

Current status of *Cucurbit aphid-borne yellows virus* in some greenhouse and open field cucumber in Iran

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(Received: December 2013; Accepted: June 2014)

Abstract

Yellowing and thickening are the most prevalent viral symptoms on cucumber grown in the greenhouses and open fields in Iran. Surveys were conducted from 2010 to 2012 in the major cucumber growing areas in the nine provinces, targeting especially the greenhouse plantations. Cucumber leaf samples showing symptoms like *Cucurbit aphid-borne yellows virus* (CABYV, genus *Polerovirus*, family *Luteoviridae*) infection, were collected from greenhouse (n=439) and open field- grown cucumbers (n=106) and tested for the presence of CABYV by DAS-ELISA and RT-PCR methods. 44% and 43% of collected samples from the greenhouses and open fields were infected to CABYV, respectively. Furthermore, CABYV positive samples were tested for the simultaneous infection of *Cucurbit chlorotic yellows virus* (CCYV) and *Cucurbit yellow stunting disorder virus* (CYSDV). Mixed infections with either CCYV or CYSDV were also confirmed in 9.4% of the CABYV-infected samples. The CABYV and CCYV combination was more prevalent than CABYV and CYSDV combination. Strain-specific RT-PCR confirmed the presence of common strain (CABYV-C) in all tested samples. Nine Iranian CABYV isolates were partially sequenced and sequence analysis has confirmed their assignment to the CABYV-C strain. The phylogenetic analysis revealed that although belonging to the CABYV-C, the Iranian cucumber CABYV isolates clustered within this strain in two phylogenetically distinct subgroups which might have an independent origin and/or introduction in Iran. This work provides further information on the natural occurrence of CABYV in Iran, including yet unreported characterization of CABYV-C strain on cucumbers grown in greenhouses and fields.
Key words: Cucumber, CABYV, RT-PCR, Strain-C.

وضعیت ویروس زردی شته‌زاد کدوئیان در برخی گلخانه‌ها و مزارع خیار در ایران

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چکیده

زردی و ضخیم شدن برگ‌ها از شایع‌ترین نشانه‌های آلودگی‌های ویروسی در گلخانه‌ها و مزارع خیار در ایران می‌باشند. در سال‌های ۱۳۸۹-۱۳۹۱ از مناطق عمده کشت خیار در نه استان کشور، نمونه‌های برگ خیار با نشانه‌های شبیه به آلودگی به ویروس زردی شته‌زاد کدوئیان (*Cucurbit aphid-borne yellows virus*, CABYV) از گلخانه‌ها و مزارع خیار (به ترتیب ۴۳۹ و ۱۰۶ نمونه) جمع‌آوری و آلودگی آنها به ویروس CABYV با آزمون‌های سرلوژیکی الیزا (DAS-ELISA) و نسخه برداری وارونه (RT-PCR) بررسی شد. به ترتیب ۴۴ و ۴۳ درصد نمونه‌ها از گلخانه‌ها و مزارع خیار آلوده به ویروس CABYV بودند. آلودگی هم‌زمان ۹/۴ درصد از نمونه‌های آلوده به CABYV، به ویروس زردی سبزرز کدوئیان (*Cucurbit chlorotic yellows virus*, CCYV) و ویروس کوتولگی زرد کدوئیان (*Cucurbit yellow stunting disorder virus*, CYSDV) مشاهده شد. میزان آلودگی هم‌زمان نمونه‌ها به دو ویروس (CABYV+CCYV) بیش از ترکیب دوتایی (CABYV+CYSDV) بود. نمونه‌های آلوده به ویروس CABYV در آزمون DAS-ELISA و RT-PCR با دو آغازگر اختصاصی برای دو استرین (C and R) این ویروس بررسی شد. نتایج حاکی از آلودگی جدایه‌های ایرانی به استرین (C) و عدم آلودگی آنها به استرین (R) بود. آلودگی نمونه‌های مورد بررسی به استرین (C) ویروس زردی شته‌زاد کدوئیان (CABYV) از طریق تعیین توالی قطعه تکثیر یافته نه جدایه ایرانی تأیید گردید. بر اساس نتایج حاصل از آنالیز تبارزایی جدایه‌های ایرانی CABYV مورد بررسی در این تحقیق در گروه استرین (C) ولی در دو زیر گروه مستقل از یکدیگر قرار گرفتند که نشانگر وجود دو منشا آلودگی مستقل از یکدیگر به ویروس زردی شته‌زاد کدوئیان در ایران می‌باشد.
واژه‌های کلیدی: خیار، ویروس زردی شته‌زاد کدوئیان (CABYV)، RT-PCR، نژاد C ویروس زردی شته‌زاد کدوئیان.

