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Molecular Characterization of *Lettuce mosaic virus* Isolates in Ankara Province

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Abstract **– Lettuce (*Lactuca sativa*) is an important vegetable crop with increasing economic value in Ankara province of Turkey. Many viruses cause diseases and economic losses in lettuce production and among them Lettuce mosaic virus (LMV), is one of the most common virus infection. In this present research, 324 symptomatic and asymptomatic lettuce samples were collected from five districts of Ankara province (Beypazarı, Çubuk, Ayaş, Nallıhan and Centrum) in May-July, 2015 and all of the collected samples were subjected to serological test of double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and among them, 25 samples were identified as solely infected with LMV. All of the selected isolates were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) assay. Coat protein gene specific primer pairs were used and amplified products were 800 bp long. Amplified cDNA fragments of Beypazari, Cubuk, Ayas isolates were sequenced for molecular characterization and the detection of genetic diversity of LMV Ankara isolates. Sequence comparisons showed 89-99%, 93-100 % and 57-100% sequence identity at nucleotide, amino acid level and N-terminus regions, respectively, of the CP gene of LMV isolates. The CP gene of the LMV Ankara isolates were clustered with Western European, Latin American, North African isolates.**

Keywords **– Ankara, Coat Protein (CP) Gene, Lettuce, LMV, Phylogenetic Analyses**

I. INTRODUCTION

Lettuce (*Lactuca sativa*) is taxonomically a member of *Asteraceae* family, mainly used as in salads contains very rich contents of vitamins and minerals [1]. Although Ankara has a terrestrial climate, fruit and vegetable cultivation have been done intensively in different regions of the province. Lettuce is considered as an indispensable vegetable of diets and meals. In addition to being a diet vegetable, the economic value is increasing day by day in the world. In Turkey, an average of 478.442 tons of lettuce is produced annually and 15% of this production takes place in Ankara Province area [2]. *Lactuca sativa* var. *capitata* (type of iceberg) is the most commonly cultivated lettuce variety and the other varieties, *L. sativa* var. *longifolia* and *L. sativa* var. *crispa* are also grown in Ankara. Particularly, Beypazari district, is the most important lettuce production area in the Central Anatolia region. While lettuce production and plantation has been increasing recently, fungal pathogens and viruses, cause diseases that reduce the quality and yield of lettuce leading to economic losses. It has been reported distinct viral pathogens belonging to different families and genuses infect lettuce [3].

Lettuce mosaic virus (LMV), which is one of the most important among many viral pathogens was described first time in 1920 by Jagger in Florida, USA [4] and has been subsequently reported in many parts of the world. LMV belongs to the *Potyvirus* genus which contains a large number of members within the family Potyviridae [5].

The genome of LMV consist of 10.080 nucleotides (nt) long, has a monopartite positive ssRNA [6] and its genomic RNA is covalently bound to a virus-encoded protein named Vpg at the 5' terminus and has a poly-(A) tail at its 3' terminus [5]-[7]. The Potyviruses genome contains a unique open reading frame (ORF) expresses an extensive 340–370 kD polyprotein [8]. LMV viral genome encodes a single polyprotein containing 3,255 amino acids which is self-cleaved into functional proteins [6]. The amino acid and nucleotide similarities of the CP gene sequences are used in the identification and classification of species and strains of *Potyvirus* genus [9]-[10]. However, recent studies have shown that while species can be distinguished from potyviruses by their CPs [11], [6] and an entire polyprotein [12]-[13], strain could not be distinguished and their geographical distributions can be determined according to the sequence data of the CPs and N-terminus [11], [6]. It has been identified as the most suitable criterion for the assignment of 76-77% nt consubstantiality species of coat proteins of potyviruses [14]. LMV-RoW, LMV-Greek and LMV-Yar, which are LMV strains were phylogenetic ally clustered in regions containing NIb and part of the N terminus of the CP rather than entire CP region [6]. The sequence data of CP proteins of potyviruses have become very important in the development of highly useful and easily applicable a serological techniques and in the taxonomical classification [10]. The N-termini of the N and C terminuses, which contains the most immune dominant regions in the particles of potyviruses and is present in the coat proteins, is quite large and virus-specific antibodies can be formed in this region [15]. By cloning the CP of LMV and using recombinant technology, polyclonal antisera have been developed for high sensitivity and rapid serological diagnosis [16].

