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Cloning and sequencing of coat protein gene of *Zucchini yellow mosaic virus* isolated from squash and muskmelon in Turkey

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Abstract: The coat protein (CP) genes of the genomic RNA of 2 severe Turkish isolates of *Zucchini yellow mosaic virus* (ZYMV) from squash and muskmelon [ZYMV-Adana (Ad) and ZYMV-Ahlat (Ah), respectively] were cloned, and their complete nucleotide sequences and deduced amino acids were determined. The analysis revealed that both Turkish ZYMV-CP genes contained 837 nucleotides encoded for a CP of about 31.2 kDa. Phylogenetic trees based on nucleic acid sequences were constructed by the neighbor joining and unweighted pair group mean arithmetic (UPGMA) methods with 100 bootstrap replicates. A high degree of homology was detected between the 2 Turkish isolates on the nucleotide sequences (97%). The CP sequence of ZYMV-Ad and ZYMV-Ah varied among the 23 isolates with overall identity of 93%-98% and 94%-99%, respectively, at the nucleotide level. Comparison of the nucleotide sequences of 23 isolates from different geographical regions worldwide showed the ZYMV-Ad isolate clustered with isolates from Middle Eastern countries (Israel, Jordan, and Syria); ZYMV-Ah isolate was clustered with isolates from Far Eastern countries (Korea and Taiwan). The N-terminal of the Turkish ZYMV-Ah CP contained a distinctive sequence at nucleotide positions 9324-9328, which distinguished the Turkish ZYMV-Ah isolate from all previously reported ZYMV isolates. The CP cistron of the ZYMV-Ad and Ah isolates contained 279 amino acid residues. Pairwise nucleotide sequence comparison revealed sequence similarities of 58%-70% between ZYMV Turkish isolates and 22 other potyviruses. Mechanical inoculations showed that ZYMV-Ah produced faster systemic symptom induction than ZYMV-Ad on squash, suggesting that ZYMV-Ah was a more severe isolate than ZYMV-Ad (GenBank accession JF317296-JF317297).

Key words: ZYMV, characterization, coat protein, phylogenetic analysis

Introduction

Zucchini yellow mosaic virus (ZYMV) was first described in northern Italy in 1973 (1). Later, it was identified throughout the world in cucurbit-cultivating areas, including Mediterranean countries, China, Australia, and the continental United States (2-7).

In general, the virus causes a light green to yellow mosaic on the leaves of infected plants. Then the symptoms can become more severe, appearing as deep foliar serration, blisters, deformations, and plant stunting. These symptoms may lead to completely unmarketable fruits. Different isolates of this virus have been disseminated in cucurbits throughout the world. The virus itself is highly infectious and can be transmitted by aphids and infected seeds or mechanically (8,9).

Potyviruses including ZYMV contain a genome that is translated as a large polyprotein that is subsequently cleaved by virus-coded proteolytic enzymes into 8 proteins. The coat protein (CP) is

located at the C-terminus of the polyprotein (10). CP amino acid sequences have recently become a useful tool for taxonomic classification of distinct potyviruses and related strains (11,12).

In Turkey ZYMV was first isolated from diseased squash from the Mediterranean area by Yılmaz and Davis (13). Recently, we obtained other isolates from commercial cucumber fields in Adana Province (ZYMV-Ad) and from muskmelon fields in Ahlat (Bitlis Province), Turkey (ZYMV-Ah). Although ZYMV has been recorded in Turkey since 1984, no detailed molecular analysis of its genome sequence variability has been reported. In the current study we focused on the characterization of 2 geographically distant ZYMV Turkish (Adana and Ahlat) isolates by cloning and sequencing full length CP genes of the purified particles used to compare these isolates with other ZYMV strains and isolates described from other areas of the world.

Materials and methods

Source of isolates

The 2 selected ZYMV isolates comprised 2 cucurbits (squash and musk melon) from, respectively, Adana and Bitlis provinces, Turkey that were collected in 2009-2010. They were identified as ZYMV by RT-PCR, and the infected leaves were mechanically inoculated on leaves of squash seedlings for use as a viral source. These 2 selected isolates showed obvious, different mosaic symptoms on squash and were named ZYMV-Ad (from Adana Province) and ZYMV-Ah (from Ahlat District, Bitlis Province). The isolates were maintained in *Cucurbita pepo* 'Sakız' under climate chamber conditions with a mean maximum temperature of 28 °C and mean minimum temperature of 18 °C and processed for gene cloning by RT-PCR. Host, geographical origin, date of isolation, and sequence database accession numbers of ZYMV isolates are shown in the Table.