Introduction

The *Cucurbitaceae* represent economically important crop species having a high impact to human nutrition in temperate, tropical, and subtropical regions. Cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* L.), and different squash species (*Cucurbita pepo*, *C. moschata*, and *C. maxima*) are four major cucurbit species which are cultivated in the Mediterranean region. Virus diseases are the most important limiting factors of cucurbit production wherever they are grown. Cucurbits are affected by at least 59-well characterized viruses belonging to the major plant virus groups (Lecoq and Desbiez, 2012).

Yellowing symptoms on older leaves of melon, cucumber, and squash are the most prevalent symptoms in open fields and greenhouses, worldwide (Abou-Jawdah *et al.*, 2000). CABYV is one of the several viruses causing yellowing symptoms in cucurbit crops. CABYV was first reported in France (Lecoq *et al.*, 1992) and then has been reported from different countries such as United States (Lemaire *et al.*, 1993), Lebanon (Abou-Jawdah *et al.*, 1997), Spain (Juarez *et al.*, 2004), Tunisia (Mnari-Hattab *et al.*, 2005), Greece (Boubourakas *et al.*, 2006), Iran (Bananej *et al.*, 2006), Italy (Tomassoli and Meneghini, 2007), Turkey (Yardimici and Ozgonen, 2007), China (Xiang *et al.*, 2008a), Slovakia (Bananej *et al.*, 2009), and Egypt (Omar and Bagdady, 2012). CABYV is a member of the genus *Polerovirus* in the family *Luteoviridae* (Mayo & D'Arcy 1999). CABYV is phloem-limited and transmitted by the aphid species *Aphis gossypii* Glover, *Macrosiphum euphorbiae* Thomas and *Myzus persicae* Sulzer in a persistent, circulative nonpropagative manner (Lecoq and Desbiez, 2012). CABYV was the first polerovirus reported to infect cultivated cucurbits naturally and to cause a severe disease (Abou-Jawdah *et al.*, 1997; Lecoq *et al.*, 1992). The yield reduction can reach up to 50% of the marketable production in cucumber, but in melon, losses are more generally in the range of 10–15% (Lecoq, 1999). In contrast to mosaic inducing viruses, CABYV does not affect fruit quality, but rather induces flower abortion and reduces the

number of fruits per plant (Lecoq *et al.*, 1992). The isometric virions of ca. 25 nm in diameter contain a single-stranded RNA of 5.6 kb. Seven complete genome sequences have been determined, originating from China (EU000535, EU636992, GQ221223), Japan (GQ221224), Taiwan (JQ700305, JQ700306) and France (NC_003688). Additional partial sequences of Spanish (Juarez *et al.*, 2004), Italian (Tomassoli and Meneghini, 2007), Chinese (Shang *et al.*, 2009), Tunisian (Mnari-Hattab *et al.*, 2009), Slovak and Iranian (Bananej *et al.*, 2009) CABYV isolates have been reported too. Recently, two strains of CABYV, common strain (C) and recombinant strain (R) were identified in Taiwan (Knierim *et al.*, 2010).

Cucurbits are the major vegetable crops in Iran, ranking first in economic value, second in yield, and third in acreage. Watermelon, melon, and cucumber are cultivated in ~300,000 hectares in different provinces of Iran (<http://www.maj.ir>, <http://faostat3.fao.org/home/index.html>). In recent years, cucumber cultivation in greenhouses has been developed in many regions of Iran. Yellowing symptoms are frequently found in many cucumber greenhouses of Iran (Bananej, unpublished results).

