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Araştırma Makalesi

Bingöl İlinde Tanımlanan Yonca Mozaik Virüs (AMV) İzolatının Filogenetik İlişkisi

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Öz

Yonca mozaik virüsü (AMV), yem bitkileri içerisinde yonca bitkisinin en önemli viral hastalık etmenlerinden biridir ve yıllık olarak önemli ekonomik kayıplara neden olur. Etmen ayrıca domates, patates ve biber gibi diğer kültür bitkileri için de potansiyel bir öneme sahiptir. Bu çalışmada, AMV'nin tanımlanması ve izolatların filogenetik ilişkileri, ters transkripsiyon-polimeraz zincir reaksiyonu (RT-PCR) ve ardından bakteriyel klonlama ile gerçekleştirilmiştir. Bu amaçla yonca örneklerinin (12 adet) cDNA'sı AMV'nin kapsid protein genine (CP) özgü primer çiftleri kullanılarak RT-PCR testlerine tabi tutulmuş ve beklendiği gibi yaklaşık 700 bp'lik bir DNA fragmenti elde edilmiştir. Amplikonlar doğrudan klonlanarak elde edilen diziler genbankasına kaydedilmiştir (Erişim No: MW962977 ve MW962976). Her iki sekansın BLASTn analizi, yoncadan elde edilen AMV izolatlarının, dünyadaki farklı bitkilerden izole edilen diğer AMV izolatlarına oldukça benzer olduğunu ve nükleotit benzerliğinin %97 ile %99.37 arasında değiştiğini göstermiştir. CP gen dizilerine dayanarak elde edilen filogenetik dendrogramın sonuçları, bu çalışmadan elde edilen izolatların Türkiye'deki yonca bitkilerinden izole edilen dört AMV izolatı ile yakından ilişkili olduğunu açıkça ortaya koymuştur. Bu çalışma, Bingöl ilinde sararma, beneklenme ve yaprak anormallikleri gösteren yonca bitkilerinde belirlenen AMV virüsünün ve moleküler filogenisinin ilk raporu niteliğindedir.

Anahtar kelimeler: Filogenetik Ağaç, Klonlama, Kapsid Proteini, RT-PCR, Yonca mozaik virüsü

Phylogenetic Relationship of Alfalfa Mosaic Virus (AMV) Isolate Identified in Bingöl Province of Turkey

Abstract

Alfalfa mosaic virus (AMV) is one of the most important viral diseases of alfalfa plant among the forage crop, causing significant annual economic losses. The agent is also of potential importance to other cultivars such as tomatoes, potatoes, and peppers in most cases. The identification and phylogenetic relationships of AMV were carried out by reverse-transcription polymerase chain reaction (RT-PCR), following by bacterial cloning. The cDNA of alfalfa samples (12) were subjected to RT-PCR tests using primer pairs, specific for the capsid protein gene (CP) of AMV, resulting in a DNA fragment of approximately 700 bp as expected. The amplicons were directly cloned and then resulting sequences were deposited in GenBank (Acc. No: MW962977, MW962976). The BLASTn analysis of both sequences demonstrated that AMV isolates from alfalfa were highly similar to other AMV isolates from various crops in the world, with nucleotide identity ranging from 97 to 99.37%. The results of phylogenetic dendrogram based on CP gene sequences clearly suggested that our isolates are closely related to four AMV isolates from alfalfa in Turkey. To our knowledge, this study is the first report of molecular phylogeny and AMV presence in alfalfa exhibiting yellowing, mottling, and leaves abnormalities in Bingöl province, Turkey.

Key words: Phylogenetic tree, cloning, coat protein, RT-PCR, *Alfalfa mosaic virus*

Introduction

Extensively, alfalfa (*Medicago sativa* L.) is first cultivated as a forage legume crop throughout the world, with an average annual cultivation exceeding 6.412.128 da in Turkey. In Bingöl province, clover production area comes first with 77.405 da and an annual production of 310.252 tons (TUİK, 2020). Alfalfa, which is highly susceptible to bacterial and fungal pathogens, can also be infected by more than 30 viruses, even the co-existence of multiple viruses is more destructive for alfalfa crops, both in terms of quality and productivity (Erwin et al., 1990; Brunt et al., 1990).