LMV is efficiently transmitted by aphids species in a non-persistent manner, and some strains are also seed borne [17]-[19]. The transmission of the virus with both aphids and seeds can lead to epidemics, especially in areas where lettuce is grown continuously [20]. Specification to such environmental conditions, virus strain, infection stage and genotype are very influential on the type of symptoms caused by LMV [16]. The characteristic symptoms on infected lettuce varieties are mosaic, dwarfing, defective heading, yellowing of the leaves, mottling, leaf distortion, necrotic spots, vein- clearing, have a frilly appearance and systemic wilting followed by plant death [19]-[23].

In this study, lettuce isolates of LMV from Ankara province, which is one of the most important regular market and lettuce production in Turkey, were determined by DAS-ELISA and RT-PCR assays. The portion CP gene of

isolates representing divergent districts which are Beypazari, Ayas, Cubuk later on were sequenced and compared to previously identified CP genes sequences of LMV isolates from divergent geographical origins and several hosts. Besides that, different types of LMV symptoms in lettuce botanical varieties were also observed.

A preliminary result of this study has been published [24].

II. Materials and Methods

A. Virus Sources

Surveys were performed during in May - July in 2015, and surveys were conducted to Ankara province. Symptomatic and non-symptomatic samples were collected from lettuce fields, showing chlorosis, necrosis, mild and severe mosaic symptoms on leaves.

B. DAS-ELISA

All samples collected from the fields were tested against LMV by the DAS-ELISA method (Clark and Adams, 1977) [25]. DAS-ELISA test was implemented with ELISA kits (Bioreba, Switzerland) according to the manufacturer's instructions.

C. Total RNA Isolation

Three different methods of RNA isolation involving Astruc et al. (1996) [26], Foissac et al. (2001) [27] and GeneJET RNA Purification Kit (Thermo Scientific, Lithuania) were applied to obtain RNA at optimum rates qualitatively and quantitatively from the samples. Total RNAs were put in nuclease free water and stored at -80°C. The RNAs quality and yield were measured using Nano Drop 2000 Spectrophotometer (Thermo Sci., Waltham, MA, USA).

D. RT-PCR Amplification

The RT-PCR method was performed in two steps in order to amplify the CP gene of LMV. First, cDNAs were synthesized in a 25 µl reaction mixtures including 3 µl of total RNA, 0.15 mM each of dNTP, 6 µl of 5X RT-buffer (250 mM Tris-HCI, 250 mM KCI, 20 mM MgCl2, 50 mM DTT), 40 units of MMLV-RT enyzme (Thermo Scientific, Lithuania), 16 units of RNase inhibitor, 1.2 mM reverse primer (1087) and then kept for 1 h at 42 °C. Second, PCR was performed in 25 µl reaction mixture containing 3.5 µl of 10X PCR buffer (100 mM Tris-HCI, 500 mM KCI, 0.8% Nonidet P40), 1.5 units of Taq DNA polymerase (Thermo Scientific, Lithuania), 0.4 mM dNTP, 2 mM MgCl2, 2 µM each of LMV-specific primers 1196 (5'-AAG GCA GTA AAA CTG ATG-3') and 1087 (5'-TTT ATA CTA CAG TCT TTA-3') [21] and 2 µl of cDNA. PCR amplification specifications consisted of 3 min denaturation at 94 °C, followed by 35 cycles of 95 °C for 1 min, primer annealing at 42 °C for 1 min and primer extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. The PCR amplified products were separated by electrophoresis in 1.2% agarose gel with 100 bp DNA marker (Thermo Scientific, Lithuania) and stained with ethidium bromide.

