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Original Article

Citrus Bent Leaf Viroid from Fars Province of Iran

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Abstract

Background and Aims: CBLVd has been reported from northern of Iran previously. The aim of this study was evaluating new viroid variant from asymptomatic citrus trees of Fars province and comparing with its other isolates.

Materials and Methods: In this study a number of citrus trees without symptoms were sampled and subjected to RNA extraction followed by RT-PCR, cloning and sequencing of PCR products. Molecular properties of viroid variants were compared.

Results: A novel variant of CBLVd from Fars was achieved and used for comparison with a number of other CBLVd sequences from GenBank. It was shown that on the basis of sequence homology, CBLVd isolate from Fars province and two CBLVd isolates reported from Pakistan (Punjab) clustered in one group. It is supposed that these variants possibly will have same origin and probably imported from Pakistan to Iran by infected plant tissues or transplants.

Conclusions: Therefore, we propose that the government regulates the importation of plants and plant products under the precise and rigorous authority of the plant protection institutes.

Keywords: Citrus viroids, CBLVd, phylogeny, plant products importation

Introduction

Viroids are the smallest phytopathogens containing circular single stranded RNA molecules with a length of 246 to 401 nucleotides, with numerous secondary structures and without any coat protein and open reading frame (1; 2). So far some citrus viroid is reported belonging to the Pospiviroidae family and its four genuses are Pospiviroid, Apscaviroid, Hostuviroid, and Cocaviroid (3).

The number one citrus viroid (CVd-I) was firstly reported from citron which induces highly specific instability symptoms such as midvein and petiole necrosis and small pores in the skin (4). Host range of CVd-I is restricted to citrus and is transferred to avocado through grafting, just under the laboratory conditions (5).

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CVd-I includes two discrete groups CV-Ia and CV-Ib, representing two separate bands in sequential polyacrylamide-gel electrophoresis (6). Deep cavities with their bumps against the trifoliate orange rootstocks along with canopy decrease of the plant are defined as the symptoms of CVd-Ia infected trees. CVd-Ib (CBLVd; citrus bent leaf viroid) induces medium epinasty, point necrosis of mid vein in Etrog citrons and mild stunting in the citrus trees grown on the trifoliate orange rootstocks (7). In terms of sequence, CVd-Ia variants have 327 – 329 nucleotides and CVd-Ib variants contain 315 – 319 nucleotides (3).

CBLVd was firstly reported from the citrus trees in Mazandaran by Alavi and coworkers. They transplanted grafts of some Unshiu citrus trees grown on the rootstocks of trifoliate orange with bark scaling on the rootstocks and stunting symptoms on Etrog citrons (Arizona 861-S1) and kept in greenhouse. They could observe mild to severe symptoms of leaf bending in the grafted plants during the next plant growth seasons. Moreover, using the RT-PCR test and sequencing definition of the

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proliferated band they prove the existence of the mentioned viroids in citrus of Mazandaran (8). In this study, a new viroid variant is extracted from asymptomatic citrus trees of Fars province and is compared with its other isolates.

Methods

Sampling. In the summer 2008, different areas of citrus planting in Jahrom city were visited and five sweet lime trees were sampled. The samples were of no specific symptoms. Some young foliage was picked up from the trees and after freezing in liquid nitrogen, they were maintained at temperature of -70OC for the further tests.

RNA extraction. Extracting RNA from plant by Yang and his associates (9) in a way that Alavi and his colleagues (8) performed was conducted with a little change. All steps were done in sterile conditions and all used solutions were prepared by sterile water treated by DEPC. Five microliters of extracted RNA was utilized in 25 microliters volume of polymerase chain reaction (PCR).

Reverse transcription polymerase chain reaction and polymerase chain reaction. PCR and RT- PCR reactions were conducted applying a specific primer pair of citrus bent leaf viroid (10) consisting of reverse primer (5′-TTCGTCGACGACGACCAGTC-3′) and forward primer (5′-GGCTCGTCAGCTGCGGAGGT-3′) which amplify fragments with about 230 base pair size. Reverse transcription was performed in a 20 µl volume containing four µl extracted RNA, one µM of reverse primer, one µM of each four deoxynucleotide triphosphates, ten mM of dithiothreitol, 4.5 µl MMuLV specific buffer (5X) and one µl of MMuLV-Reverse transcriptase enzyme (200 U/µl). For this purpose the mixture of RNA and reverse primer at the temperature of 70°C was placed in a thermocycler with a cover temperature of 105°C for ten minutes so that RNA denaturized and then quickly it was put in ice water for three minutes. After centrifuging for a few seconds, the rest of material was added to the micro-tube. After two or three times pipetting smoothly and centrifuging for a few seconds, the mixture was maintained in the thermocycler at 42 OC for two hours. Afterwards it was kept at 94 OC for ten minutes and then quickly cooled on ice for three minutes. Five µl of the mixture with one µl of each primers (10 µM), 0.5 µl of the four deoxynucleotide triphosphates (10 mM), one µl of dithiothreitol (10 mM), 0.75 ml MgCl2 (50mM), 2.5 ml of Taq DNA polymerase specific buffer (10 X) and 0.25 ml of Taq DNA polymerase enzyme (5U/ml), were applied in the 25 ml polymerase chain reaction.

