Phytoparasitica (2009) 37:477–483
DOI 10.1007/s12600-009-0058-z

Characterization of a new strain of *Eggplant mottled crinkle virus* (EMCV) infecting eggplants in Israel

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Received: 29 July 2009 / Accepted: 8 September 2009 / Published online: 20 October 2009

Abstract Eggplant (*Solanum melongena*), which is grown worldwide, is cultivated all year round in Israel. We report on the outbreak of a new viral disease infecting eggplants in Israel, which causes significant damage due to plant stunting, leaf mottling and narrowing, accompanied by fruit malformation. Virus purification from the infected plants yielded isometric particles, 37–40 nm in diameter. Biological and molecular characterization of the viral agent indicates that it is a new strain of the *Eggplant mottled crinkle virus* (EMCV-Is) that has been assigned to the *Tombusvirus* genus. Phylogenetic analysis indicated a close relationship of EMCV with *Pear latent virus* (PeLV) and *Lisianthus necrosis virus* (LNV), which can be considered strains of EMCV rather than distinct viruses.

Keywords *Lisianthus necrosis virus* (LNV) · *Pear latent virus* (PeLV) · *Solanum melongena* · *Tombusvirus*

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Introduction

The ancient ancestors of eggplant grew wild in India (Daunay and Janick 2007) and nowadays the crop is grown worldwide. In Israel eggplants are grown during summer in open fields in coastal areas whereas during the winter months it is grown in warmer parts of the country, viz. the Jordan and Arava valleys, where the crop is kept under polyethylene covers in greenhouses or walk-in tunnels. In the summer of 2005, a devastating outbreak of a new disease was observed on eggplants grown in an open field near Rehovot, Israel, in the coastal plain. The disease symptoms were reminiscent of a viral infection; disease incidence reached nearly 100%. The resulting damage was heavy, due to plant stunting accompanied by leaf and fruit malformation. The present study was carried out to identify the viral causal agent with biological and molecular tools. The viral agent which was isolated from infected eggplants was identified as a strain of the *Eggplant mottled crinkle virus* (EMCV). The virus was first described in Lebanon (Makkouk et al. 1981), then in India (Raj et al. 1989), and more recently in Iran (Rasoulpour and Izadpanah 2008). It has been assigned to the Tombusviridae family, genus *Tombusvirus* (Lommel et al. 2000). The present paper describes some biological and molecular characteristics of the Israeli strain of EMCV, including a comparative analysis of selected viral genes to related members of the *Tombusvirus* genus.

Table 1 Partial laboratory host range and symptoms induced by the Israeli isolate of *Eggplant crinkle mottled virus* (ECMV-Is)

Host	Symptoms[a]
Capsicum annuum cv. Maor	Necrosis of leaf veins spreads systemically to adjacent tissues to form diffuse necrosis
Chenopodium amaranticolor	Small LL
C. morale	Irregular brown LL on inoculated leaves
C. quinoa	LL
Citrullus lanatus cv. Mallali	Pinpoint LL on cotyledons
Cucumis sativus cv. Beit Alpha	LL on cotyledons; brown center with a yellow halo
Cucurbita pepo cv. Mayan	Pinpoint LL on cotyledons and yellowing of true leaves
Datura stramonium	Starts with vein yellowing turning later to restricted necrosis of veins and leaf distortion. At late stages apical leaves develop severe mosaic symptoms followed by plant collapse
Emilia sonchifolia	Concentric LL on inoculated leaves
Eustoma russellianum (Lisianthus)	Starts with necrotic spots and rings on the leaf, later spreading systemically
Gomphrena globosa	Starts with small LL (after one day) that become diffuse
Lycopersicum esculentum	No symptoms
Nicotiana benthamiana	Systemic infection leading to plant collapse
N. clevelandii	LL on inoculated leaves followed by systemic spread and plant collapse
N. glutinosa	LL on inoculated leaves
N. rustica	Small LL on inoculated leaves
N. tabacum Samsun	LL on inoculated leaves
N. tabacum Samsun NN	LL on inoculated leaves
N. tabacum Xanthi	LL on inoculated leaves
Ocimum basilicum	Brown LL on inoculated leaves
Physalis floridana	Starts with diffuse LL on inoculated leaves followed by necrosis of growing tips
Vigna unguiculata cv. Black Eye (cowpea)	Brown LL on inoculated leaves