Table. Designation, geographic origin, and GenBank accession numbers of ZYMV Ad and Ah isolates and the ZYMV isolates used for CP sequence comparison.

Isolate name	Country	GenBank accession no.	Host
Berlin 1	Germany	AJ420019	*C. pepo* L.
SYZY 1	Syria	AB458595	*Cucurbita pepo* L.
Azr. Mak.W	Iran	FJ705272	*Citrullus vulgaris* L.
SYR 32	Syria	EU999757	*C. pepo* L.
SYR 3	Syria	AB458596	*C. pepo* L.
Hor. Min. S	Iran	FJ705263	*Cucurbita maxima* Duch. E Lam
Jor B5	Jordan	EU999760	*C. pepo* L.
AG	Israel	EF062583	*Cucurbita pepo* L. 'Ma'ayan'
Pak	Pakistan	AB127936	*Lagenaria siceraria* Standl.
128-08	Serbia	HM072431	*C. pepo* L. 'Olinka'
Austria 12	Austria	AJ420017	*C. pepo* L.
Kuchyna	Slovakia	DQ124239	*C. pepo* L
NA	Japan	AB004641	*C. maxima* Duch. 'Hokoseihi'
ZTRICH	Brazil	GU586790	*Trichosanthes cucumerina*
India 1	India	GQ482976	*Cucumis anguria*
Cu	Poland	EU561043	*Cucumis sativus* L.
CH99/116	China	AY611021	*C. pepo* L.
Italy 1	Italy	AJ420020	?
14spno1-5	Mali	HM005312	*C. vulgaris* L.
Chile 1	Chile	AF308732	*C. pepo* L.
ZYMV C	USA	L31350	*C. pepo* L.
NA	Korea	AF062518	*C. sativus* L.
TW-TN3	Taiwan	AF127929	*Luffa cylindrica* Roem.
ZYMV-Ah	Turkey	JF317297	*C. melo* L.
ZYMV-Ad	Turkey	JF317296	*C. pepo* L.

NA: not applicable.

RNA extraction, reverse transcription, and PCR amplification (RT-PCR) of CP genes

Total RNA was isolated from 100 mg of leaf tissue according to the silica capture method as described by Foissac et al. (14). Reverse transcription and PCR were by 2 step RT-PCR kit (Fermentas). Based on the nucleotide sequence of the CP coding region of ZYMV Japanese isolate (access. no.: AB188116), primers were made that corresponded to the N- and C-terminal regions. The specific primers for production of CP were (on the 5' terminus) Z-HindIII-F-5'-*CAGT*AAGCTTTCAGGCACTCAGCCAACT-3' and (on the 3' terminus) Z-*Sac*I-R-5-*GT*GAGCTC CTGCATTGTATTCACACCTAGT-3'; they were designed to encompass the unique endonuclease restriction sites *Hind* III and *Sac* I (underlined) and a further 4 additional unrelated residues at their 5' end (italicized) for cloning into the pSPT18 vector (Roche) at *Hind*III and *Sac* I sites on the polylinker. The complete CP genes were generated by RT-PCR with the following thermal cycling scheme: 3 min at 94 °C, 40 cycles of 30 s at 94 °C, 1 min at 62 °C, and 45 s at 72 °C followed by a final incubation of 10 min at 72 °C with 40 cycles.

Cloning and sequencing of the CP genes

Full-length double stranded cDNA was separated on 2% agarose gel, recovered using a Zymoclean™ Gel DNA recovery kit (Zymo Research), and purified with the DNA Clean & Concentrator™ kit (Zymo Research) according to manufacturer instructions. The purified DNA fragments were ligated into the pSPT18 vector for transformation into *Escherichia coli* JM 109 by electroporation (BioRad, USA). The cDNA clones were sequenced by automated DNA sequencer (Applied Biosystems) at Iontek Research and Biotechnology Company.

