Occurrence and Characterization of Coat Protein Gene of Zucchini yellow mosaic potyvirus (ZYMV) Isolate Infecting Pumpkin (Cucurbita pepo L.) in Bingol Province (Turkey)

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Abstract

Zucchini yellow mosaic potyvirus (ZYMV) belongs to family Potyviridae, which causes serious economic losses in many cucurbits from worldwide. In 2018 (September), leaf samples of pumpkin exhibiting typical viral symptoms including mosaic, blistering and wrinkling and healthy pumpkin leaf samples were collected from Bingöl and screened by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) against ZYMV infection. Tested leaf samples were reacted positive resulting in an expected about 840 bp DNA fragments of partial coat protein (CP) gene of ZYMV. ZYMV-CP gene was further inserted into a pGEM-T Easy prokaryotic cloning vector and their partial nucleotide sequences and deduced amino acid were ascertained. The provided ZYMV-CP gene sequence consisted of 837 nucleotides in length coded for 279 amino acid residues of approximately 31.2 kDa. The isolate was denominated as ZYMV-Bingol and registered with MK689858 accession number in the NCBI. The sequence of ZYMV-Bingol CP gene were aligned with 21 isolates deposited in GenBank from different geographical location and its phylogenetic relationships were determined. Molecular analysis of the ZYMV CP gene sequence indicated the highest similarity with 100% Turkish isolate (JF317296) and the lowest with 91.64% of Korea isolate (AF062518), at nucleotide level. Moreover, phylogenetic analyses revealed that ZYMV Bingöl isolate is clustered with the Turkish-Adana isolate (JF317296) and Pakistan isolate (AB127936). ZYMV has been reported for the first time in the pumpkin plant from Bingöl province of Turkey by this study.

Key words: ZYMV (Zucchini yellow mosaic potyvirus), RT-PCR, cloning, characterization.

Bingöl İlinde Kabak Bitkisini (Cucurbita pepo L.) İnfekteleyen Zucchini Yellow Mosaic Potyvirus (ZYMV) Virüs İзолatının Belirlenmesi ve Moleküler Karakterizasyonu

Özet

Kabak sanı mozaik potivirüsü (ZYMV), Potyviridae ailesinesine ait bir virüs olup dünya çapında birçok kabakgil bitkide ciddi ekonomik kayıplara neden olmaktadır. 2018 yılı Eylül ayında, Bingöl iline bağlı kabak yetiştiriciliği yapılan alanlarda kabarıklık ve buruşukluk gibi tipik viral hastalık belirtisi gösteren bitkiler ile sağlıklı bitkilerden örnekler alınarak ZYMV enfeksiyonu bakımından Reverse Transkripsiyon Polimeraz Zircir Reaksiyonu (RT-PCR) ile testlenmiştir. RT-PCR test pozitif reaksiyon vererek ZYMV’nin kılıf proteinine karşılık gelen yaklaşık 840 bp DNA fragmenti oluşturulmuştur. Ayrıca, çoğaltılan ZYMV’nin tam kılıf protein (coat protein, CP) geni, prokaryotik klonlama vektörü olan pGEM-T Easy vektörüne klonlanmış ve genin tam nükleotid ve amino asit dizisi tespit edilmiştir. Elde edilen ZYMV-CP gen dizisinin 279 amino asit kodladığı (yaklaşık 31.2 kDa) ve 837 nükleotidi içerdiği ortaya çıkaranmış ve bu dizi ZYMV-Bingöl izolatı olarak isimlendirilerek MK689858 erişim numarası ile gen bankasına (NCBI) kaydedilmiştir. ZYMV-Bingöl CP geninin dizisi, farklı çoğrafî bölgelerden elde edilen 21 izolat ile analiz edilerek filogenetik ilişkileri belirlenmiştir. Nükleotid düzeyinde moleküler analizlere göre ZYMV CP gen dizisi, en yüksek % 100 oranında Türkiye izolati (JF317296) ile ve en düşük % 91.64 oranında Kore izolati (AF062518) ile benzerlik göstermiştir. Ayrıca, filogenetik analizler, ZYMV Bingöl izolatının Türkiye-
Introduction

Pumpkin (Cucurbita pepo L.) is a cultivated plant belonging to the family Cucurbitaceae, which can grow elsewhere in different agricultural ecosystems. Thirty-two different viruses and virus-like agents have been identified, including Zucchini yellow mosaic potyvirus (ZYMV), which infects Cucurbitaceae plants (Lovisolo, 1980). Since this plant pathogen was first discovered in Italy in 1973 and was provisionally called as MYSV (Muskmelon yellow stunt virus) (Lisa et al., 1981), it has been reported in many countries in the continents of Europe, Africa, Asia, Oceania, America and Middle East (Desbiez and Lecoq, 1997).