Alfalfa mosaic virus (AMV) was first detected in 1931 and has been considered to be the most important viral pathogen of alfalfa ever since (Weimer, 1931; Avgelis, 2008; Parella, 2000). Having a wide range of hosts, the virus is potentially infectious to about 600 species of plants, most of which belong to the Fabaceae family, as well as woody plants and weeds worldwide (Jaspars and Bos, 1980; Xu and Nie, 2006). AMV, a member of the *Bromoviridae* family, has an icosahedral capsid structure of 30−57 nm in length and 18 nm in diameter (Hiruki and Miczynski, 1987). The RNA-structured genome, which is a positive-sense single-strand, consists of 4 genetic parts. RNA1 and RNA2 are responsible for genome replication and internal transcription of sgRNA4, while RNA3 and sgRNA4 are responsible for movement and capsid proteins (Bol, 1999; Van der Vossen et al., 1994). The viral pathogen is efficiently transmitted by about 40 aphid species, especially *Myzus persicae* known as Green peach aphid, in a non-persistent manner, as well as seed, various parasitic plants, and mechanical inoculation (Jeffries, 1998; Ragsdale et al., 2001; Fletcher, 2001; Sertkaya et al., 2017).

The presence of AMV in alfalfa specimens was commonly accomplished by serological and DNA-based methods using AMV specific primer pairs (Mohammed and Mahmoud, 2013; Al-Shahwan et al., 2017). To date, AMV has been recorded throughout the world, including Africa, Asia, Europe, North America, Oceania, and South America continents (Colimba et al., 2016; Hulse et al., 1993; Fisher and Nameth, 2000; Finetti-Sialer et al., 2005; Zadjali et al., 2002; Chalam et al., 2008). In Turkey, AMV infection causing a serious threat in terms of agricultural aspects, has been detected in different crops and insects (Sertkaya et al., 2017; Çetinkıran and Baloğlu, 2011), but studies on its molecular properties are insufficient.

In this work we aimed to isolate the capsid protein (CP) gene of AMV in alfalfa plants, determine the nucleotide sequence by amplifying by RT-PCR, and reveal its phylogenetic relationship using molecular techniques in naturally infected alfalfa plants that had not been reported from Bingöl province of Turkey.

Material and Methods

Sampling, Total RNA Isolation and cDNA Synthesis

The observation and sample collection were performed in September of 2020 in the central district of Bingöl province. A total of 26 plant specimens were gathered from 3 alfalfa fields. Alfalfa plants were visually inspected and virus-indicative alfalfa leaves as well as apparently healthy plants were sampled. All specimens were stored at -80C in the phytopathology laboratory of Bingöl University until further use.

Total RNA extraction from specimens was carried out via the silica-based method of Foissac et al. (2001) with a slight difference. 0.5 g of frozen tissue was excised and ground with ball-bearing grinding homogenizer in 1 ml of Grinding Buffer containing 1% ME (2-Mercaptoethanol). Approximately 1000µl of homogenate with an addition of 100µl of sarcosyl was transferred to 2 ml collection tube and next kept at 70 °C for 10 min by occasional inversion, then incubated on ice for 5 min, and finally centrifuged for 10 min at 13.000 rpm. The supernatants (~500 µl) were transferred to new tubes with EtOH (150µl), NaI (300µl), and resuspended silica (25 µl, pH 2) and inverted gently. The mix obtained was incubated for 10 min on an intermittent shaker, than centrifuged at 6.000 rpm for 1 min. The aqueous phase was discarded and the bottom precipitate was dissolved in a wash buffer (500 µl), then centrifuged for 1 min at 6.000 rpm. The washing step was repeated twice. Precipitate was resuspended in 100 µl of nuclease-free water and incubated at 70 °C for 4 min, then centrifuged at 13.000 rpm for 3 min. The extracted RNA concentration was substantiated by the nano drop method and stored at -80 °C for further molecular analysis.

Total RNAs were submitted to cDNA synthesis. For reverse transcription, the 12 µl reaction mix consisted of total RNA (5 µl), dNTP (1 µl), antisense primer (1 µl), and nuclease-free water (5 µl). The reaction mix was incubated at 65 °C for 5 min, and the other reaction formulation of 8 µl was prepared, which was 4 µl 5X RT buffer, 3 µl nuclease-free water, and 1 µl RT enzyme. The reaction mix was kept at 42 °C for 50 min, and then incubated at 70 °C for 15 min for enzyme inactivation.

