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Molecular and Biological Characterization of the Iranian Isolate of the *Australian Grapevine Viroid*

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ABSTRACT

***Australian grapevine viroid* (AGVd), an apscaviroid of the family *Pospiviroidae*, was recently identified in vineyards of southern Iran. It had a relatively wide host range and caused stunting, leaf deformation, mottling and vein clearing in experimental hosts upon mechanical inoculation of nucleic acid extracts or agroinfiltration of the viroid infectious cloned DNA. Predicted secondary structure of the AGVD-Ir showed a difference from the predicted structure of the type isolate in the viroid pathogenicity domain. Mutational analyses showed sequence changes introduced into that domain of the AGVD-Ir clone decreased the viroid's replication efficiency *in planta* but did not show any effects on its movement.**

Keywords: Grapevine viroids, Mutagenesis analysis, Host range, Replication efficiency, Viroid movement.

INTRODUCTION

Viroids are small covalently closed circular single stranded RNAs that infect many higher plants. Among them, *Grapevine yellow speckle viroid* 1 (GYSVd-1), *Grapevine yellow speckle viroid* 2 (GYSVd-2), *Hop stunt viroid* (HSVd) and *Citrus exocortis viroid* (CEVd) are reported to infect grapevine with worldwide distribution (Hadidi *et al.,* 2003). GYSVd-1, GYSVd-2 and HSVd were recently found in vineyards of southern Iran (Zaki-aghl and Izadpanah, 2004, 2005, 2006). *Australian grapevine viroid* (AGVd) was first reported from Australia in 1990. It was restricted to grapevine in nature (Rezaian, 1990). Analysis of the nucleotide sequence of this viroid suggested that it was a natural chimera between CEVd and GYSVd-1 (Rezaian, 1990). Based on the sequence of the central conserved region, AGVd was classified in the genus *Apscaviroid* of the family *Pospiviroidae* (Owens *et al*., 2011). Members of this family form rod-like secondary structure with five domains (Keese and Symons, 1985), which are involved in pathogenicity, replication and movement of the viroid in the plant (Gozmanova *et al., 2003*; Gora-Sochacka, 2004, Hadidi *et al*., 2003; Hammond and Owens, 1987; Koltunow and Rezaian, 1988; Owens *et al.,* 1995; Owens *et al.,* 1996; Qi and Ding 2002; Sano *et al.,* 1992; Zhong *et al.,* 2008).

AGVd was recently reported from China (Jiang *et al.,* 2009), Tunisia (Elleuch *et al.,* 2002, 2003) and the United States (Al Rwahnih *et al.,* 2009). This paper is a report of molecular and biological characterization and mutagenesis studies of an isolate of AGVd (AGVd-Ir) recently found in the Fars province in southern Iran.

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MATERIALS AND METHODS

Viroid Source and Mechanical Inoculation

Vines in the vineyards of Fars province in southern Iran were randomly sampled and examined for the presence of AGVd by RT-PCR using AGVd specific primers (Wan Chow Wah and Symons, 1997, Table 1). AGVd positive samples were used to inoculate cucumber and tomato seedlings. Initial transmission of AGVd was achieved by injection of stems with nucleic acid extracts from infected grapevines. For further mechanical inoculation of these plants, purified nucleic acid extracts from cucumber were rubbed on carborundum dusted leaves of test plants. The infected cucumber plants as well as the original grapevine samples were used for nucleic acid extraction and molecular studies.

cDNA Synthesis, Cloning and Sequence Analysis

Nucleic acid was extracted from tissues using a method described by Wan Chow Wah and Symons (1997) with slight modification. cDNA was initially generated from viroid RNA using Agv-C1 primer (Table 1). Two µL of the primer (10 µM) was mixed with 4 µL of nucleic acid preparation, heated at 70°C for 10 minutes and chilled on ice. Reverse transcription mixture (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 10 mM dithiothritol, 1mM each dNTP) and 200 units of MMuLV reverse transcriptase (Fermentas, Lithuania) were incubated at 42°C for 60 minutes to generate the first strand cDNA. Two µL of the first strand suspension was added to 9.3 µL PCR mixture of 10 mM Tris-HCl, 50 mM KCl, 1.76 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pM of each primer, 3% dimethyl sulphoxide (DMSO), 10% glycerol and one unit of *Taq* DNA polymerase (Cinagene, Iran). The mixture was subjected to an initial denaturation step at 94°C for 5 minutes and 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for one minute. The final cycle was followed by 5 minutes incubation at 72°C.