All materials used in the reverse transcription polymerase chain reaction and the polymerase chain reaction were provided from Fermentas and CinaGen companies.

Temperature cycle for the polymerase chain reaction was a program cycle consisting of 90°C for 30 seconds and 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for one minute. After the last cycle, the mixture was maintained at a temperature of 72°C for five minutes.

PCR product was analyzed by electrophoresis in agarose gel 1% in TBE buffer (10.8 gr Tris, 5.5 gr boric acid and 0.73 gr EDTA in 1000 ml distilled water, pH=8.3). After gel staining with ethidium bromide, nucleic acid bands were observed by the UV transilluminator and the gel was photographed by gel documentation equipment.

Cloning. The PCR product was purified utilizing High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions. The refined product of PCR reaction and was placed in plasmid vector pTZ57R/T then was transferred to the E. coli bacteria according to the Fermentas Kits instructions. Therefore the mixture of plasmid and PCR product containing three µl plasmid vector pTZ57R/T, four µl of PCR product, three µl of Ligation buffer 10 X, three µl PEG solution 4000 (50w/v), one µl of T4 DNA Ligase (five U/µl) and 16 µl distilled sterile water for inducting cDNA into the plasmid vector, was kept at 22°C for one night.

Applying Ins T/A Clone PCR Product Cloning Kit (Fermentas) the recombinant plasmid was transferred to the E. coli bacteria according to

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the instructions. After being cultured in solid LB medium including 100 µg/ml ampicillin antibiotics, 20 µg/ml IPTG and 20 µg/ml X-gel for one night, white colonies which are supposed to contain recombinant plasmid were taken and cultured separately in five ml of liquid LB medium including 100 µg/ml ampicillin, and maintained in shake incubator at 37°C for a night. Boiling method (11) was applied to extract DNA of recombinant plasmid from the bacterial cells. For approval of the details entered into plasmid, one µg of the extracted DNA of the recombinant plasmid was added to the PCR reaction mixture, according to the mentioned conditions, and the results were analyzed by electrophoresis after conducting PCR.

Nucleotide sequencing. After verification of the cloned pieces, bacteria containing the recombinant plasmid was selected and re-cultured and its plasmid was extracted using High Pure Plasmid Isolation Kit (Fermentas) according to the manufacturer's instructions and was re-analyzed with the enzymatic digestion method. All cases, after screening for each cloned product which contains the desired components were sent to Macrogen Company in South Korea in order to sequence using the specific primer of citrus bent leaf viroid (10). Sequencing was performed in two opposite directions. The obtained sequence(s) was (were) compared with the NCBI sequences in GenBank, via BLAST software.

Sequence analysis. Phylogenetic analysis, sequence homology of multiple alignments of the genomes and calculation of the amount of genetic variations among the CBLVd-Iran isolate(s) of Fars province and other parts of the world (Table 1) was performed using the Vector NTI 9 software (InforMax, Bethesda, MD). Applying the neighbor-joining method (12) phylogenetic tree was depicted via 10 000 replicates.

Table 1: Characteristics of CBLVd isolates used in this study.

Isolate	Accession no.	Host	Origin	Number of nucleotides
CBLVd Ia-Jp	AB006734	Citrus clementina	Japan	328
CBLVd Ia-P2	AB006735	Citrus madurensis	China	329
CBLVd-E83	AB054636	Citrus	Japan	328
CBLVd-201	AF428052	Citrus	Uruguay	318
CBLVd-205	AF428055	Citrus	Uruguay	318
CVd Ia-20	AY226156	Citrus	Spain	327
CVd Ia-21	AY226162	Citrus	Spain	328
CBLVd-225	CBU21125	Citrus	Israel	315
CBLVd-CTB	M74065	Citrus	Israel	318
CBLVd-CQ	EU382212	Citrus	China	330
CBLVd-E117	EU872278	Citrus	Spain	328
CBLVd-A33	FJ773265	Citrus	Pakistan	328
CBLVd-A	FJ773267	Citrus	Pakistan (Punjab)	328
CBLVd-NC	NC-001651	Citrus	Israel	315
CEVd-1 (out group)	FJ626865	Sweet lime	Iran (Fars)	370

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Results and discussion

Reverse transcription polymerase chain reaction. Conducting the reverse transcription polymerase chain reaction with a specific primer of citrus bent leaf viroid led to amplification of 230 nucleotides fragments in size, in both sweet lime trees from Jahrom (Fig. 1, lanes one and three to five, repetition of each samples). The resulting segments were successfully cloned and sequenced in two directions. The outcomes revealed that the nucleotide sequences in primers joining locations of the sequenced clones are the same as the utilized primers. By comparing the obtained sequence with the GenBank database using BLAST software its proximity to different variants of citrus bent leaf viroids was proved.