PCR tests for AMV determination and DNA sequencing

The resulting cDNA solution were screened for the presence of viral CP genes. The PCR mix reaction (50 µl) contained 5 µl of 10× Taq Buffer, 3 µl of $MgCl_2$, 3 µl of total cDNA, 1 µl dNTP mix (10 mM), 1 µl sense and antisense primer (20 pmol), 0.4 µl of Taq DNA polymerase, 35,6 µl of Nuclease-free H_2O. All the molecular procedures were performed in same thermal cycler (Eppendorf, USA). PCR cycling conditions and primers were adopted as used by Martinez et al. (2004) as follows: the sense primer AMV-CP-S: 5'-GTGGTGGGAAAGCTGGTAAA-3' and antisense primer AMV-CP-A: 5'-CACCCAGTGGAGGTCAGCATT-3'. 15 µl of cycled PCR-amplified yields were run in agarose gel and evaluated electrophoretically in TAE buffer, then viewed in transilluminator apparatus and photographed (Syngene™ UV Transilluminator 2020LM). 1 Kb molecular size of DNA ladder (Fermentas) was used as a standard marker. The previously characterized alfalfa-AMV isolate (MT210179, Alakoy Y9) was used as a positive control, and cDNA from healthy plants was used as a negative control.

In DNA sequencing, two of the randomly selected visible DNA fragments were purified from agarose gel, and directly submitted to prokaryotic cloning vector pGEM T-Easy plasmid using T4 DNA Ligase enzyme (Promega, USA). Ligation products were transferred electrically into competenet cell *E. coli* JM 109 strain. Electroporation was performed by MicroPulser Electroporator (Bio-Rad, USA). Purified recombinant plasmids containing the CP gene of the two isolates were sent to the company for DNA sequencing (Sentebiolab-Turkey) and the associated AMV sequences were recorded in NCBI.

Phylogenetic analysis of AMV-CP gene

Phylogenetic relationships were assessed using the CP sequences of AMV identified from alfalfa and from 20 other CP sequences from GenBank reference isolates using the Neighbor Joining method of MEGA software version 7 (Table 1). The phylogeny inference was conducted from 1000 resampling using an isolate (KC900900) of *Barley yellow mosaic virus* as the outsource.

Results and Discussion

AMV is one of the major destructive viruses in plants of economic value in the *Solanaceae* and *Fabaceae* families. In field conditions, this infection reduces yield by about 20% on both fresh and dry weight basis. AMV-infected alfalfa specimens exhibite growth retardation and even the re-shooting rate of the alfalfa plants is low after cutting. On the other hand, the green part, seed production, and nitrogen fixation as well as nodule formation in alfalfa roots are negatively affected by AMV infection. This means economic and time loss for the producer (Bailis and Ollennu, 1986; Edwardson and Christie, 1986). Another markedly effect of AMV is that the essential mineral content such as Fe, Cu, Zn, and Mn in alfalfa leaves is significantly reduced as a result of plant metabolism and cell integrity disruption by AMV infection (Yardımcı et al., 2007).

Symptoms reminiscent of virus infection were observed in fields of alfalfa plants in Bingöl province of Turkey. AMV diseased alfalfa plants are frequently associated with characteristic symptoms such as yellow patch of leaves, intervenial chlorosis, development abnormalities, and mottling (Rubies- Autonell and Turina, 1994; Parrella et al., 2010; Martínez-Priego et al., 2004), which was also noticed on alfalfa plants in Bingöl province (Fig 1).

Figure 1. Symptom diversity in alfalfa plants showing virus-like symptoms in Bingöl province

Until now, indicator plant tests, serological and DNA-based molecular methods have been widely preferred to diagnose the viral pathogen. The presence and incidence of AMV infection was successfully detected by ELISA assays using antibodies specific to the CP from Solanaceous

products and weeds by Fidan et al. (2012), Pourrahim and Farzadfar (2016), and Sofy et al. (2021). In the present study, we identified two different AMV isolates, cloned and molecularly characterized the CP genes from naturally infected alfalfa in Turkey in 2020. Positive results were obtained from 12 symptom-bearing samples of all specimens tested (26) by PCR assays. Amplicons of desired size (about 700bp) was evaluated as a positive result. No PCR product was detected for the healthy ones (Fig 2).