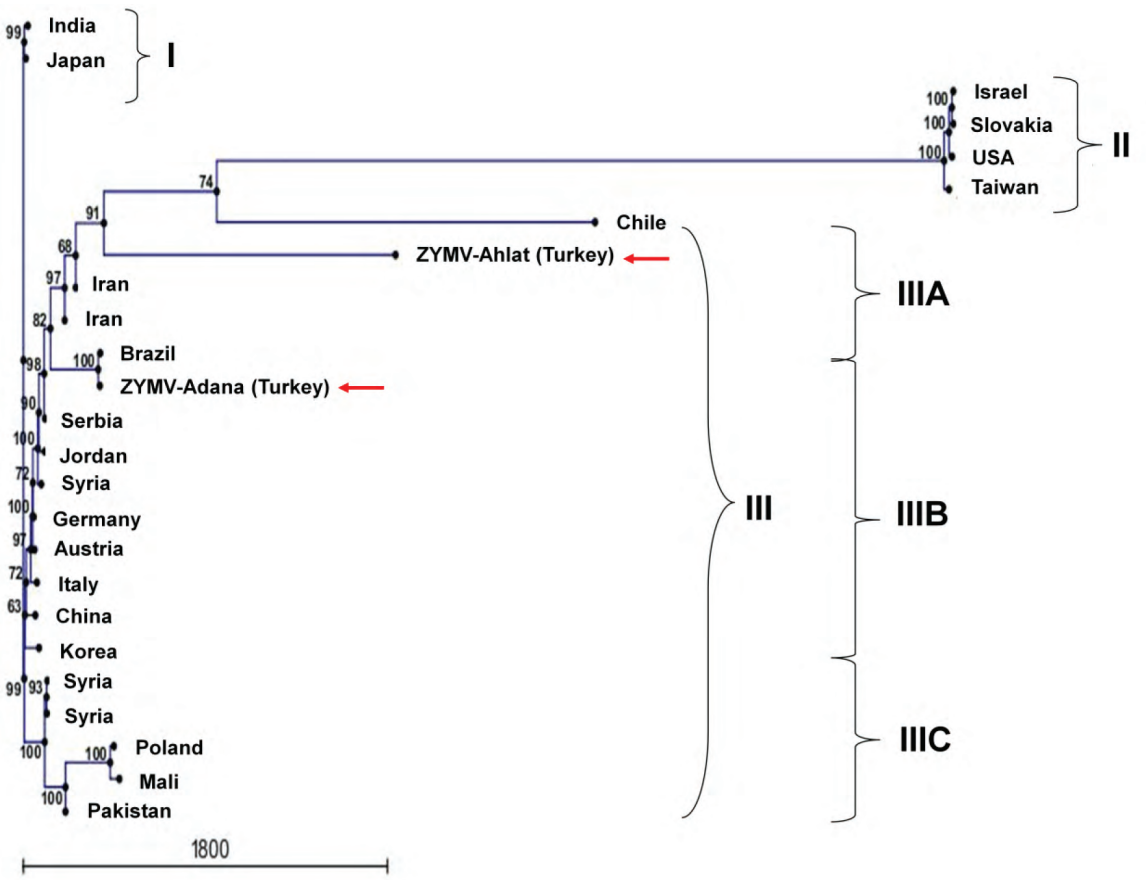

Figure 1. Phylogenetic tree of ZYMV isolates reconstructed from the 837 nt long CP gene. Phylogenetic tree was generated by the neighbor joining method. Bootstrap values of 100 resamplings as percents are indicated at key nodes. Only bootstrap values >63% are shown. Turkish isolates are highlighted by arrows.