The agent belongs to the Potyvirus which is the greatest genus among plant viral pathogen, has an infectious (+) ssRNA genome characterized by 750 nm long and 12 nm in diameter contained about 9600 nucleotides in size (Lisa et al., 1981; Balint et al., 1990). The viral genome also has the VPG protein (genome-linked protein) at 5' position and poly (A) tail at 3' position. ZYMV forms tubular scroll-like cytoplasmic cylindrical inclusion bodies within infectious cells (Shukla et al., 1994; Adams et al., 2012).

ZYMV is a worldwide plant pathogenic virus that parasitizes cucurbit plants including Cucurbita pepo, C. pepo, C. moschata, C. maxima, Cucumis sativus, Cucumis melo, Citrullus lanatus, and can causes substantially product losses up to 94% every year in all seasons except winter (Wang et al., 1992). It has also been reported that some weeds and cultivated plants such as Ranunculus sardous, Lamium amplexicaule, Sesamum indicum, Ranunculus sardous, Chenopodium amaranticolour, C. quinoa, Phaseolus vulgaris, Nicotiana benthamiana, Cucurbita pepo, and Cucumis melo are the experimental hosts, Melothria pendula and Moluccella laevis, which are wild cucurbit species are natural hosts of ZYMV, can also serve as inoculum resources in the pathogen life table (Mahgoub et al., 1997; Kheder et al., 1997; Harmer et al., 1995; Harper and Creamer, 1995). Nowadays, many viruses in the genus Potyvirus including ZYMV can be unequivocally detected by PCR procedure, which is broadly performed targeting the gene sequence of the viral CP protein (Simmons et al., 2011; Zheng et al., 2010).

In this study, the presence of ZYMV in symptomatic pumpkin plants was confirmed by the molecular techniques in Bingol province located in the east of Turkey and the phylogenetic relationships with other isolates around the world were revealed based on the ZYMV-CP sequence.

Material and Methods

Plant material and biological cloning

Young leaves showing virus-like symptoms in pumpkin were obtained from the Beyaztoprak village of Bingöl province on September 2018. The symptoms triggered by ZYMV were also evaluated by mechanical inoculation as the conventional method in the C. pepo. The ZYMV suspension was empirically treated with carborundum powder and potassium phosphate buffer (PPB) (pH 7.2) on the cotyledon leaves after the seedling phase. Symptom development was observed in the climate chamber for 3 weeks. Inoculated plants were tested by RT-PCR for ZYMV infection.

Total RNA extraction, and cDNA synthesis

Because the ZYMV has an RNA genome, total RNA was extracted from nearly 0.25 g leaf...
tissue according to the silica-based method described by Foissac et al. (2001). The asymptomatic pumpkin plant was also gathered as a negative control in the laboratory tests.

The cDNA mixture was prepared in 20 µl volume. The following components were introduced into an Eppendorf tube for the synthesis of complementary DNA (cDNA): 5 µl of purified RNA (as a template), 1 µl of 10 mM dNTP mix, 1 µl of 20 pmol/µl the antisense primer (ZA), 5 µl of RNase free water, followed by 65 °C for 5 min and afterwards immediately immersed in ice for 5 minutes. 4 µl of 5X RT reaction buffer, 2 µl of 0.1M DTT, 1 µl of RNase inhibitor and 1 µl of RT enzyme were put onto the resulting mixture and hold at 42 °C for 50 min. Finally, the mixture was incubated at 70 °C for 15 min to discontinue the reaction.

Table 1. Accession number, location, isolate name and host of ZYMV isolates retrieved from GenBank used for phylogenetic analysis in this study