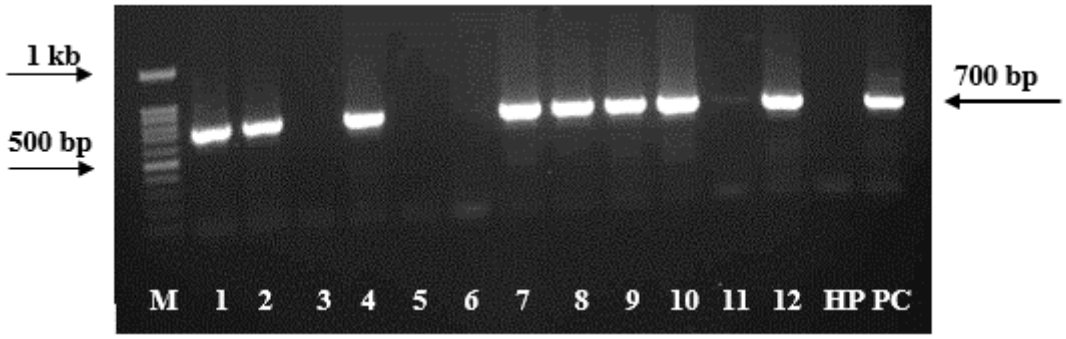

Figure 2. Agarose gel image showing the molecular size of RT-PCR amplification products applied to some symptomatic and asymptomatic alfalfa plants. M: 1kb DNA Marker; 1, 2, 4, 7, 8, 9, 10: Amplified DNA fragments of AMV CP gene from alfalfa; 3, 5, 6, 11: AMV-negative specimens; HP: Healthy plants; PC: Positive control

Two isolates randomly selected from positive-reacted DNAs were successfully cloned, sequenced, and characterized. Two viral CP sequences of 700 bp length were recorded in the GenBank with Acc. No. MW962977 and MW962976, named as Bingöl 11 and Bingöl A8 isolates. This result is in agreement with the findings reported by various researchers using the same primer set in alfalfa and other plant species (Al-Saleh and Amer, 2013; Abdalla et al., 2019; Usta and Güller, 2020). Different primers can also be used in the diagnosis of AMV. Stanković et al. (2014) detected the AMV by obtaining a DNA band of 751 nucleotides with different primer sets designed for the CP gene in the lavandin plant in Croatia. Similarly, 351bp, 780 bp, and 620 bp PCR amplification products were obtained by Xu and Nie (2006), Massumi et al. (2012), and Zitikaitė and Samuitienė, (2008), who used CP specific primer pair in alfalfa and tomato plants.

AMV is infectious for edible crop and other weeds including potato, lucerne, eggplant, snap bean, cowpea, pepper, chickpea, sugar beet, tomato, lavandula, *Tecoma capensis*, mung bean, *Viburnum tinus*, *Teucrium fruticans*, *Carica papaya, Hibiscus cannabinus* (Shepherd et al., 1964; Rubies-Autonell and Turina, 1994; Hajimorad and Francki, 1988; Al-Shahwan et al., 2017; Abdalla et al., 2019; Mangeli et al., 2019; Peña et al., 2011; Parrella et al., 2012; Moreira et al., 2010). Based on the pair wise comparison, a high degree of nucleotide similarity was determined among the 21 AMV sequences given in Table 1. Two AMV isolates (MW962977 and MW962976) from alfalfa in Bingöl province showed 99.86% sequence identity among themselves. The homology of CP gene sequences of both AMV isolates ranged from 97 to 99.37% with that of other members of the *Alfamovirus* genus. The phylogenetic interrelationship created from 21 CP sequences of AMV using the Neighbor-Joining algorithm indicated that both AMV sequences are closely related to alfalfa isolates from Van province (MT210179 and MT210178) and Iğdır province of Turkey (MW882262 and MW882261) (Fig 3).