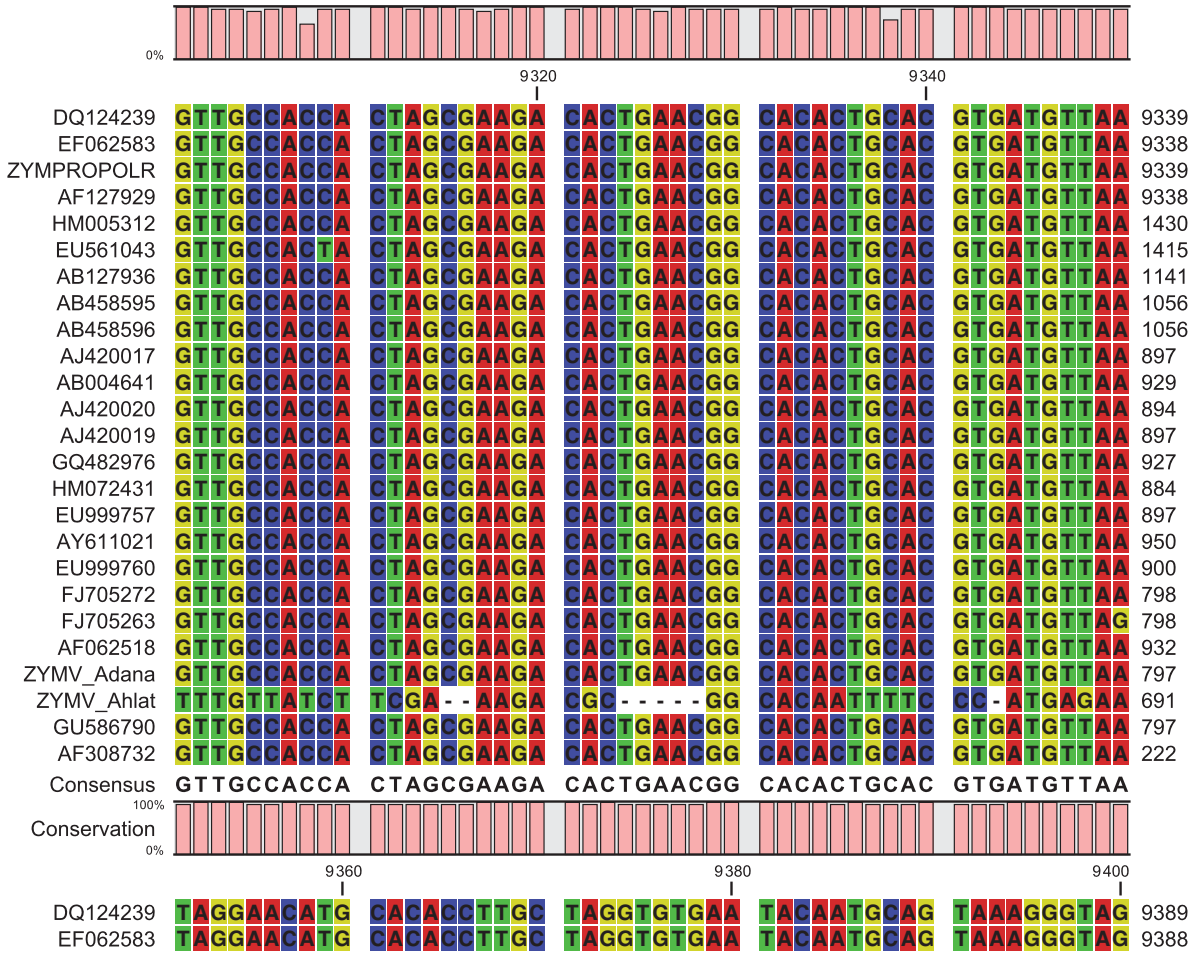

Figure 2. Sequence alignment of Turkish ZYMV isolates with available world isolates based on 837 bp CP gene.

Determination of sequence homology and phylogenetic analysis

For sequence similarity and phylogenetic analysis, GenBank accessions for ZYMV sequences from different hosts were used listed (Table). Multiple sequence alignments were generated using CLC Main Workbench program. Phylogenetic trees were produced according to neighbor joining (100 replicates) and the unweighted pair group mean arithmetic (UPGMA) methods using CLC Workbench 4.4 software. The latter algorithm assumes that evolution occurs at a constant rate in different lineages.