PCR products were visualized in 1.2% agarose gel containing 0.5 µg ml^{-1} ethidium bromide in TBE buffer. Purified PCR fragments were cloned into pTZ57R/T plasmid using InsT/A clone PCR cloning kit (Fermentas, Lithuania) and sequenced in both directions using an ABI PRISM system (Tech Dragon, Hong Kong). The data were analyzed by Vector NTI 9.1 package and aligned with other viroid sequences deposited in GenBank using BLAST program from the National Center for Biotechnology Information (NCBI). Phylogenetic analysis was performed using Neighbor Joining Method and the Molecular Evolutionary Genetics Analysis software ver.

Table 1. Oligonucleotide primers used for amplification of Iranian isolate of *Australian grapevine viroid*.

Primer	Sequence 5′ to 3′[b]	REN[c] site
Agv-H1[a]	*GTCGACGAAGGGTCCTCAGCAGAGCACC*	--
Agv-C1[a]	*CTCGACGACGAGTCGCCAGGTGAGTCTT*	--
AgvdetF	*GGCCCTGGGCACCAACTAGTGG*	--
AgvdetR	*TCCAAACAGGGGGTTCCAGGG*	--
Agv- F1	*AA**TCTAGA**GAAGGGTCCTCAGCAGAGCACCG*	*Xba*I
Agv- R1	*TT**GTCGAC**GACGAGTCGCCAGGTGAG*	*Sal*I
Agv- F2	*TT**GTCGAC**GAAGGGTCCTCAGCAGAGC*	*Sal*I
Agv- R2	*AA**AAGCTT**GACGACGAGTCGCCAGGTG*	*Hind*III
Mutation-F	*GAAGGCCGCGAAGCAGGGAAAGAAAAAG*	--
Mutation-R	*CTTTTTCTTTCCCTGCTTCGCGGCCTTC*	--

[a] Primer pair selected from Wan Chow Wah and Symons (1997); [b] Bold letters show restriction site of enzymes; underlined letters show mutant nucleotides in the primer, [c] Restriction endonuclease.

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4 (MEGA4) (Tamura *et al*., 2007). The predicted RNA secondary structure of the AGVd sequences was obtained using RNA draw v 1.1 b2 program.

Construction of Dimer Clone of the Viroid

Full length dimer of AGVd-Ir (GenBank Acc. No. FJ940923) DNA was constructed by the amplification of two complete monomer DNAs with primer pairs Agv-F1/R1 and Agv-F2/R2 (Table 1) in a buffer containing 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO4$, 10 mM KCl, 1.5 mM $MgSO_4$, 0.2 mM dNTPs, 0.5 µM of each primer and 1 U of *Pfu* DNA polymerase (Fermentas, Lithuania). PCR parameters were described earlier. The PCR product was electrophoresed in 1.2% agarose gel. The DNA fragments which shared a *Sal*I site present in the upper CCR of AGVd, were separately digested with *Xba*I/*Sal*I or *Sal*I/*Hind*III and purified using the PCR Purification Kit (Bioneer) as per the manufacturer's protocol. Further, the fragments were ligated into pTZ57R vector previously digested with *Xba*I/*Hind*III. The resulting plasmid containing the dimer DNA of AGVd-Ir, designated as pTAGVd-Ir2.0, was sequenced. The dimer construct was released from pTAGVd-Ir2.0 by digestion with *Eco*R1/*Hind*III and sub-cloned into the corresponding sites of pGreen0029 binary vector (Hellens *et al.,* 2000) to form pGAGVd-Ir2.0. The resulting plasmid was transformed into *Agrobacterium tumefaciens* strain C58C1 by electroporation (Gardner *et al*., 1986; Wang, 2006). Recombinant clones were incubated in liquid SOC in the presence of 50 µg ml^{-1} rifampicin and kanamycin at 28°C with agitation until the OD_{600} reached 1.5-2, then agroinfiltrated to the test plants to verify infectivity of the constructs.