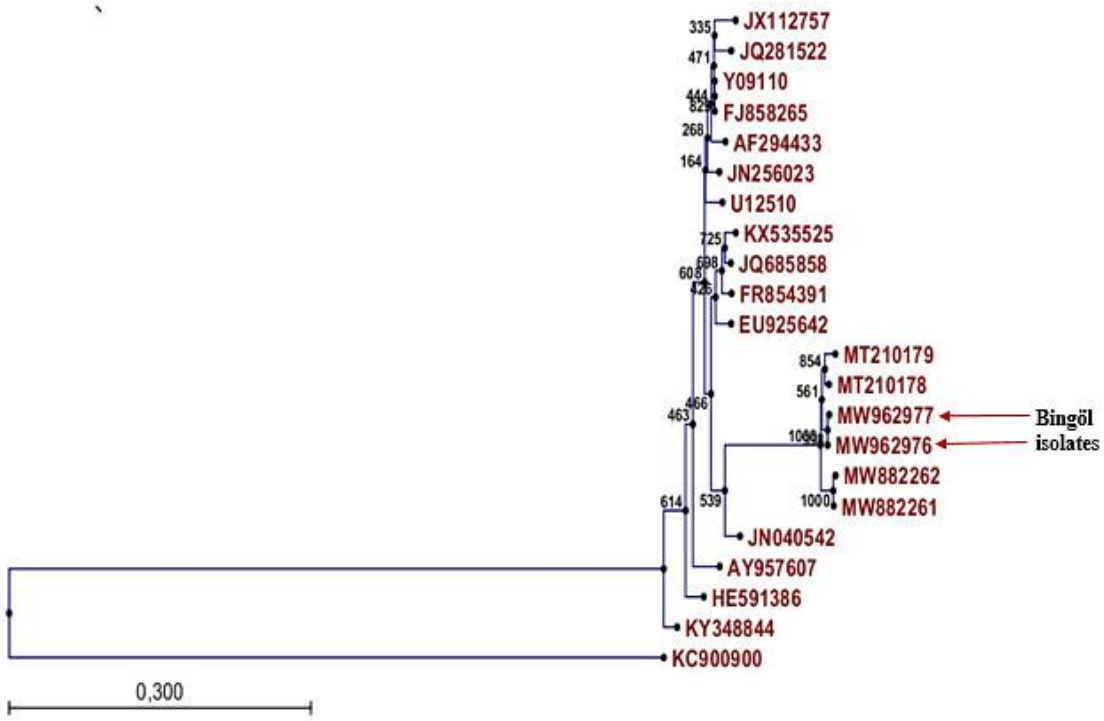

Figure 3. Alfamovirus phylogenetic dendrogram. A Neighbor-joining tree was constructed based on 1000 bootstrap resampling. Numbers on branches refer to bootstrap values associated with viral CP sequence.

Earlier literature suggested that AMV has existed for forty years in Turkey and various crops are highly susceptible to this infection (Erdiller and Lesemann, 1985; Çalı, 1990; Yardımcı and Açıkgöz, 1997; Çıtır, 1982; Usta and Güller, 2020). The presence of AMV has been reported in agroeconomic crops and weed such as potato, eggplant, *Datura stramonium*, bean, alfalfa, husk tomato, and pepper from agricultural region nationwide (Özdemir and Erilmez, 2007; Sertkaya et al., 2017; Ozdemir et al., 2011; Çulal Kılıç and Yardımcı, 2015; Çetinkıran and Baloğlu, 2011; Topkaya, 2020). On the contrary, eggplant plantation regions were screened against AMV infection using RT-PCR technique in Antalya province in 2018-2019, but no AMV infection was detected in eggplant (Fidan and Sarıkaya, 2020). It seems that the spread of the viral pathogen and the resulting crop loss will increase from year to year. It is highly likely that the virus will spread from alfalfa, which is the main inoculum source, to sensitive economic crops in adjacent fields causing epidemics if the necessary control measures are not taken.

Conclusion and Recommendations

In this study, AMV infection was detected in symptomatic alfalfa plants by using molecular tools in Bingöl province. Other AMV host vegetables such as potatoes, tomatoes and peppers are also widely grown in this area. Considering the spread of this virus by aphids, seeds and weeds, farmers should be informed about this agent in order to minimize potential crop losses and prevent epidemics, and a comprehensive survey should be carried out to detect viral distribution in other vegetable crops.

Conflict of Interest Statement: The authors declare that there is no conflict of interest between them.

Contribution of Researchers: The authors contributed equally to this article.

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