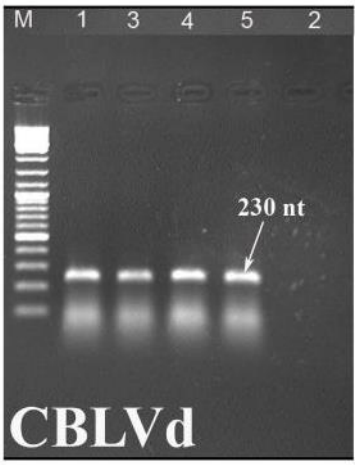

Fig. 1. Electrophoresis pattern of DNA fragments amplified in RT-PCR with a specific CBLVd primer pair. 1-5 sweet orange trees from Jahrom; No band was observed in healthy control sample (lane 2). M= marker.

Analysis of nucleotide sequence and RNA secondary structure. In all cases, the amplified segments were about 230 nucleotides and the relevant sequence was established in GenBank (EMBL, GenBank and DDBJ databease) with accession number of GQ166528 and a name of CBLVd-Iran. The primary structure and comparison of multiple alignments of the nucleotide sequence of CBLVd-Iran with other isolates of this viroid (Table 1) are illustrated in Fig. 2.

Since the designed primers for CBLVd in this study cannot amplify whole genome of this viroid, different analysis were performed only based on 230 nucleotides of the 330 genome nucleotides of the available isolates of CBLVd. Based on the comparison and multiple alignments of incomplete genome sequences of CBLVd-Iran with other variants of this viroid (Table 2 and Fig. 2), it was demonstrated that CBLVd-Iran is mostly like (98.3%) CBLVd-A33 (reported from Punjab Pakistan) and in phylogenetic terms belongs to a group with the reported isolates from Punjab Pakistan (Fig. 3) and this may indicate similar origin for these isolates.

It is assumed that CBLVd-Iran perhaps imported from Pakistan to Iran by the infected plant tissues. Therefore, with the increase in global trade, there is a corresponding increase in awareness of the need to prevent the introduction of potentially damaging plant pathogens by plant products importation. For this responsibility, government departments have been perform free of charge inspection and/or testing in accordance with appropriate official procedures and certify that the plants, plant products or other regulated articles are free from the quarantine pathogens.

Although there aren't too much differences among CBLVd-Iran and other isolates of CBLVd introduction of this isolate as a new variant of citrus bent leaf viroid is essential, considering high effect of a change even in one nucleotide in viroids (13).

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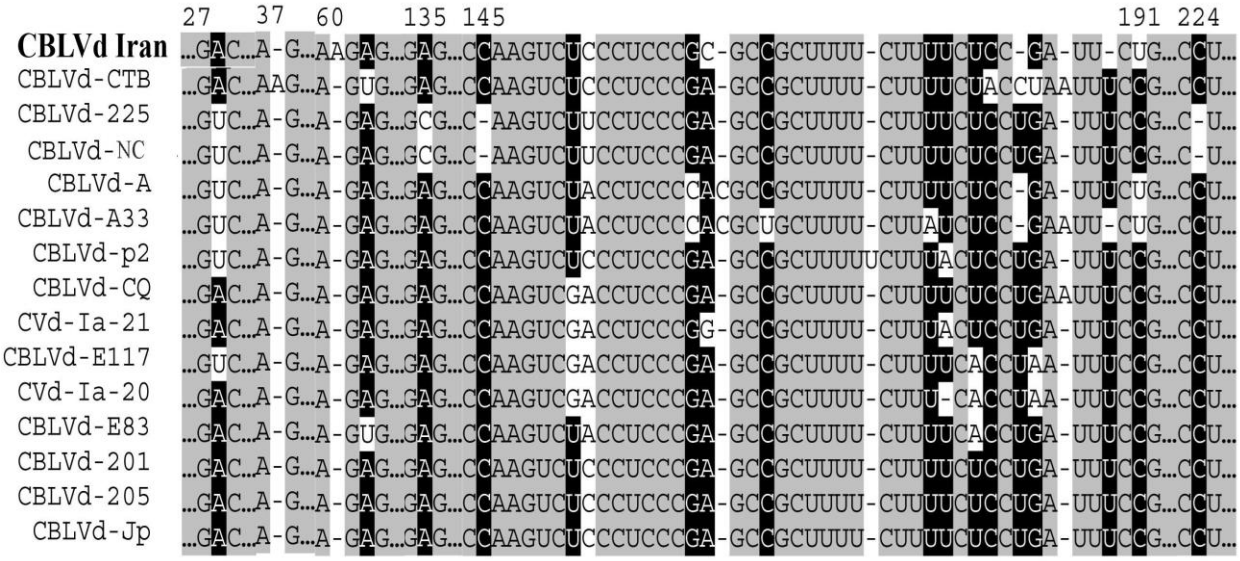