Infectivity Test and Host Range Determination

Cucumber (*Cucumis sativus*) and tomato (*Solanum lycopersicum*) plants were used in infectivity tests as suggested by Rezaian (1990). In addition, squash (*Cucurbita pepo*), purple passion (*Gynura aurantiaca*), tobacco (*Nicotiana tabacum* var. Turkish and *N. glutinosa*) and pot marigold (*Calendula officinalis*) were inoculated for host range determination. The plants were inoculated by the agroinfiltration of dimer construct into the leaves. Nucleic acids were extracted from new leaves of inoculated plants as described earlier and examined for the presence of the *de novo* populations of the viroid in non-inoculated leaves. RT-PCR using Agv-H1/Agv-C1 primer pair (Table 1) and dot blot hybridization using a full length DIG-labeled AGVd-Ir specific probe (Mumford *et al*., 2000; Nakahara *et al*., 1998) were used to detect the viroid in inoculated plants at 4 and 5 weeks postinoculation (wpi), respectively. Hybridization results were analyzed by TotalLab V1.10.

Point Mutation Analysis

The extra loop in the P-domain of AGVd-Ir secondary structure was disrupted using Quick-change® II XL site directed mutagenesis kit (Stratagene) (Sanjuan and Daros 2007). Mutant PCR products were prepared using a PCR mixture of 2 µL (10 ng) pGAGVd-Ir2.0 as DNA template, 2.5 µL (125 ng) of each mutation primer (Table 1), 1 µL of dNTP mix (10 mM), 1 Ml (2.5 U μL^{-1}) of *PfuUltra* HF DNA polymerase, 3 µL of QuikSolution and 5 µL of 10× reaction buffer. PCR conditions were incubated 1 min at 95°C as initial denaturation followed by 18 cycles at 95°C for 50 seconds, 60°C for 50 seconds and 68°C for 5.24 minutes, with a final extension of 7 minutes at 68°C. One µL (10 U μl^{-1}) of the *Dpn* I restriction endonuclease was added directly to the PCR Product and incubated at 37°C for 2 hours to digest the parental (i.e., the non-mutated) supercoiled dsDNA. Mutated product was transformed into *E. coli* XL10-Gold competent cells for nick repair and plasmid propagation. Transformed cells were spread on an LB plate containing 10 µg mL^{-1} tetracycline and 50 µg mL^{-1} kanamycin and incubated at

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37°C for 12 hours. The integrity of the mutant was confirmed by sequence analysis. The resulting construct was introduced into *Agrobacterium* using electroporation and was inoculated to cucumber plants as described earlier.

Replication of the mutant was assayed at 2 wpi in inoculated cucumber cotyledons using a quantitative RT-PCR system (Hayward-Lester *et al*., 1995; Qi and Ding 2002; Wang *et al.,* 1989). RNA was extracted using Invisorb® spin virus RNA mini kit (Invitek) as outlined by the manufacturer. The preparations were treated by *DNase*I to confirm elimination of injected plasmids. RT-PCR was carried out using AgvdetF/AgvdetR primers (Table 1). Modified Solaris qPCR gene expression assays protocol (Thermo Scientific) was used to assess the replication of AGVd-Ir. PCR products were resolved in 3% agarose gel and intensity of the bands was determined using MCID® software to quantify the replication efficiency of the viroid. Five replicates were made for each sample. Normalization of the data and calibration were carried out by comparison with healthy and template dilution series (Hayward-Lester *et al*., 1995; Qi and Ding, 2002; Wang *et al.,* 1989).

The accumulation of the mutant viroid was also assayed in young expanding leaves of inoculated cucumber plants at 4 wpi to verify systemic infection and movement of the viroid by dot blot hybridization using an AGVd-Ir specific probe (Mumford *et al*., 2000; Nakahara *et al*., 1998). Hybridization results were analyzed by TotalLab V1.10.

The mutant construct was agroinoculated to tomato and *N. glutinosa* plants to determine their reaction to the mutant.

RESULTS

Occurrence and Mechanical Transmission of AGVd

Australian grapevine viroid (AGVd-Ir) was found in 6 of 32 samples (18.7%) analyzed for the presence of the viroid in the Fars province of Iran. No specific symptoms could be attributed to the viroid in the grapevines.