Among the cucurbit viruses in Iran, *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), *Tomato spotted wilt virus* (TSWV) (Shaabani *et al.*, 2004; Samei *et al.*, 2004; Massumi *et al.*, 2007), *Tomato leaf curl Palampur virus* (ToLCPMV) (Hessari *et al.*, 2010), and *Cucurbit aphid-borne yellows virus* (CABYV) (Salehi *et al.*, 2012), have been reported from greenhouse-grown cucumbers. During a survey conducted from 2011-12, CCYV and CYSDV, two cucurbit infecting *Crinivirus* (family *Closteroviridae*) were identified in greenhouse-grown cucumber (*C. sativus* L.) and field-cultivated cucumber, squash (*Cucurbita* sp.) and melon (*C. melo* L.) in Iran (Bananej *et al.*, 2013).

CABYV has been previously reported from the major cucurbit growing areas in Iran and was detected from four cucurbit species: melon, cucumber, squash, and watermelon (Bananej *et al.*, 2006; Bananej and Vahdat, 2008), and has also been reported from greenhouse-grown cucumbers in Tehran and Alborz provinces in Iran (Salehi *et al.*, 2012).

In this study, cucumber leaf samples showing

symptoms like CABYV infection were collected from cucumber greenhouses and also open fields from different regions of Iran, and tested for CABYV infection, using double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) and reverse transcription (RT)-PCR experiments. The molecular variability was assessed by partial sequencing of Iranian CABYV isolates and their comparison with sequences available in the GenBank database.

Materials and Methods

Virus isolates: During two years from 2010 to 2011, regarding on different type of yellowing symptoms and different geographical regions, sampling was done from the major cucumber growing areas (greenhouse condition) in nine provinces Tehran (Varamin, Pishva, Kolein), Semnan (Ivankey, Damghan), Yazd (Taft, Rastagh, Akram-Abad, Chah-e-Shahrdar), Isfahan (Mobarakeh, Shahr-Reza, Falavar-Jan), Fars (Jahrom, Shiraz), Kerman (Jiroft, Manojan), Bushehr (Bushehr, Borazjan), Hormozgan (Haji-Abad) and Khorasan-Razavi (Chenaran, Neishabur, Faiz-Abad), located in the central, southern and eastern of Iran. Cucumber leaf samples with the suspicious symptoms of CABYV including yellows and thickening (Fig. 1), frequently on older leaves (Lecoq *et al.*, 1992) were collected from greenhouse (n=439, 7-10 greenhouses in each province) and open field (n=106, 3-5 open fields in each province) grown cucumbers (Table 1). The presence of CABYV was ascertained by DAS-ELISA (Clark and Adams, 1977), using polyclonal antibody, kindly provided by Dr. H. Lecoq (INRA Avignon, France).



Fig. 1. Yellowing and thickening symptoms on older leaves of greenhouse cucumber (Tehran-Varamin)

Cucumber leaf samples in which CABYV was detected were also checked for the presence of CCYV and CYSDV by a DAS-ELISA using polyclonal specific antisera against CYSDV and CCYV (DSMZ, Germany).

RT-PCR: Total RNAs were extracted from DAS-ELISA-positive samples (10-15 samples from each province) using TRI-Reagent (Sigma Chemical, St Louis, MO, USA). The cDNA synthesis was made using CAB reverse primer (see specification below). RT-PCR were carried out using CAB forward (5'-CGCGTGGTTGTGGTCAACCC-3') and CAB reverse (5'-CCYGCAACCGAGGAAGATCC-3') primers designed from the conserved region of the coat protein gene (nt 3580–4058 numbered according to the sequence of a CABYV-N reference isolate) (NC_003688, Guilley *et al.*, 1994; Bananej *et al.*, 2006) under following cycling conditions: initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and a final extension step for 7 min at 72°C. All PCR products were electrophoresed on a 1% agarose gel (70 V, 30-40 min), stained with ethidium bromide, and photographed under UV light.