[a] LL represents local lesions

Materials and methods

Virus source, plant inoculation and maintenance Symptomatic eggplants were collected in an open field near Rehovot. The virus was transmitted to test plants by sap mechanical inoculation using 0.01 M phosphate buffer and carborundum (silicon carbide) dust. The isolate was passed twice through a single local lesion (LL) on

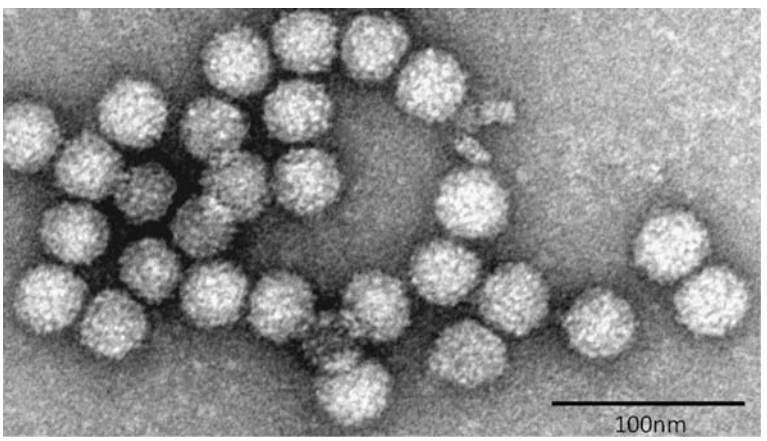

Fig. 1 Electron micrograph of purified particles of the Israeli isolate of *Eggplant mottled crinkle virus* (EMCV-Is). The modal diameter of the particles is 37–40 nm

leaves of *Nicotiana glutinosa*. Plants were held in an insect-proof greenhouse and were sprayed routinely with insecticides to avoid insect infestation.

Virus purification The virus was propagated in *Datura stramonium* plants that served as a source plant for virus purification. The purification of virions was carried out according to the procedure described by Hibi and Furuki (1985). Samples containing the purified viral particles were analyzed by transmission electron microscopy (TEM) (Tecnai G2, FEI-Philips, the Netherlands).

Characterization of the viral coat protein The molecular weight (MW) of the viral coat protein (CP) subunit was determined by SDS-PAGE of purified virus preparations fractionated in 12% acrylamide gels containing 0.4% sodium dodecylsulfate (SDS), according to Laemmli (1970). The resulting protein bands were visualized by staining with Coomassie Brilliant Blue (Sigma-Aldrich Corp., St. Louis, MO, USA).

RNA extraction Purified virus preparations served as a source for virion RNA extraction as described previously (Rosner et al. 2006).

Characterization of virion RNA Virion RNA was separated on 1% agarose denaturing gel containing formaldehyde (Sambrook 1989) and ethidium bromide. RNA samples were denatured by mixing with a solution containing MOPS running buffer, formaldehyde and formamide. The denaturing solution was incubated at 65oC for 15 min, and loaded on the gel. Electrophoresis was carried out at 75 V for 2 h.