Total nucleic acid extracts from AGVd infected grapevines were found to be infectious when mechanically inoculated to cucumber and tomato seedlings and induced stunting, leaf deformation and mottling (data not shown). Systemic symptoms appeared at 4 wpi. Infection of inoculated plants was verified by RT-PCR analyses.

Molecular Characterization of AGVd-Ir

PCR products obtained from grapevine were cloned and four independent full length clones were sequenced. The data confirmed that Iranian isolates of AGVd consisted of either 369 or 371 nucleotides.

Sequence analysis showed that full length sequence of Iranian isolates of AGVd had 95-97% nucleotide sequence identity when compared with other AGVd sequences deposited in GenBank. Phylogenetic analyses showed that despite minor differences, the Iranian isolates were closely related, but could be distinguished from other isolates of AGVd reported from Australia, China and Tunisia. As shown in Figure 1, AGVd variants of Iran are similar to Chinese isolates. A 371 base isolate designated as AGVd-Ir (GenBank Acc. No. FJ940923) was used in further analyses.

Analysis of predicted secondary structure of the AGVd-Ir and Australian (type) isolates of AGVd showed that both isolates were identical in the right hand portion of the CCR except for a change of U211A. However, the AGVd-Ir differed from the type isolate in pathogenicity domain where the two additional nucleotides caused formation of an extra loop (Figure 2). The required free energy for secondary structure formation at 37°C was -106.22 and -100.2 kcal for type strain and AGVd-Ir, respectively. The secondary structure of the Iranian isolates with 369 bases was similar to that of type strain. No difference was

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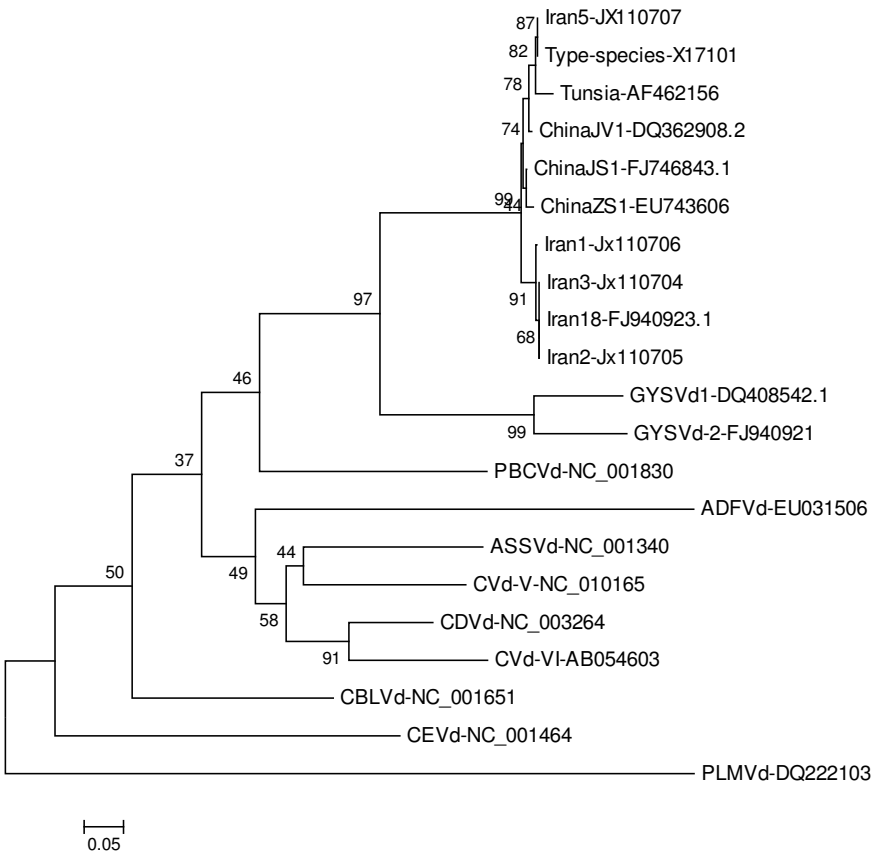

Figure 1. Phylogentic tree of Iranian and other isolates of AGVd. The tree was constructed by neighbor joining (NJ) method using MEGA 5 program. Numbers in the branches indicate bootstrap support from NJ (1,000 replicates, 10,000 seeds). AGVd variants from Iran are clearly distinguished from other variants and show a closer relationship with Chinese isolates. *Citrus exocortis viroid* (CEVd) and *Peach latent mosaic viroid* (PLMVd) are used as outgroups.