Strain identification and phylogenetic analysis: RT-PCR was carried out for positive samples, 5-7 samples from each province, which amplified in above section, using primers specifically detecting strain C; CA-C-2891-F (5'-GAYGGAACATTATTAGCGCAGAGA-3, forward) and CA-3372-R (5'-AATCTATTGKTGGACTCTTDGTAACG A-3', reverse) and strain R; CA-R-3050-F (5'-ACCTAGCGAAATACGCTGAGCTA-3', forward), and CA-3372-R (5'-AATCTATTGKTGGACTCTTDGTAACGA-3', reverse) (Knierim *et al.*, 2010). The PCR products (n=9) were purified using a Wizard PCR Preps DNA Purification System (Promega) and directly sequenced (BIONEER, South Korea) with the same oligonucleotides as used for PCR. The sequence analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA v. 5.1; Tamura *et al.*, 2011) and DNA Sequence Polymorphism software (DnaSP v. 5; Librado and Rozas, 2009). For comparison, the available CABYV sequences were retrieved from Genbank (www.ncbi.nlm.nih.gov).

The nucleotide sequences reported in this paper have been deposited at GenBank under accession numbers KF425566 - KF425574.

Results and Discussion

Yellowing diseases have been observed worldwide in recent decades. However the disorders were first attributed to nutritional deficiencies, the use of appropriate diagnostic tools revealed that they were mostly caused by viruses (Kassem *et al.*, 2007). Different plant viruses can cause yellowing symptoms in cucurbit crops (Wisler *et al.*, 1998).

Recently, cucurbit cultivation in greenhouses has increased in different regions of Iran. It was shown previously, that CABYV is one of the most prevalent viruses in the major cucurbit crops in open fields of Iran (Bananej *et al.*, 2006). To complete the picture of CABYV prevalence, 545 cucumber leaf samples from the cucurbit plantations in the greenhouses (n=439) and open field (n=106) throughout Iran were tested using serological and molecular methods. DAS-ELISA results showed that ~44% (n=193) and ~43 % (n=46) of the collected samples from greenhouses and open fields (Table 1) were infected with CABYV, respectively. CABYV was identified in cucumber in all of the nine surveyed provinces (both in the greenhouses and open fields). The DAS-ELISA results were in perfect correlation with RT-PCR, which yielded the product of expected size (~480-bp, using CAB-primer) from ELISA positive samples (10-15 samples from each province), but not from the negative ones. Moreover, the serological tests revealed a single infection (n=175) with CABYV and a mixed infection by CCYV and CYSDV with 9.4% (n=18) of the CABYV-infected plants. Our results also supported that other symptomatic samples (CABYV negative samples) could be probably infected by whitefly transmitted virus species (CCYV and CYSDV) causing this type of yellowing symptoms (data not shown).

Understanding the molecular variation of cucurbit viruses is an essential step to design knowledge-based management strategies. Kassem *et al.* (2013) found that in spite of limited geographical area and a relatively short sampling period, CABYV population in Spain appeared to be genetically quite diverse and recombination has had an important role in diversity generation and maintenance. Recently, an increased genetic variability of CABYV was

detected by identification of recombinant CABYV isolates in Taiwan (Knierim *et al.*, 2010). These isolates were shown to arise by recombination between ancestors of CABYV and MABYV (*Melon aphid-borne yellows virus*) in the 3'-terminal part of the RdRp and a 5' part of the intergenic region. To exclude the possibility that such isolates are present in the Iranian sample set, an RT-PCR using strain-specific primers was carried out. Only CABYV-C specific fragment were amplified (~530-bp) from all the examined samples in this work and not any amplifications were occurred using primers specific for strain R.

To further study the genetic variability of CABYV in Iran, the RT-PCR fragment amplified from nine Iranian isolates, selected to represent the open field and greenhouse plantations in nine provinces (Table 2) have been partially sequenced (480bp after primer removal, corresponding to nt position 2886-3365 of NC_003688). Sequence analyses showed that the nucleotide identities in this genome portion range from 93.7 to 100% among Iranian isolates (the within group divergence reached 3.7%). Interestingly, amino acid sequences were strictly identical, with the exception of 1 amino acid substitution (Val to Ala), due to one nt difference at position 3013.

