG	E, LD	M, LJ
	Solanum tuberosum	LJ	--	SL
	Capsicum annum	SL	--	--
	Petunia hybrid	SL	SL	SL
	Nicotiana glutinosa	Th	SL	NT
	Nicotiana tabacum var. Turkish	SL	SL	SL
Leguminosae	*Phaseolus vulgaris*	LC, LD	V, C	CT
Asteraceae	*Gynura aurantiaca*	SL	--	--

E: epinasty; BG: bushy growth; LC: leaf curl; LD: leaf deformation; M: mottling; C: crinkle; CT: crazy top; V: vein banding; Th: increased in leaf thickness; LJ: leaflet joining; SL: symptomless; --: nonhost plant.

Nicotiana glutinosa, *Nicotiana tabacum* and *Petunia hybrida* showed no symptoms.

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

Citrus plants are harboring several viroid species belonging to the Pospiviroid, Hostuviroid, Cocadviroid and Apscaviroid genera. Naturally, members of the genus Apscaviroid have been reported to be restricted only 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 genetic information for apscaviroids is rather scant. To date, there have been no reports for CVd-V CBLVd and CDVd transmission to any herbaceous plant species. Infectivity of AGVd, ASSVd and GYSVd1 in herbaceous host plants (Lee *et al.,* 2015, Rezaian, 1990; Zakiaghl and Izadpanah, 2010; Walia *et al.,* 2014) showed that apscaviroids have wider host range.

To identify herbaceous hosts of citrus infecting apscaviroids, we constructed the infectious clones of CVd-V, CBLVd and CDVd in order to fulfill the Koch's postulates and to compare the host range of the viroids.

Numerous studies gave evidence that longer viroids or monomers of the viroids regulated by artificial promoter are infectious in plants (Daros and Flores, 2004; Gomez and Pallas, 2006; Gora-Sochacka *et al.*, 1997; Podstolski *et al.,* 2005; Rezaian 1999; Tabler *et al.,* 1992). In this study, we made monomeric construct under control of the 35S promoter for CVd-V CBLVd and CDVd. All of the DNA constructs were infectious in *Poncirus*, cucumber and tomato 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 CDVd, CBLVd and CVd-V. Past attempts had failed to transmit CVd-V to non-citrus species (Serra *et al.,* 2008).

In this study, eleven different herbaceous plant species are reported for the first time as experimental hosts for CVd-V, CBLVd and CDVd. However, only *Solanum lycopersicum* and *Phaseolus vulgaris* plants displayed visual symptoms (Table 3). These plant species were also symptomatic hosts for AGVd (Zakiaghl and Izadpanah, 2010) and ASSVd (Walia *et al.,* 2014). CVd-V showed mottling and leaf deformation but CBLVd and CDVd were symptomless in cucumber. Cucumber is also known as symptomless assay host plant of three other apscaviroids, such as Pear blister canker viroid (Flores *et al.,* 1991), AGVd (Rezaian *et al.,* 1990; Zakiaghl and Izadpanah, 2010) and ASSVd (Walia *et al.,* 2014). It seems that cucumber serves as a relatively good host plant for apscaviroids. In the case of *Nicotiana tabacum*, *Nicotiana glutinosa* and *Petunia hybrida,* replication of CDVd, CBLVd and CVd-V induced no symptoms. Similar results were obtained for ASSVd (Walia *et al*., 2014) and AGVd (Zakiaghl and Izadpanah, unpublished data).

In conclusion, we analyzed experimental host range of three apscaviroids naturally infecting citrus species. We have fulfilled the Koch's postulates in order to show that several herbaceous plants belonging to *Solanaceae, Fabaceae, Cucurbitaceae* and *Asteraceae* families, are systemic hosts of CVd-V, CBLVd and CDVd.

This study shows that apscaviroids infecting citrus have wide experimental host range. The availability of an infectious clone and wide host range in herbaceous plants will provide the basis for carrying out studies on the molecular biology, interaction and functional genomics for these viroids in the future

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