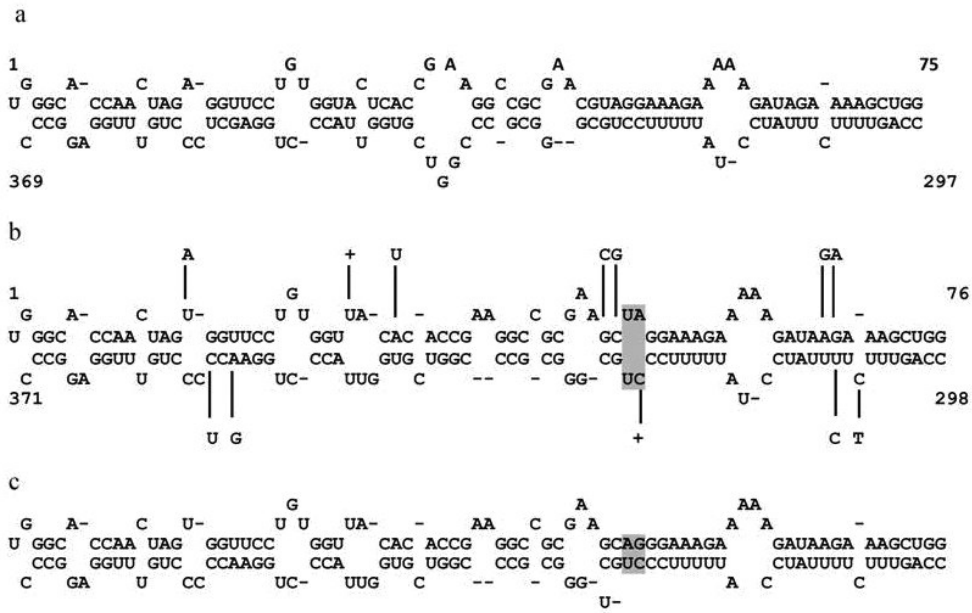

Figure 2. P-domain of predicted secondary structure of Type (A) (Rezaian 1990) and Iranian isolate (B, intact and C, mutant) of *Australian grapevine viroid*. Vertical bars show points of difference of Iranian isolate from the type isolate. Gray box in C shows nucleotide changes in the mutant. Extra loop in AGVd-Ir is shown as gray box in B.

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observed in the terminal conserved region (TCR) between Iranian and other isolates.

Infectivity of AGVd-Ir Cloned Genome

PCR products of expected size were obtained with AGVd specific primers when extracts from naturally infected grapevine or mechanically inoculated cucumber were used as template (data not shown). AgvdetF/AgvdetR primer pair was used to detect AGVd-Ir in agroinoculated cucumber and tomato plants (Figure 3-a). The viroid was not detectable in agroinoculated cucumber cotyledons at 1 wpi. However, it was readily detected at 2 wpi. In non-inoculated true leaves, the viroid was hardly detectable at 3 wpi but positive results were obtained at 4 wpi (Figure 3-b). Sequencing of PCR products from cucumber and tomato confirmed that *de novo* populations of AGVd-Ir were generated in those hosts, and the extra loop was present in *de novo* populations.

AGVd-Ir induced stunting, but no other obvious symptoms in cucumber plants. It induced stunting, leaflet deformation and mottling in inoculated tomato plants (Figures 4-a and 4-b). Symptoms developed in infected tomato at 6 wpi. Infected plants showed symptoms similar to those observed in plants inoculated with purified nucleic acid extracted from infected grapevine.

AGVd-Ir replicated in squash (2/3) (infected plants/inoculated plants), purple passion (2/2), pot marigold (2/5) and *N. glutinosa* (4/4) as confirmed by RT-PCR (Fig. 3c) and dot blot hybridization. Despite replication in squash and pot marigold, AGVd-Ir induced no obvious symptoms in these plants. Twisting and leaf edge sharpening were observed in infected purple passion (Figure 4-c); infected *N. glutinosa* showed mottling and faint vein clearing (Figure 5-d). No infections were found in inoculated *N. tabacum* var. Turkish plants (0/4).