Results

Two ZYMV isolates collected from naturally infected cucurbits from geographically distinct locations in Turkey were analyzed. DNA sequences for the CP protein genes after molecular cloning were reported for the first time for both isolates of ZYMV from Turkey. The 2 CP-cDNA clones, ZYMV-Ad from squash and ZYMV-Ah from muskmelon, both contained 837 nucleotides. The CP molecular weight was calculated to be approximately 31.2 kDa for both isolates. Comparison of the 837 nucleotide sequences of the 2 cloned CP genes, ZYMV-Ad (JF317296) and ZYMV-Ah (JF317297), showed that they were 97% identical. The phylogenetic tree, reconstructed from the sequences of the CP genes (Figure 1), clearly shows clustering of the 2 Turkish ZYMV isolates within 2 distinct groups.

Although bootstrap support values were high and branch lengths were short, one of the isolates, ZYMV-Ah, clustered away from the other Turkish isolate. When compared at the nucleotide level,

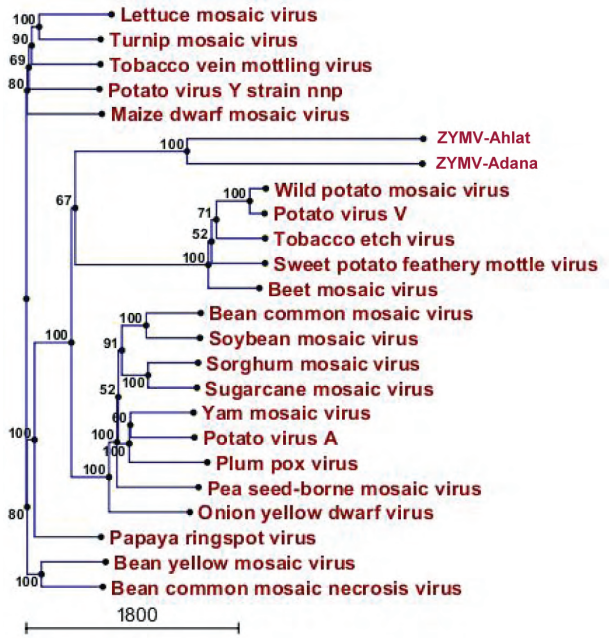

Figure 3. Evolutionary relationships of CP gene of ZYMV Turkish isolates compared to 22 genetically-related potyviruses. Phylogenetic tree was generated by UPGMA method. Default value of 100 was used for bootstrap analysis, and corresponding values are shown on individual branches. UPGMA cluster analysis was performed assuming constant population size and mutation rate. Turkish isolates are highlighted with bold letters, and representative isolates referred to in Table are underlined. Branch lengths are relative indicators of time since divergence.

ZYMV-Ah and Ad isolates exhibited 93%-98% and 94%-99% similarity with the selected world isolates, respectively.

From the analysis of other ZYMV isolates, high sequence conservation was observed between ZYMV-Ad and previously sequenced ZYMV Middle East isolates (Iran, Syria, and Jordan) in the highly conserved region, with nucleotide identity levels reaching 98%-99%.

The alignment of amino acid sequences with 23 CP amino acid sequences from several ZYMV isolates in databases revealed that the Turkish isolates differed from these isolates mostly in the N-terminal region of CP. When compared with the other 22 potyviruses the amino acid sequences deduced in ZYMV-Ad CP and ZYMV-Ah CP were 58%-70% identical. For bootstrap analysis the default value of 100 was used. Bootstrap values are attached to each branch. When 23 selected ZYMV isolates together with 2 Turkish isolate sequences were analyzed, bootstrapping analysis by CLC RNA Workbench software produced a phylogenetic tree with 3 main clusters (I, II, and III). Cluster III, the larger one, was separated by its original hosts (i.e. *C. pepo*). On the phylogenetic tree 3 branches can be clearly recognized (Figure 1). The ZYMV-Ad isolate from *C. pepo* formed a clear branch containing isolates from squash isolates from Syria and Jordan. Cluster III comprised 3 branches of ZYMV from different regions of the world: IIIA, IIIB, and IIIC. Branch IIIB contained *C. pepo*-ZYMV, mostly from Europe (i.e. Germany, Austria, and Italy), the Far East (Korea and China), and the Middle East (Syria and Jordan); branch IIIA contained isolates from Iran and Brazil. Cluster II contained squash-ZYMV isolates from Israel and the USA.