The central region of the polerovirus genome is suitable for comparison and phylogenetic analyses, as shown by Knierim *et al.* (2010). Therefore, using the sequence data generated during this study and data available from the GenBank database, a phylogenetic analysis has been carried out (Fig. 2). The analysis clearly discriminates CABYV isolates from two other related species - MABYV (Xiang *et al.*, 2008b) and SABYV (*Suakwa aphid-borne yellows virus*, Shang *et al.*, 2009). CABYV isolates have been further divided into two strains (separation supported by 100% bootstrap value), representing the common strain (CABYV-C) and a recently reported recombinant strain (CABYV-R). The same topology has been obtained using other algorithms (minimum evolution, maximum likelihood, data not shown). As expected from the specific RT-PCR analysis, all nine Iranian isolates were assigned to the CABYV-C, although into two different sub clusters.

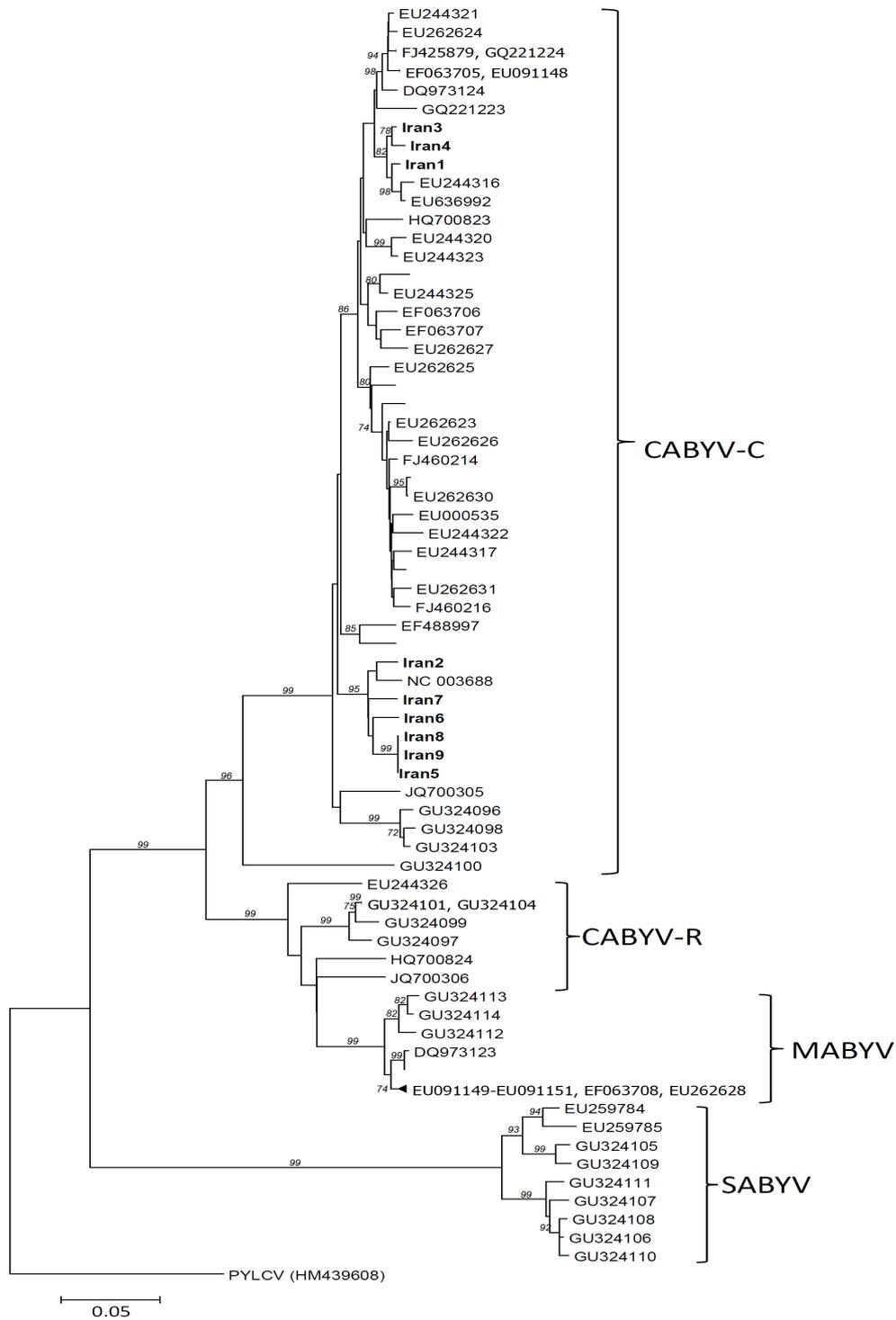


Fig. 2. Phylogenetic tree generated from partial CABYV sequences (3'-terminal part of the RdRp and a 5' part of the intergenic region, nt position 2886-3365 of the NC_003688) from 65 polerovirus isolates retrieved from Genbank and the 9 cucumber CABYV isolates determined in this study (in bold). The position of the CABYV-C, CABYV-R, MAYBV and SABYV is indicated. The scale bar indicates a genetic distance of 0.05. The phylogenetic tree was reconstructed using the neighbour-joining algorithm implemented in MEGA v.5 and a strict nucleotide distance model. The isolate of Pepper yellow leaf curl virus (HM439608) was used as an outgroup.

Table 1. The rate of CABYV infection in cucumber samples which were collected from greenhouses and open fields

CABYV (open field)	CABYV (greenhouse)	
7/12	37/82(45%)	Tehran
6/11	21/40(53%)	Semnan
5/11	11/27(41%)	Hormozgan
4/12	17/55(31%)	Yazd
7/10	54/82(66%)	Isfahan
5/13	10/28(36%)	Bushehr
3/12	16/25(64%)	Fars
4/12	12/47(26%)	Kerman
5/13	15/53(28%)	Khorasan Razavi
46/106(43%)	193/439(44%)	Total