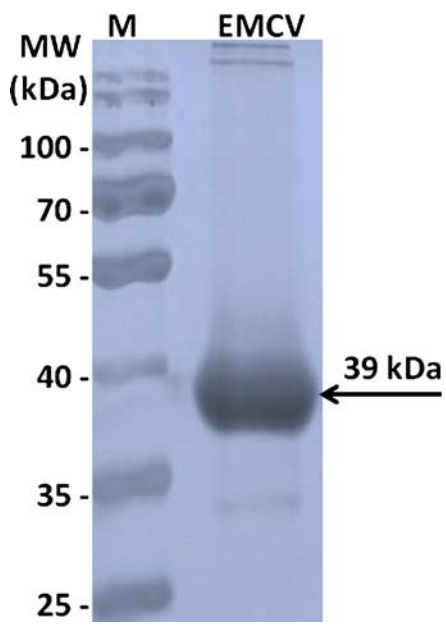

Fig. 2 Elecrophoretic separation of the coat protein subunit of the Israeli isolate of *Eggplant mottled crinkle virus* (EMCV-Is). Electrophoresis was performed in the presence of 12% sodium dodecyl sulfate (SDS). Gel staining was carried out with Coomassie brilliant blue. M represents the protein marker ladder

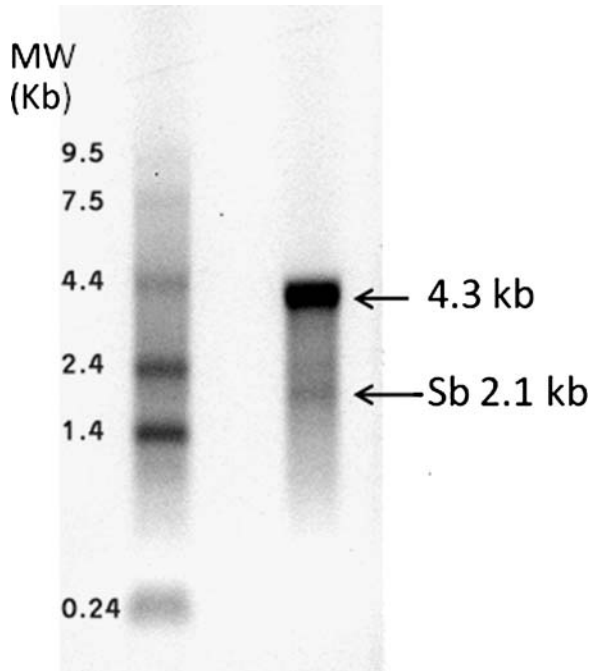

Fig. 3 Separation of virion RNA of the Israeli isolate of *Eggplant mottled crinkle virus* (EMCV-Is) on 1% agarose denaturing gel containing formaldehyde and ethidium bromide (*Right lane*); RNA molecular weight marker ladder (*Left lane*). The subgenomic RNA1 is labeled by Sb

Serological analysis DAS ELISA (Clark and Adams 1977) was used to study the serological relationships with *Tomato bushy stunt virus* (TBSV), the type species of the *Tombusvirus* genus. Polyclonal antibodies against TBSV were purchased from Agdia (Elkhart, IN, USA). Optical densities of virus samples were recorded at 405 nm by a plate reader (Anthos Labtec Instruments, Salzburg, Austria).

PCR amplification Virion RNA was used as a template for reverse transcription (RT) reactions using specific complementary primer (R-CP 5′ TATCCAT GAACTGGTCTGTTC 3′) derived from the published CP sequence of EMCV (accession No. AY500890). Alternatively, random hexamers primers were used for the RT reaction. All the RT reactions were performed with the VersoTM cDNA KIT- Thermo