Mutation Analysis

Sequencing data and secondary structure analysis showed that U50A and A51G changes in the genome of AGVd-Ir, resulted in disruption of the extra loop and increased base pairing in P-domain of the viroid (Figure 2-c). These changes increased the required free energy of secondary structure formation in the mutant up to -109.39 kcal at 37°C compared to the wild type.

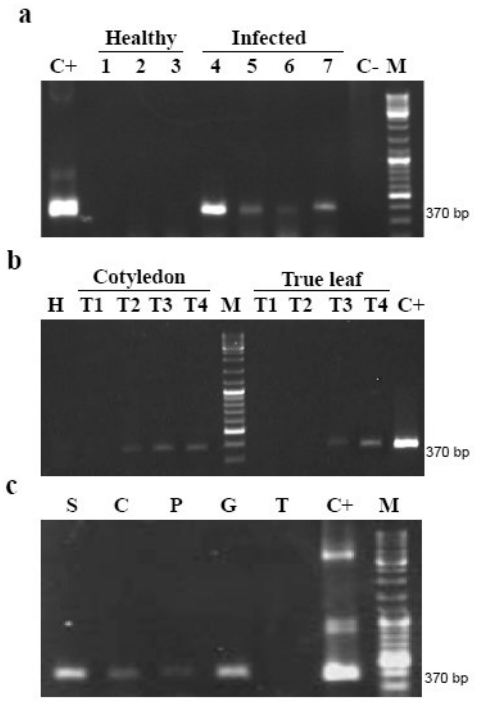

Figure 3. Electrophoresis pattern of PCR products from: (a) Healthy (lanes 1-3) and AGVd-Ir agroinfiltrated (lanes 4-7) cucumber plants at 4 wpi; (b) AGVd-Ir agroinoculated cotyledons and non-inoculated true leaves of cucumber plants in time course infectivity assay (T1, T2, T3 and T4 are sampling times at 1, 2, 3 and 4 wpi, respectively), and (c) AGVd-Ir agroinoculated squash (S), pot marigold (C), purple passion (P), *Nicotiana glutinosa* (G) and *Nicotiana tabacum* var Turkish (T) plants using AgvH1/AgvC1 primer pair. C-: Negative control (water); C+: Positive control (AGVd-Ir infectious cloned DNA), M: the Generuler ™ 1k base DNA ladder (Fermentas).

Infectivity assay showed that the mutant construct was still infectious as verified by RT-PCR of inoculated plants. The symptoms of the mutant construct on cucumber were similar to those induced by the wild type construct. Tomato plants, in addition to stunting and leaf deformation, showed faint vein clearing (Figures 5a-c). However, the symptoms appeared less severe in tomato plants infected with the

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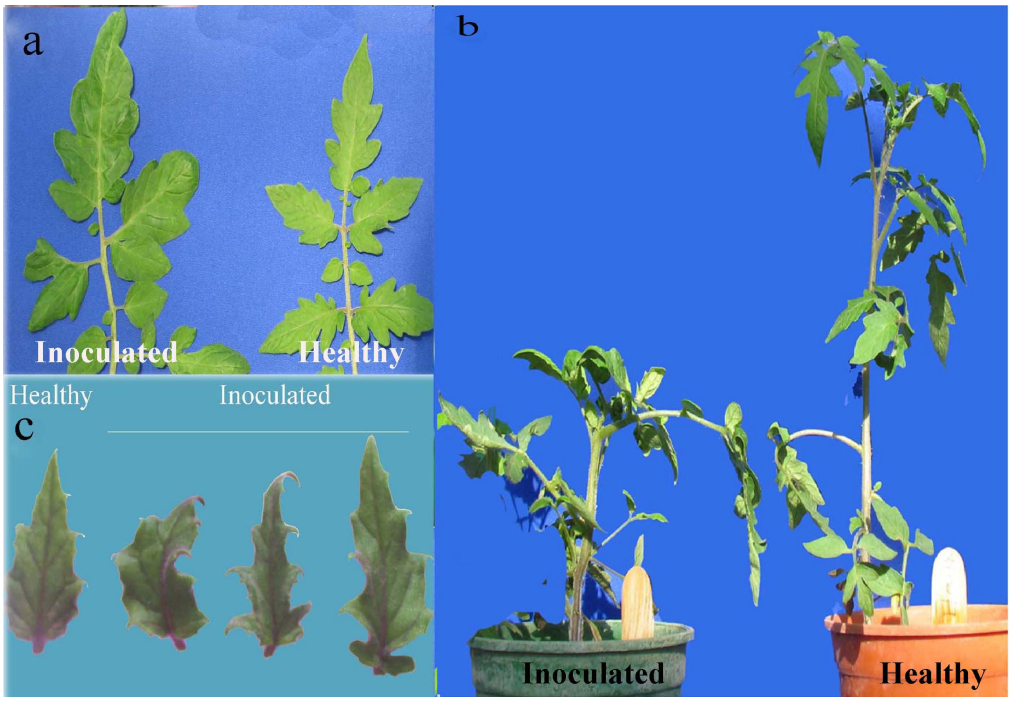