While Iran-1, -3, -4 (all originating from the Khorasan-Razavi province) are most closely related to isolates from China (EU244316 and EU636992), isolates Iran-2, -5, -6, -7, -8, -9 are placed in the same sub cluster as the French isolate (NC003688). Contrary to the situation in Spain (Kassem *et al.*, 2013), the genetic diversity of the Iranian CABYV populations from cucumber seems to be low in Iran, assuming the sampling has covered all the crop production areas.

In conclusion, CABYV was found prevalent in cucumber crops in Iran, including the recently established cucumber plantations in the greenhouses. The specific RT-PCR detection from a representative set of nine cucumber samples confirmed only the presence of the common (CABYV-C) strain in Iran.

Table 2. List of CABYV isolates characterized from cucumber in this study

Acc. Numbers	Specific RT-PCR		RT-PCR (CP gene)	DAS- ELISA	Type of plantation	Province/locality	Host	Isolate
	R strain	C strain						
KF425566	-	+	+	+	greenhouse	Khorasan- Razavi/Chenaran	cucumber	Iran-1
KF425567	-	+	+	+	greenhouse	Tehran/Varamin	cucumber	Iran-2
KF425568	-	+	+	+	open field	Khorasan-Razavi/Sarakhs	cucumber	Iran-3
KF425569	-	+	+	+	greenhouse	Khorasan-Razavi/Neishabour	cucumber	Iran-4
KF425570	-	+	+	+	greenhouse	Alborz/Karaj	cucumber	Iran-5
KF425571	-	+	+	+	greenhouse	Kerman/Jiroft	cucumber	Iran-6
KF425572	-	+	+	+	open field	Semnan/Ivankey	cucumber	Iran-7
KF425573	-	+	+	+	greenhouse	Yazd/Ashkzar	cucumber	Iran-8
KF425574	-	+	+	+	greenhouse	Isfahan/Mobarakeh	cucumber	Iran-9

However, the phylogenetic analysis revealed that although belonging to the CABYV-C, the Iranian cucumber CABYV isolates clustered within this strain in two phylogenetically distinct subgroups (84% bootstrap support, Fig. 2), which might have an independent origin and/or introduction in Iran. Symptom severity varied greatly according to season, being more pronounced in summer than in winter; it also differed with cultivar (Lecoq *et al.*, 1992). The use of cultivars that carry genetic resistance to the virus

is the key role in control of the disease. Several resistance sources for CABYV in melon have been described (Dogimont *et al.*, 1997; Dogimont *et al.*, 1996). However, cultivars with resistance to CABYV have not yet been produced on a commercial basis.

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