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

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**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 *Poterovirus*, 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.
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 (TSVW) (Shaabanian 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 CAYBV 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 CAYBV 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 CAYBV 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 CAYBV was ascertained by DAS-ELISA (Clark and Adams, 1977), using polyclonal antibody, kindly provided by Dr. H. Lecoq (INRA Avignon, France).

Cucumber leaf samples in which CAYBV 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'-CGCGTGGTTGTGTGTTGTCACCC-3') and CAB reverse (5'-CCYGCAACCCGGAGGATCC-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'-AATCTATTGTGGACTCTTDGAACG A-3', reverse) and strain R; CA-R-3050-F (5'-ACCTAGCGAAATACGCTGAGCTA-3', forward), and CA-3372-R (5'-AATCTATTGTGGACTCTTDGAACG A-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 CAYBV 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

<table>
<thead>
<tr>
<th>CABYV (open field)</th>
<th>CABYV (greenhouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tehran</td>
<td>37/82 (45%)</td>
</tr>
<tr>
<td>Semnan</td>
<td>6/11 (53%)</td>
</tr>
<tr>
<td>Hormozgan</td>
<td>11/27 (41%)</td>
</tr>
<tr>
<td>Yazd</td>
<td>17/55 (31%)</td>
</tr>
<tr>
<td>Isfahan</td>
<td>54/82 (66%)</td>
</tr>
<tr>
<td>Bushehr</td>
<td>1/28 (36%)</td>
</tr>
<tr>
<td>Fars</td>
<td>16/25 (64%)</td>
</tr>
<tr>
<td>Kerman</td>
<td>12/47 (26%)</td>
</tr>
<tr>
<td>Total</td>
<td>193/439 (44%)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Acc. Numbers</th>
<th>Specific RT-PCR</th>
<th>Type of plantations</th>
<th>Host</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>KF425566</td>
<td>-</td>
<td>greenhouse</td>
<td>Khorasan-Razavi/Chenaran</td>
<td>cucumber</td>
</tr>
<tr>
<td>KF425567</td>
<td>-</td>
<td>greenhouse</td>
<td>Tehran/Varamin</td>
<td>cucumber</td>
</tr>
<tr>
<td>KF425568</td>
<td>-</td>
<td>greenhouse</td>
<td>Khorasan-Razavi/Sarakhs</td>
<td>cucumber</td>
</tr>
<tr>
<td>KF425569</td>
<td>-</td>
<td>greenhouse</td>
<td>Khorasan-Razavi/Neishabour</td>
<td>cucumber</td>
</tr>
<tr>
<td>KF425570</td>
<td>-</td>
<td>greenhouse</td>
<td>Alborz/Karaj</td>
<td>cucumber</td>
</tr>
<tr>
<td>KF425571</td>
<td>-</td>
<td>greenhouse</td>
<td>Kerman/Jiroft</td>
<td>cucumber</td>
</tr>
<tr>
<td>KF425572</td>
<td>-</td>
<td>greenhouse</td>
<td>Semnan/Ivankey</td>
<td>cucumber</td>
</tr>
<tr>
<td>KF425573</td>
<td>-</td>
<td>greenhouse</td>
<td>Yazd/Ashkzar</td>
<td>cucumber</td>
</tr>
<tr>
<td>KF425574</td>
<td>-</td>
<td>greenhouse</td>
<td>Isfahan/Mobarakeh</td>
<td>cucumber</td>
</tr>
</tbody>
</table>

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.

References

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