Fig. 2. Primary structures of CBLVd variants from various sources. Sequences are aligned for maximum homology. Nucleotides: Conserved sequences; Nucleotides: Identical sequences; -: lack of nucleotide.

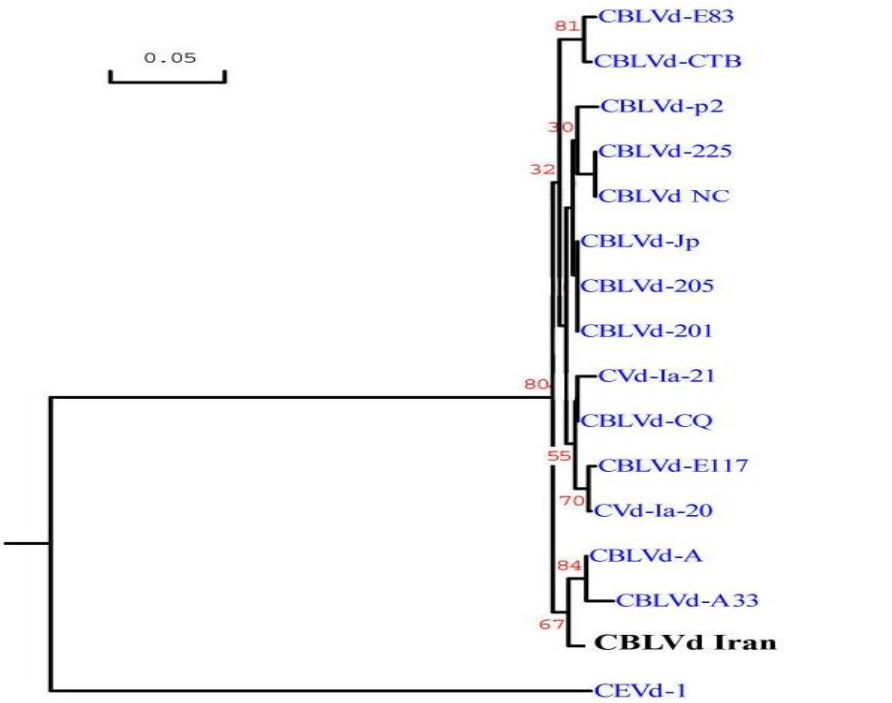

Fig. 3. Phylogenetic tree constructed from the alignment of nucleotide sequences of 14 CE isolates using the neighbor-joining method based on 10 000 replicates. The numbers indicate bootstrap percentage. See Table 1 for viroid accession numbers.

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Table 2: Percent sequence identity between selected CBLVd isolates used in this study.

	CBLVd Iran	CBLVd-A	CBLVd-A33	CBLVd-225	CBLVd-NC	CBLVd-CQ	CVd-Ia-21	CBLVd-E117	CVd-Ia-20	CBLVd-E83	CBLVd-CTB	CBLVd-p2	CBLVd-201	CBLVd-205	CBLVd-Jp
CBLVd Iran	100	98.3	97.4	96	96	96	97	96	96	97	95	97	97.8	97.8	97.8
CBLVd-A		100	99	96	96	96	97	97	96	97	94	97	97	97	97
CBLVd-A33			100	95	95	96	95	95	95	95	94	95	96	96	96
CBLVd-225				100	100	97	97	97	96	97	95	97	98	98	98
CBLVd-NC					100	97	97	97	96	97	95	97	98	98	98
CBLVd-CQ						100	98	98	98	98	97	97	98	98	98
CVd-Ia-21							100	98	98	98	95	98	98	98	98
CBLVd-E117								100	99	98	95	97	98	98	98
CVd-Ia-20									100	98	95	97	98	98	98
CBLVd-E83										100	97	97	99	99	99
CBLVd-CTB											100	96	97	97	97
CBLVd-p2												100	99	99	99
CBLVd-201													100	100	100
CBLVd-205														100	100
CBLVd-Jp															100

Conclusion

With regards to the importance of viroids as the factors reducing citrus growth, investigation of these pathogens especially in southern parts of the country by constructing their infectious clones, conducting pathogenicity tests, observing the symptoms and preventive measures for preventing mechanical transmission or withdrawal and transfer of pathogens from infected areas would be necessary.

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