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Location</th>
<th>Isolate name</th>
<th>Host</th>
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</thead>
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<tr>
<td>AJ420019</td>
<td>Germany</td>
<td>Berlin 1</td>
<td>C. pepo</td>
</tr>
<tr>
<td>AB458595</td>
<td>Syria</td>
<td>SYZY 1</td>
<td>Cucurbita pepo L.</td>
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<td>Iran</td>
<td>Azr. Mak.W</td>
<td>Citrullus vulgaris L.</td>
</tr>
<tr>
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<td>Syria</td>
<td>SYR 3</td>
<td>C. pepo</td>
</tr>
<tr>
<td>FJ705263</td>
<td>Iran</td>
<td>Hor. Min. S</td>
<td>Cucurbita maxima Duch. E Lam</td>
</tr>
<tr>
<td>EF062583</td>
<td>Israel</td>
<td>AG</td>
<td>Cucurbita pepo L. ‘Ma’ayan’</td>
</tr>
<tr>
<td>AB127936</td>
<td>Pakistan</td>
<td>Pak</td>
<td>Lagenaria siceraria Standl.</td>
</tr>
<tr>
<td>HM072431</td>
<td>Serbia</td>
<td>128-08</td>
<td>C. pepo L. ‘Olinka’</td>
</tr>
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<td>Austria 12</td>
<td>C. pepo L.</td>
</tr>
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<td>NA</td>
<td>C. maxima Duch. ‘Hokoseihi’</td>
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<td>India</td>
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<td>Cucumis anguria</td>
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<td>?</td>
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<td>Turkey</td>
<td>C5</td>
<td>Cucurbita moschata</td>
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<td>Turkey</td>
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<td>C. pepo L.</td>
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<td>MK689858</td>
<td>Turkey</td>
<td>ZYMV-Bingol</td>
<td>C. pepo L.</td>
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</table>

**Molecular detection of ZYMV- infecting pumpkin and primer pairs**

The ZYMV infection was examined by RT-PCR tests in pumpkin. Primers used in the detection of ZYMV in this study were retrieved by formerly research (accession number: AB188116) and were synthesized employing the Sentegen company (Ankara/Turkey). Employed primers used were designed as sense (ZS-5’-TCAGGCACCTCAGCCAACCT-3’) and antisense (ZA-5’-CTGCATTGTATTCCACCTAGT-3’), amplifying a CP gene region about to 837 bp.

The RT-PCR thermocycling program that amplifies the CP gene using Eppendorf Mastercycler (Hamburg, Germany) is as follows: 3 min at 94 °C (pre-denaturation), 40 cycles of 30 s at 94 °C (denaturation), 1 min at 62 °C (annealing), and 45 s at 72 °C (elongation), lastly 10 min at 72 °C for final elongation. 5 µl of cDNA were submitted to RT-PCR test in a total volume of 50 µl: 31.6 µl RNase free water, 5µl 10X PCR Buffer, 3µl 25mM MgCl2, 1µl 10mM dNTP, 1µl 20µM of each primer pairs and using the proofreading 0.4 µl Taq DNA polymerase enzyme (5U/µl) (Thermo, USA).

The molecular size of PCR yield and 1 kb DNA ladder as the marker (Fermentas, Vilnius, Lithuania) was evaluated by 1.5% agarose gel electrophoresis stained with EtBr using Tris-Acetate EDTA buffer (TAE1X). The monitored DNA fragments were documented by photography with UV light (Syngene™ UV Transilluminator 2020LM). ZYMV-Ahlat isolate (GenBank accession JF317297), from previous studies, served as a positive control, and symptomless pumpkin plant and PCR yield- free mixture were used as a negative control for diagnosis of the ZYMV.

**T-A cloning and sequencing**

The amplified CP gene of the expected size was recovered by using a DNA gel extraction kit.
(Fermantas) according to manufacture's directions. The purified PCR products were ligated into the pGEM-T Easy plasmid vector by DNA ligase enzyme. Accordingly, a sterile PCR tube containing the following components; 2x Rapid Ligation Buffer of 5 µl, pGEM-T Easy vector of 1 µl, purified PCR product of 5 µl and T4 DNA ligase enzyme of 1 µl (Promega, USA) and was incubated at 4 °C overnight, followed by introduced into E.coli (Promega, USA) via electroshock wave (BioRad, USA). Potential recombinant plasmids were purified with the commercially supplied kit (GeneJET Plasmid Miniprep Kit, Thermo, USA). Sequencing was carried out in both directions using the above primer sets by Iontek Company for recombinant plasmid involving the CP-DNA sequence of the ZYMV Bingöl isolate. The DNA sequence was blasted against GenBank data, was registered in NCBI (The National Center for Biotechnology Information).

**Alignment and cladistic analysis**

Twenty-one different ZYMV isolates were used to determine phylogenetic relationships. The sequence corresponding to 837 bp from Bingöl isolate with other isolates from the diverse host that was online available in GenBank as illustrated in Table 1 were assembled and assessed by using the Mega 7 program. The phylogenetic dendrogram was inferred using the Neighbor-joining algorithm by 100 repetition bootstrap analysis. The evolutive interrelationships were computed with the Tamura-Nei method. The CP gene of Barley yellow dwarf virus isolate (KC900900) was designated as an outgroup for branching.