The CP sequence of a ZYMV-Ah isolate had deletions of 8 bp at different positions of the CP gene (positions 9315-9316, 9324-9328, and 9343) that differed from the same nucleotide positions in ZYMV world isolates (Figure 2). A BLAST search of these deletions revealed no similar sequence in GenBank. However, the downstream of the gene contains several substitutions specific to ZYMV-Ah isolate (positions 9159, 9162, 9163, 9164, 9167, 9170, 9175, 9178, 9179, 9180, 9186, 9195, and 9199).

Computer analysis of the 837 nucleotide sequence showed that the CP of both isolates would translate into a protein of 279 amino acids. The nucleotide length and protein size are consistent with the CP of other potyviruses. The overall identity of CP nucleotides of the Turkish isolates compared with 22 other potyviruses was 58%-70% (Figure 3).

ZYMV-Ad and ZYMV-Ah isolates recovered from different original cucurbit hosts were transmitted by mechanical inoculation to *C. pepo* seedlings. Although both isolates exhibited characteristic symptoms on *C. pepo* (leaf malformation, mosaics, and chlorotic mottling), ZYMV-Ah created faster systemic symptom induction than ZYMV-Ad, suggesting that ZYMV-Ah is a more severe isolate than ZYMV-Ad (Figure 4).

Discussion

The present survey presents 2 complete CP sequences of ZYMV from Turkey. The nucleotide sequence data

Figure 4. Characteristic symptoms of ZYMV-Ad and ZYMV-Ah on *C. pepo* (leaf malformation, mosaics, blisters).

reported in this study were deposited in the EMBL GenBank (accession numbers: JF317296 for isolate ZYMV-Adana; JF317297 for isolate ZYMV-Ahlat). Phylogenetic trees were constructed according to the nucleotide sequences of the CP gene of the 2 ZYMV isolates and 23 additional ZYMV isolates reported elsewhere. All 25 ZYMV isolates were classified into 4 groups in phylogenetic trees based on CP nucleotide sequences (Figure 1).

Despite the large distance between the 2 collection sites (up to 900 km), the CP sequences of ZYMV (Ad and Ah) showed very little genetic diversity, regardless of origin and other biological and symptomatological characteristics.

DNA distance analysis using CLC Workbench software and successive clustering by the neighbor joining method showed the ZYMV-Ad isolate grouping with isolates of the virus from Middle Eastern countries (Figure 1). Although they were separated into different branches, results show close similarity between the Middle Eastern and ZYMV-Ad isolates. Geographically, Adana Province is close to Middle Eastern countries, suggesting a possible origin and route for the isolate. Pfosser and Baumann (8) indicate that a specific virus isolate can spread quickly to geographically adjacent areas; however, it may not be directly related to isolates found in neighboring countries.

Similarly, a slightly lower correspondence was seen between the ZYMV-Ah and Asian isolate. As a result, it may be necessary to compare the CP sequence of this isolate with sequences from all the Asian isolates in order to address the origin the virus.

The nucleotide sequences of CP genes of Turkish ZYMV isolates were compared with those of previously reported ZYMV isolates. ZYMV-Ah showed 93%-98% nucleotide similarity to other ZYMV isolates; ZYMV-Ad showed 94%-99% nucleotide similarity.

The analysis revealed that both Turkish ZYMV-CP genes contained 837 nucleotides. This encodes a protein 279 amino acids long with a predicted M_r of 31,225 for Ahlat and 31,339 for Adana isolate. The predicted M_r obtained from the CP gene is consistent with value estimates from Lisa et al. (1); however, it is somewhat lower than their estimate of 36,000.

The amino acid sequence of the CP from both ZYMV isolates was compared with the published amino acid sequences of other potyviruses. The overall homology was between 58% and 70%. The results are in agreement with the 38%-71% range in homologies observed among distinct potyviruses (11,12).

In this study we characterized the ZYMV CP region of 2 Turkish isolates. This is the first report of the full length ZYMV CP sequences from Turkey. Comparing the nucleotide sequences of CP regions of ZYMV isolates revealed the presence of a Middle East cluster in the phylogenetic tree. These findings are in good agreement with the recent results of Desbiez et al. (15) and Tóbiás and Palkovics (16), suggesting effective worldwide dissemination of ZYMV.

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