E. Sequencing and Phylogenetic Analyses

Nucleotide sequences of the LMV CP genes were determined using a pair of specific primers of CP [21]. In order to represent the isolates of LMV CP genes in Ankara province, PCR products were selected from each infected regions and directly sequenced (Ankara, Turkey). Phylogenetic analyses were performed by selecting LMV isolate sequences collected from different hosts and geographical regions of the world that were available on GenBank databases of National Center for Biotechnology Information (NCBI) (Table 1.), and then accordingly three different analyses were performed based on nucleotide, amino acid and N-terminal sequences differences of those LMV isolates. Phylogenetic analyses were performed using MEGA 7 software [28], using multiple sequence alignments in Clustal W sequencing and phylogenetic trees were described by statistical method of the neighbor-joining using Kimura-two-parameter model. Bootstrap analyses with 1000 replicates were implemented to anticipate the corroboration for inferred phylogenies. Finally, the phylogenetic trees were displayed by M7: Tree Explorer software. After all LMV isolates were aligned with Clustal W, similarity ratios of these isolates were calculated using Sequence Demarcation Tool, version 1.2 program [29].

III. Results

A. Virus Sources and Field Surveys

Lettuce leaf samples were collected from 5 divergent districts of Ankara province located in Central Anatolia of Turkey and they were stored in deep freeze at -20°C. Totally 324 samples were collected from the research areas. Mosaic symptoms and necrosis were generally observed on the leaves collected from lettuce fields but there was no dwarfing, no systemic wilting and a frilly appearance on lettuces. Furthermore, the symptoms observed among the different varieties of lettuce were differentiated. Severe mosaic with necrosis and mild mosaic symptoms were observed on these botanical varieties of *L. sativa* var. *longifolia* and *L. sativa* var. *crispa,* respectively but no symptoms on *L. sativa* var. *capitata* (type of iceberg) variety. Symptoms observed on some of the collected *L. sativa* var. *longifolia* and *L. sativa* var. *crispa* samples infected with LMV is shown in Fig.1

B. Determination of LMV by DAS-ELISA Test

All of the collected samples were subjected to DAS-ELISA test and LMV was detected from 25 of 324 lettuce samples collected from three districts of Ankara province, except the districts Nallihan and Merkez. The LMV infection rates were calculated for all of Ankara districts and Cubuk had the highest rate of LMV infection with %40, followed by Ayas (%11,1) and Beypazari (7,1).

Fig. 1. Severe mosaic and necrosis symptoms on leaves of cos lettuce infected with LMV (Beypazari, Ankara).

C. Total RNA Isolation and RT- PCR

Confirmation of DAS-ELISA test results were done by RT-PCR assay. Total RNA isolation was performed from samples giving positive results according to the ELISA test and therefore a total of 25 lettuce samples detected LMV positive by DAS-ELISA were selected for RNA isolation together with some non-infected isolates. In terms of workforce, time and productivity, the values obtained with the use of commercial kit were found to be more efficient and the works were then continued using the isolation kit. RT-PCR assay was performed in two steps; cDNAs containing the CP gene of LMV obtained at the first step with the reverse primer and these were amplified at the second step. As a consequence of RT- PCR assays, fragments of 800 bp were obtained from all of LMV infected samples and no amplification was observed with non-infected samples.

D. Phylogenetic Analysis

The nucleotide sequences of CP gene of LMV isolates were converted to amino acid sequences and analyzed using the Mega7 program [28]. These analyses were performed to N-terminus, nucleotides and amino acid sequences of the CP genes of 32 previously sequenced LMV isolates from different geographical origins and various hosts and the portion CP gene of 3 isolates sequenced in this study. The coat protein of the Plum pox virus isolate was included as an out group in analyses in order to enhance the comparison of isolates.

The Beypazari (Acces.no. MG517452), Ayas (Acces.no. MG517454), Cubuk (Acces.no. MG517453) isolates and other isolates obtained from GenBank were divided into 2 major branches in phylogenetic tree according to nucleotide sequences of the CP. The first major branch was not divided into subgroup and only single isolate named YAR [30] was placed there. The second major branch contained 34 isolates obtained from different hosts and countries, however, the Greek isolates and the Spanish isolate named ES16 [30] separated from other 30 isolates and these seemed to be separate groups among themselves (Fig. 2).