**Results and Discussion**

**Detection of ZYMV using appropriate primer sets**

A pumpkin (Cucurbita pepo) was collected, that characteristic viral symptom associated with mottling, mosaic and lumps, leaf distortion, down curling leaves (Fig 1), compared with healthy pumpkin which using as negative control. ZYMV infection was confirmed by RT-PCR as mentioned above. Data in Fig. 2 displayed that RT-PCR resulted in an amplification yield size of 837 bp corresponding to the ZYMV-CP gene from ZYMV-infected pumpkin. No DNA fragment healthy plant or PCR yield-free mixture using as negative control were DNA fragments displayed.

**Alignment and cladistic analysis**

The sequence of ZYMV CP gene isolate was ascertained as 837 bp and was recorded in GenBank (with accession number MK689858), called ZYMV- Bingol. This data was used to determine the genetic diversity among Bingöl isolate and other chosen isolates in different plants. BLAST research (Basic Local Alignment Search Tool) revealed that the sequence of ZYMV- Bingol CP gene shared with 91.64-100% similarity, the highest score with 100% of the Turkish isolate (JF317296), with the lowest score with 91.64% of Korea isolate (AF062518) and were confirmed by cladistic analyses using computer-supported program. Phylogenetic analysis of isolate presented in this study with 21 isolates from GenBank indicated that ZYMV- Bingol was same grouped with isolates identified on Cucumis sativus and Lagenaria siceraria (JF317296, AB127936) (Fig 3).
Figure 2. Agarose gel pattern displaying DNA fragment of ZYMV from individually infected pumpkin leaf tissue by RT-PCR using CP-specific primer sets. Lane M: 1 kb DNA marker Lane 1-3-4: Healthy pumpkin plants Lane 2: ZYMV isolate Lane 5: ZYMV-positive control.

Figure 3. Phylogenetic dendrogram created by the neighbor-joining algorithm with 100 replication bootstrap value (scores at the nodes) from 21 distinct ZYMV isolates. ZYMV-Bingöl isolate is underlined.

Since pumpkin (Cucurbita pepo L.) is consumed as fresh fruit and snacks, it is an important food for many people both in terms of nutrition and livelihood. Cucurbits are very susceptible to viruses and more than 35 viruses have been reported infecting cucurbits (Zitter et al., 1996). Presently, pumpkin has substantially affected by ZYMV, followed by SqMV, CMV, WMV (Coutts et al., 2011; Simmons et al., 2011, 2013). ZYMV is a viral agent that is prevalent in almost all countries and leads to a severe yield loss, ruining Cucurbitaceae family members due to the severe epidemics. Here, we determined the ZYMV isolate which depicted yellow-greenish severe mosaic, distortion and serrated leaves in pumpkin plants by PCR-based molecular methods from Bingöl province of Turkey, 2018. Disease symptoms observed this study were consistently similar with those reported in studies in different countries such as Czech Republic (Svoboda and Polák, 2002), Trinidad (Chinnaraja et al., 2016), Sudan (Mohammed et al., 2014), Egypt (Khalifa et al., 2015), Saudi Arabia (Al-Salheh et al., 2014), Japan (Ohtsu et al., 1985), Malaysia (Fujisawa et al., 1986), Western Australia (Coutts et al., 2011), India
ZYMV has been previously reported in numerous provinces such as Konya, Aksaray and Karaman (Yeşil, 2013, 2014; Yeşil and Ertunç, 2012, 2013), Burdur (Çulal Kılıç et al., 2016), Adana and Mersin (Kamberoğlu et al., 2016; Özer et al., 2012), Afyon (Yılmaz et al., 1992), Hatay (Sertkaya et al., 2004), Erzurum, Erzincan and Artvin (Bostan et al., 2002), Gaziantep (Ozaslan et al., 2006; Dağ, 2005), İzmir, Aydın, Manisa and Balikesir (Kaya and Erkan, 2011), Ahatl (from Bitlis) (Özer et al., 2012), Samsun, Sinop and Bolu (Şevik and Arlı-Sokmen, 2003; Şevik and Balkaya, 2015), Amasya and Çorum (Çıtır et al., 1998), Tekirdağ, Edirne and Kırklareli (Köklü and Yılmaz, 2006), Tokat (Korkmaz et al., 2018), Ankara (Ertunç, 1992), Antalya (KP872577, KP872571, KP872570) (Topkaya and Ertunç, 2012; 2013; 2013, respectively) (unpublished data), Turkish Republic of Northern Cyprus (Karamanlı and Kamberoğlu, 2010).