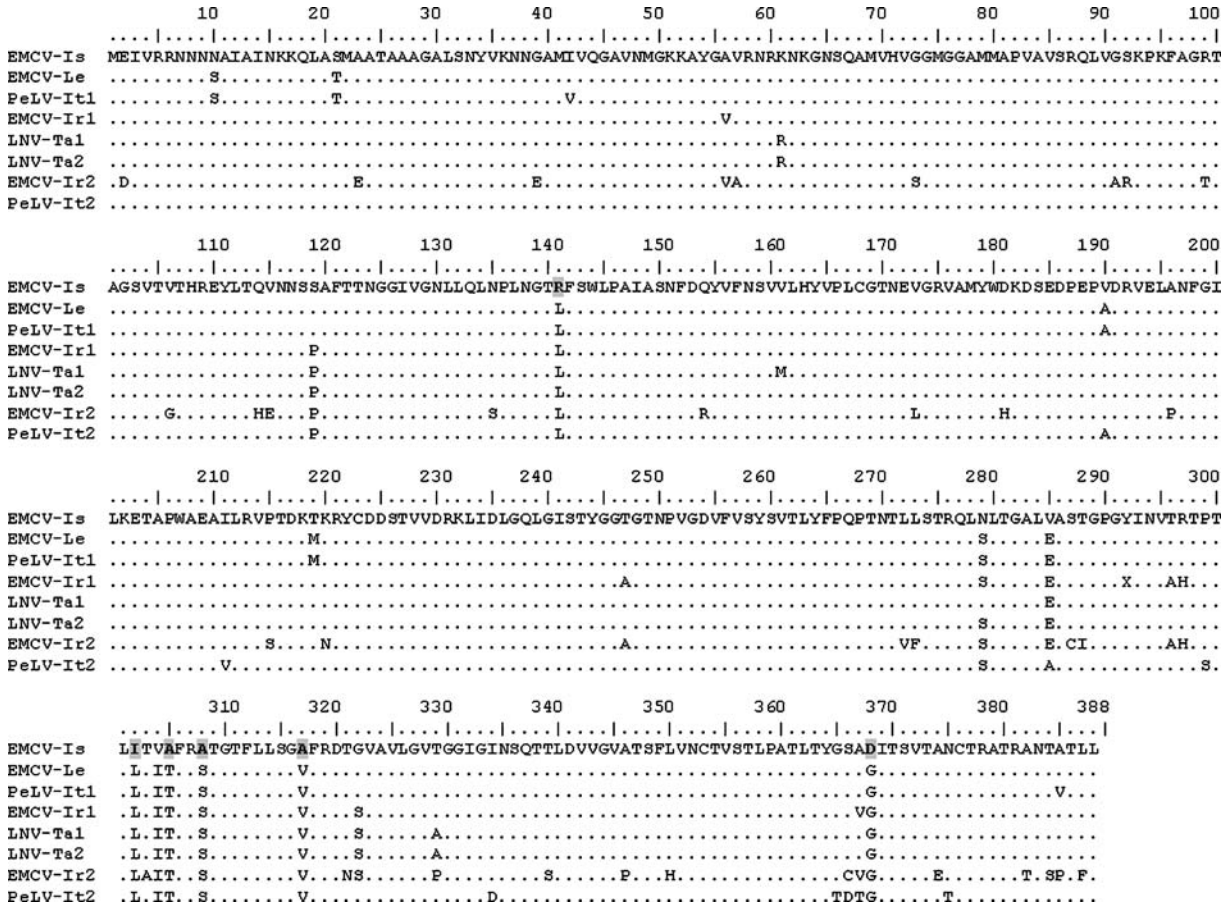

Fig. 4 Alignment of the deduced amino acids sequences of the coat protein (aa 1-388) of several related *Tombusvirus* isolates: *Eggplant mottled crinkle virus* (EMCV), *Lisianthus necrosis virus* (LNV) and *Pear latent virus* (PeLV). The geographical origin of each isolate is indicated by the two letters that follow the virus acronym: *Ta1* and *Ta2* represent the Taiwanese isolates of LNV (accession Nos. CAM98054 and YP_588432, respectively); *Ir1* and *Ir2* represent the Iranian isolates of EMCV (accession Nos. ACA49239 and ABK92203, respectively); *It1* and *It2* represent the Italian isolates of PeLV (accession Nos. NP_835241 and AAO92354, respectively); *Le* represents the Lebanese isolate of EMCV (accession No. AAT00612); and *Is* represents the Israeli isolate of EMCV (accession No. ACP21314)