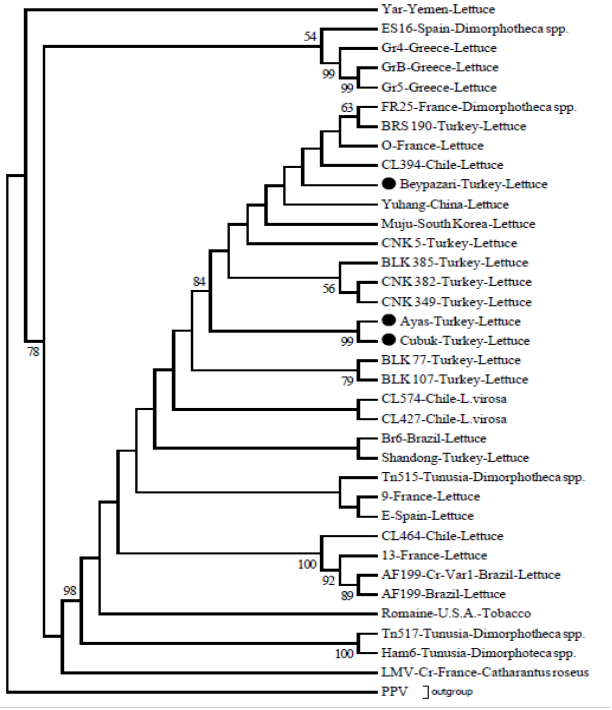

Fig. 2 Phylogenetic tree was constructed by statistical method of the neighbor-joining using the Kimura-2-parameter model, displaying the relationships between the nucleotide sequences of the LMV coat protein gene derived from lettuce to several hosts and different geographical regions in the world were selected from the GenBank database of NCBI. The CP gene of PPV (Access. no. U27652) was used as an out-group isolate (Bootstrap value higher than 50% is shown in the trees)

Three major branches were distinguished in phylogenetic tree which are constructed using amino acid sequences of the LMV CP gene containing 269 amino acids (Fig. 3a). The first major branch was not divided into subgroup and this group is composed of single branch contained the isolates of Greek, ES16 and YAR. The second major branch contained only single isolate named LMV-Cr [31] from France and *Catharantus roseus* host plant. The third major branch contained 3 lettuce isolates from Ankara and 26 LMV isolates from lettuce and different hosts including tobacco, *Lactuca virosa* and *Dimorphotheca* spp. from Turkey and different countries, though the branch including 29 isolates were not clearly divided into subgroup according to the amino acid sequences of the CP gene. However, another phylogenetic analysis was performed to determine the genetic diversity of the N-terminus amino acid sequences of the CP gene of the isolates placed in Table 1. When phylogenetic analysis was conducted with amino acid sequences of the LMV CP, similar branching was observed, namely, three major branches were distinguished in tree (Fig. 3b).