In Turkey, although the virus has been identified for more than 30 years, little is known concerning its molecular feature. Most of the studies have been performed serologically, biologically and symptomatologically, but molecular characterization for its coat protein gene is very limited, besides no genomic sequence information was reported except for 2-3 studies. ZYMV can be detected with different primer sets, referred on coat protein (CP) ve Nib gene (Karamanlı and Kamberoğlu, 2010; Wang and Li, 2017; Khaled et al., 2017; Nasr-Eldin et al., 2016; Prieto et al., 2001). We identified the ZYMV using ZS and ZA primer pairs designed specifically for the CP gene. ZYMV Bingol isolate was produced 837 bp fragment, which were equivalent to that of ZYMV-Ah (JF317297) and ZYMV-Ad (JF317297), which were harmony with that submitted Özer et al. (2012), Spadotti et al. (2015) and Topkaya and Ertunç, (2011; 2012; 2013) (unpublished data) who successfully identified ZYMV by RT-PCR.

Cucurbita pepo with together 11 families of dicotyledons is a voluntary host for ZYMV (Romay et al., 2014). The symptoms of ZYMV-Bingol isolate were characterized symptomatologically by the reaction to the pumpkin. In the surveys performed at different times, pumpkin plants were systemically infected, as expected, and exhibited foliar symptoms such as mosaic, leaf deformation and leaf blistering were exhibited, which were similar effect to naturally infected plants with ZYMV, in agreement with Wang and Le, (2017) (China) and Nasr-Eldin et al. (2016) (Egypt) (Fig 5). The presence of ZYMV was also confirmed in inoculated leaves by RT-PCR assay.

The origin of ZYMV, its mechanism of evolution and its geographic distribution is
important to understand the emergence of plant viruses. In the past studies, the sequence of the CP gene nucleotide was used to differentiate phylogenetic relationships, resulting in two or three groups (Zhao et al., 2003; Romay et al., 2014). ZYMV- Bingol sequence (MK689858), which has homology to previously proposed isolates, was analyzed with other representative isolates in NCBI for phylogenetic interrelationship. Phylogenetic assay of the partial sequences of the CP gene demonstrated the main three distinct clusters (named I, II and III). Cluster I are divided into two subclusters (A and B) and ZYMV-Bingol isolate was clustered within subclusterB of cluster I as shown in Fig3. This DNA distance analysis revealed that Bingol isolate is part of a cluster of the other Turkey (KP872566, JF317296), Syria (AB458596, AB458595), Pakistan (AB127936) and India (GQ482976) from Middle Eastern countries. Interestingly, even though Bingol and Bitlis (Ahlat) had a neighboring province (about 230 km between two locations) and shared an almost similar geographical region, both isolates obtained from these provinces (MK689858 and JF317297) were in the completely different cluster (Cluster I and Cluster II), respectively. In contrast, ZYMV-Ad (Adana) and ZYMV-Bingol (Bingol) identified in the two provinces away from each other (about 666 km between two locations) were included in the same subcluster of the same cluster (Cluster IA). This shows the variations occurring in the virus genome at the same location. This shows that rapidly ongoing variations occur in the ZYMV virus genome in the same country.

Figure 5. Symptoms induced by ZYMV in mechanical inoculated pumpkin (Cucurbita pepo). A- mosaic and mottle on leaves, B- severely foliar deformation.

Sequence analysis indicated that the 837 bp nucleotide sequences of ZYMV-Bingol isolate shared with the similarity of 91.64–100% nucleotide homology with other assembled potyvirus from GenBank. These sequences had substitutions of four nucleotides at distinct points of the CP gene-specific to ZYMV-Bingol isolate. Computational assays revealed that Timin instead of Guanine, Timin instead of Cytosine, Timin instead of Cytosine, and Adenine instead of Guanine at 22, 390, 736, and 817 positions, respectively. Also, multiple alignments showed that a conserved amino acid codon, without any change.

The cultivation of plants in the Cucurbitaceae family is not widespread and on a large scale in Bingol province. Although there is no risk situation for ZYMV at the moment, if the cucurbit crops such as watermelon, melon, pumpkin, cucumber are grown in large scale, necessary cultural and chemical measures should be taken to abstain ZYMV infection as the destruction of residues after harvest, prevention of planting ornamental plants as Malva and begonia served as an alternative host, planting certified tolerant or resistant varieties when available, and as well as using pesticides for vector insects (Lecoq et al., 2014).

Conclusion

According to our knowledge, this is the first record on the occurrence of ZYMV disease on Cucurbita pepo L. in Bingol province of Turkey. The outcome of this study will help to better understand further research on the complexity, epidemiology and hence variety of ZYMV in Turkey.
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