Figure 4. Symptoms induced by cDNA construct of AGVd-Ir in agroinoculated plants: (a) Mottling and leaf deformation in tomato; (b) Stunting in infected tomato, © Leaf deformation and sharpening of edges in infected purple passion.

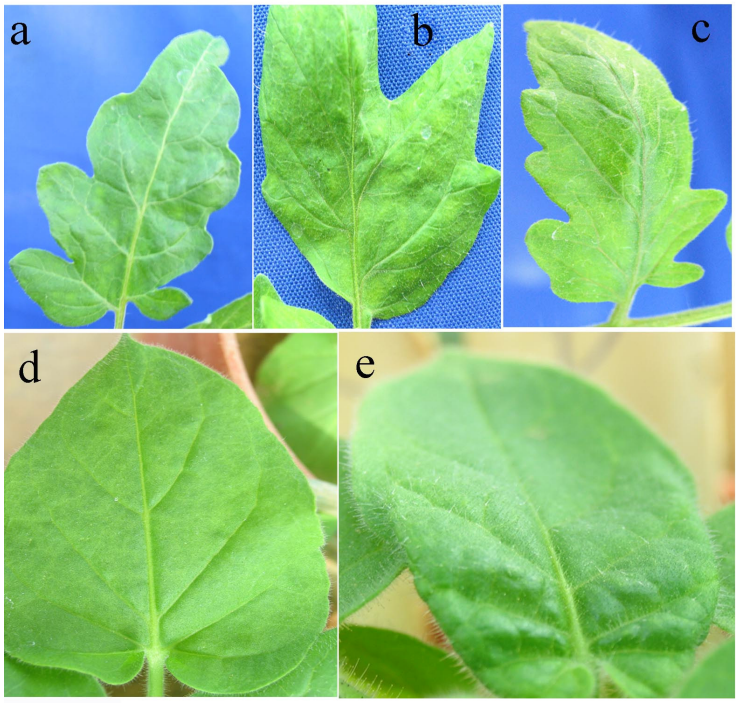

Figure 5. Symptoms induced by infectious intact (a, d) and mutant (b, c, e) constructs of AGVd-Ir in tomato (a-c) and *N. glutinosa* (d, e).

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mutant construct compared to those infected with the wild type construct. *N. glutinosa* plants showed rugosity in addition to mottling and vein clearing (Figures 5-d and 5-e) when inoculated with mutant construct.

Efficiency of replication of the mutant in cucumber was about 24% lower than that of the wild type construct, but the movement of the mutant AGVd-Ir construct was not affected significantly (at 5% level), i.e., it became systemic in most inoculated plants (Table 2).

DISCUSSION

AGVd was reported from Australia (Rezaian, 1990), and recently from China (Jiang *et al.,* 2009), Tunisia (Elleuch *et al.,* 2002, 2003) and the United States (Al Rwahnih *et al.,* 2009). It was first reported from Iran in 2009 (Zaki-aghl and Izadpanah, 2009). It seems that this viroid has worldwide distribution although it is less frequent than other grapevine viroids (Jiang *et al*., 2009, Zaki-Aghl and Izadpanah, 2009).