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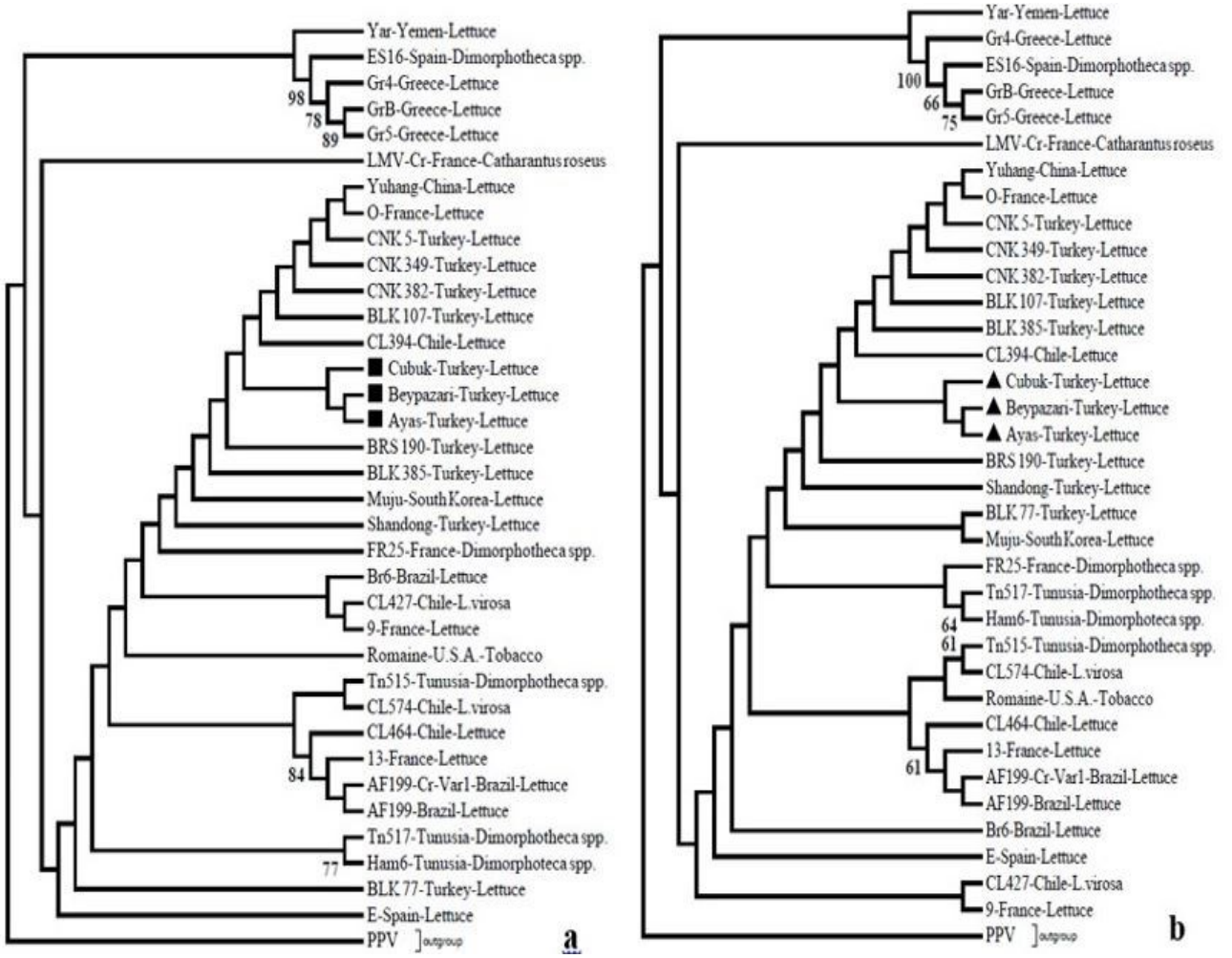

Fig. 3 Phylogenetic tree was constructed by statistical method of the neighbor-joining using the Kimura-2- parameter model, displaying the relationships between the 269 amino acids **(a)** and N-terminus **(b)** sequences of the LMV coat protein gene derived from lettuce to several hosts and different geographical regions of the world were selected from the GenBank database of NCBI. The CP gene of PPV (Access. no. U27652) was used as out-group. (Higher than 50% bootstrap value is shown in the trees)

In the first major branch including 5 isolates, the isolates of Greece, ES16 and YAR were clustered. While the second branch contained only one isolate named LMV-Cr, the third branch contained all other remaining isolates from Ankara Province and different geographical regions and various hosts. The third major branch was consisted of 29 LMV isolates from Turkey and other countries.

Sequence comparisons of the portion CP genes of LMV Ankara isolates showed 89-99%, 93-100% and 57-100% homology in nucleotide, amino acid and N-terminus sequences respectively with the other LMV isolates from different countries of the world and various hosts.

IV. Discussion

Symptomological observations were made during field surveys and the severity of LMV symptoms varied among lettuce varieties. Common LMV symptoms were not observed in *L. sativa* var. *capitata* (iceberg type) varieties in fields, moreover suspected samples collected and were tested serologically and molecularly both, gave negative results. The most severe mosaic symptoms with necrosis was observed in cos lettuce (*L. sativa* var. *longifolia* 'Yedikule') variety, while mild mosaic symptoms were observed in curly lettuce (L. sativa var. crispa).

In this study, field surveys were conducted to lettuce fields in 5 different regions of Ankara Province and 324 samples were collected from according to situations of symptomatic and asymptomoatic plants and DAS-ELISA were first applied to all samples collected from the fields. Results of serological tests were similar to serological findings obtained from previous studies related to detection of LMV in different regions of Turkey including the South Marmara [32] and the Eastern Mediterranean Region [33]. Also, different serological methods have been successfully used with different applications for the detection of LMV in infected plants such as Indirect-ELISA [21], tissue immune blotting [11] and ACP-ELISA (antibody coated plate) for the accumulation of LMV [34]. However, with the moderately immunogenic properties of the CP region of potyviruses [5], polyclonal antibodies have been developed which we can now use for more sensitive detection of LMV [16].

Table.1 A list of isolates used for phylogenetic analysis

Accession number	Isolate	Host	Origin	Reference
KJ161194	Yar	Lettuce	Yemen	Candresse et al. 2014 unpub.[30]
Z78230	GrB	Lettuce	Greece	Revers et. al. 1997 [6]
Z78228	Gr5	Lettuce	Greece	Revers et. al. 1997
Z782229	Gr4	Endive	Greece	Revers et. al. 1997
KJ161192	Tn517	*Dimorphotheca* spp.	Tunusia	Candresse et al. 2014 unpub.
KJ161185	ES16	*Dimorphotheca* spp.	Spain	Candresse et al. 2014 unpub.
KJ161188	Tn515	*Dimorphotheca* spp.	Tunusia	Candresse et al. 2014 unpub.
KJ161187	Ham6	*Dimorphotheca* spp.	Tunusia	Candresse et al. 2014 unpub.
KJ161186	FR25	*Dimorphotheca* spp.	France	Candresse et al. 2014 unpub.
KJ161183	CL574	*Lactuca virosa*	Chile	Candresse et al. 2014 unpub.
KJ161182	CL464	Lettuce	Chile	Candresse et al. 2014 unpub.
KJ161180	CL427	*Lactuca virosa*	Chile	Candresse et al. 2014 unpub.
KJ161179	CL394	Lettuce	Chile	Candresse et al. 2014 unpub.
KJ161173	13	Lettuce	France	Candresse et al. 2014 unpub.
KJ161172	9	Lettuce	France	Candresse et al. 2014 unpub.
KX378997	BLK385	Lettuce	Turkey/Balikesir	Karanfil and Korkmaz 2016 [32]
KX378993	BRS190	Lettuce	Turkey/Bursa	Karanfil and Korkmaz 2016
KX378989	BLK77	Lettuce	Turkey/Balikesir	Karanfil and Korkmaz 2016
KX378991	BLK107	Lettuce	Turkey/Balikesir	Karanfil and Korkmaz 2016
KX378987	CNK382	Lettuce	Turkey/Canakkale	Karanfil and Korkmaz 2016
KX378985	CNK349	Lettuce	Turkey/Canakkale	Karanfil and Korkmaz 2016
KX378968	CNK5	Lettuce	Turkey/Canakkale	Karanfil and Korkmaz 2016
KJ161174	Br6	Lettuce	Brazil	Candresse et al. 2014 unpub.[30]
KF268956	LMV-Cr	*Catharanthus*	France	Svanella-Dumas et al. 2014 [31]
KF955619	Muju	*roseus*	South Korea	Lim et al. 2014 [39]
AJ306288	Yuhang	Lettuce	China	Zheng et al. 2001 unpub.[37]
X977704	0	Lettuce	France	Revers et al. 1997b [36]
X97705	E	Lettuce	France	Revers et al. 1997b
EF633502	Shandong	Lettuce	China	Wang et al. 2007 unpub.
KF268955	AF199-Cr	Lettuce	Brazil	Svanella-Dumas et al. 2014
KF268954	AF199	Lettuce	Brazil	Svanella-Dumas et al. 2014
LMU24670	Romaine	Lettuce	U.S.A.	Zerbini et. al. 1995 [21]
MG517452	Beypazari	Tobacco	Turkey/Ankara	In this study
MG517454	Ayas	Lettuce	Turkey/Ankara	In this study
MG517453	Cubuk	Lettuce	Turkey/Ankara	In this study
		Lettuce		

All strains of LMV were detected with serological tests [34] and this study showed that LMV infected plants collected between May and July could also be detected at an effective level by the DAS-ELISA method.