Infectivity of AGVd to cucumber was verified previously by detection of native RNA in leaves of inoculated plants (Rezaian *et al.,* 1988; Rezaian, 1990). In this paper, we established the infectivity of AGVd-Ir by an artificial cDNA construct for the first time. This method can solve problems in studying the biology of AGVd previously hampered by CEVd and HSVd contamination (Rezaian *et al.,* 1988). It also provides a facility for reverse genetics studies of this viroid.

Infection of squash, purple passion, pot marigold and *N. glutinosa* by AGVd-Ir is reported for the first time in the present research. It shows that AGVd has a wider experimental host range than it was thought earlier.

Although AGVd isolates generally show a low level of variation (Jiang *et al.,* 2009), some Iranian isolates appear to be different from the type isolate (Rezaian 1990) in size and secondary structure (Keese and Symons, 1985). This may be the reason why AGVd-Ir induces symptoms somewhat different from those of the type isolate, especially in tomato (Owens *et al.,* 1996). Variations in P-domain in other viroids are known to affect replication efficiency, symptom expression and host range (Gora-Sochacka, 2004; Owens *et al.,* 1995; Owens *et al.,* 1996; Qi and Ding, 2002; Rigden and Rezaian 1993; Szychowski *et al.,* 1998; Zhong *et al.,* 2008). However, in pospiviroids secondary structure of VM (virulence module) region, a motif located in the P-domain of the viroid, controls symptom severity (Gora-Sochacka, 2004; Hammond and Owens, 1987; Owens *et al.,* 1995; Owens *et al.,* 1996). Mutation in this region reduces replication efficiency and abolishes the movement of *Potato spindle tuber viroid* (PSTVd) (Zhong *et al.,* 2008). This domain is not known in apscaviroids; but in GYSVd-1, a member of the genus *Apscaviroid, the* absence of speckle symptoms has been attributed to increased base pairing in the P-domain (Koltunow and Rezaian, 1988; Rigden and Rezaian, 1993;

Table 2. Comparison of the infectivity data obtained by agroinfiltration of cucumber plants with the wild type and mutant infectious constructs of AGVd-Ir.

Infections construct	Length of the first internode (millimeter)	Replication efficiency (%)[a]	% Trafficking[a]	Number of systemically infected plants/Numbers of inoculated plants
Wild type	10.58*	100	100	12/12
Mutant	10.27*	76*	92[Ns]	11/12

[a] Replication and trafficking assay were performed at 2 and 4 wpi using RT-qPCR and dot blot hybridization, respectively.
*Difference was significant at 5% level.
[Ns] No significant difference observed.

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Szychowski *et al.,* 1998). Similar to PSTVd (Zhong *et al*., 2008), sequence changes in P-domain of AGVd-Ir genome did not affect systemic movement of the viroid significantly but reduced the viroid titer and the severity of the symptoms in plants (Table 2). However, other investigators have reported no clear correlation between viroid titer and symptom severity (Ding and Itaya, 2007; Flores *et al*., 2005; Gora-Sochacka, 2004; Owens and Hammond, 2009; Tabler and Tsagris, 2004).

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تعیین خصوصیات مولکولی و بیولوژیکی جدایه ایرانی ویروئید استرالیائی مو

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چکیده

ویروئید استرالیائی مو(*Australian grapevine viroid*) یک اپسکاویروئید از تیره *Pospiviroidae* است که برای اولین مرتبه در تاکستانهای جنوب ایران یافت شد. این جدایه از این ویروئید دارای دامنه میزبانی وسیعی بود و پس از مایه‌زنی مکانیکی نوکلئیک اسید خالص سازی شده یا مایه زنی اگروباکتریومی همسانه عفونت زای ویروئید، علائمی از قبیل کوتولگی، پیچیدگی برگ، پیسک و رگبرگ روشنی را در میزبان‌های آزمایشگاهی تولید کرد. در مقایسه با سویه تیپ، جدایه ایرانی ویروئید استرالیائی مو (AGVd-Ir) دارای ساختمان ثانویه‌ای متفاوت با یک لوپ بیشتر در ناحیه بیماری زائی است. آنالیزهای موتانت با استفاده از همسانه عفونت زای AGVd-Ir نشان داد که حذف این لوپ باعث کاهش نرخ همانندسازی در ویروئید می‌شود لیکن بر میزان حرکت سیستمیک آن در گیاه تاثیری ندارد.

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