Each leaf sample collected from the fields was labeled and stored at -20 ° C within 1 and 2 days to perform RNA isolation and other tests. Different isolation methods including method recommended by Astruc et al. (1996) [26], Foissac et al. (2001) [27] and GeneJET RNA Purification Kit (Thermo Scientific, Lithuania) have been tried to obtain pure and high quality RNAs from lettuce which leaves rich in phenolic compounds [35]. In this study, RNAs was obtained from lettuce samples using all this three methods but no pure and high quality RNA was isolated from the samples with Foissac et al. (2001) [27] extraction method whereas RNA was isolated with method recommended by Astruc et al. (1996) [26], but this method has succeeded in obtaining high amounts of RNAs, although it is not effective enough to remove the organic compounds contained in the plant. Though the concentration was low with GeneJET RNA Purification Kit, pure RNAs suitable for pure and molecular studies free from organic and inorganic compounds have been obtained and also much more time efficient.

Sequence comparison between Ankara LMV isolates and other Turkey LMV isolates [32] showed the amino acid, nucleotide and N-terminus sequence similarities ratios are quite high, arriving approximately 97-100%, 97-99% and 90-100%, respectively. Zerbini et al. (1995) [21] showed that hypervariable (HV) regions of the CP genes of the 11 LMV isolates shared similarity at 88 to 98%. In this study, the HV region of the CP genes of Ankara isolates showed quite varied similarities with the world isolates determined in previous studies in Table 1. From 57 to 100%. The N-terminal part of the HV region of the coat proteins of potyviruses is highly variable in terms of amino acid sequences [9]-[10],[15] and the difference in the amino acid sequences of this immune dominant HV region was demonstrated by phylogenetic analysis and similarity analysis in this study.

Phylogenetic studies showed that Beypazari, Ayas, Cubuk isolates were clustered with Western European, North Africa and Latin American isolates and shared the highest similarities with LMV-0 [36], Yuhang [37] and Turkish isolates. However, LMV-Cr isolate [34] was clustered on a separate branch according to nucleotide, amino acid and N-terminus. Zerbini et al. (1995) [21] and [38] found that the HV region sequence of the CP gene is

not a distinguishing criterion for determining strains of isolates of LMV, the same finding were also obtained in this study. In another study, at least three groups were separated in phylogenetic analyzes based on the results of the N-terminal region of LMV isolates [11] when the CP genes of the Ankara isolates and isolates that newly loaded in Genbank were evaluated, 3 groups were conserved in phylogenetic branches, but groups of some isolates have changed and located at different branch tips.

Molecular results based on amino acid sequence of N-terminus region of the LMV CP genes showed that phylogenetic clusters differ in this study. Es16, Greek and YAR isolates clustered together, however, LMV-Cr and RoW isolates separated two main branches and Ankara isolates were placed in the biggest cluster with RoW isolates. Also, the last amino acid of the CP gene of Cubuk (Acces.no. MG517453) isolate was differed from other LMV isolates by a point mutation and this point mutation resulted in the conversion of Histidine (His) amino acid to Glutamine (Gln).

The Ankara isolates which were clustered in the same main branch with RoW isolates and new isolates obtained as a result of recent studies showed a wide genetic variation in phylogenetic studies. In particular, LMV-Cr which is a European isolate was located at the tip of a different branch than the other European isolates which has been generally clustered with each other at a main branch in the phylogenetic trees in terms of the CP sequence. In addition, the LMV pathogen isolated from lettuce and various hosts showed a wide genetic variation and were not formed sub group according to host range compared to the CP, one of the most important proteins, in phylogenetic studies.

The LMV coat protein gene, in which the point mutations occur frequently at the N-terminus region; namely different proteins formed by transition and transversion; consequently will lead to emergence of the new strains and different situations arise in the transmission of the pathogen, increment of the host range in the future.

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