Table 2 Demonstration of the amino acids mismatches that were found in the sequence of the P33 gene of the Israeli isolate of *Eggplant mottled crinkle virus* (EMCV-Is) (accession No. ACR49216) when compared with the P33 gene of foreign isolates of the virus: the Lebanese isolate of EMCV (EMCV-Le) (accession No. AAZ08583), the Italian isolate of *Pear latent virus* (PeLV-It) (accession No. NP_835239), the Taiwanese isolate of *Lisianthus necrosis virus* (LNV-Ta) (accession No. CAM98052). The unique amino acid modifications are marked in gray

Position	12	14	21	30	50	56	80	91	99	101	105	120	127	140	147	194	220
EMCV-Is	K	I	T	V	G	S	V	T	S	A	N	V	L	F	A	G	V
EMCV-Le	R	L	I	V	C	Q	A	A	S	T	R	F	L	F	L	D	A
PeLV-It	R	L	I	V	C	Q	A	A	S	T	R	F	L	F	L	D	A
LNV-Ta	K	I	I	M	C	S	A	A	G	A	R	F	F	L	A	D	A

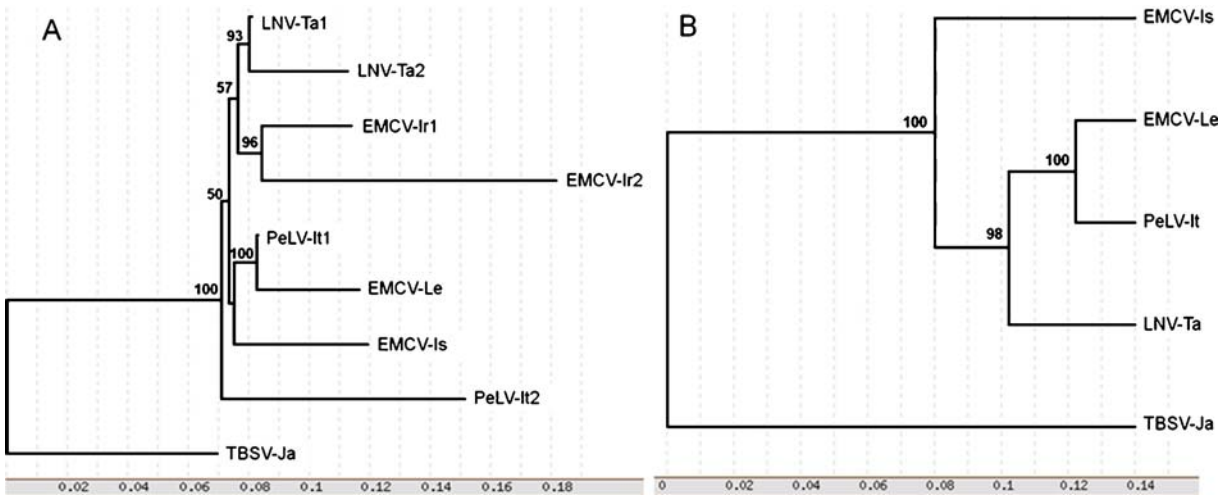

Fig. 5 A phylogenetic tree constructed on the basis of the deduced amino acid sequences of *Eggplant mottled crinkle virus* (EMCV) viral genes. (**A**). Analysis of the coat protein of several related *Tombusvirus* isolates: *Eggplant mottled crinkle virus* (EMCV), *Lisianthus necrosis virus* (LNV), *Pear latent virus* (PeLV) and *Tomato bushy stunt virus* (TBSV). The geographical origin of each isolate is indicated by the two letters that follow the virus acronym: *Ta1* and *Ta2* represent the Taiwanese isolates of LNV (accession Nos. CAM98054 and YP_588432, respectively); *Ir1* and *Ir2* represent the Iranian isolates EMCV (accession Nos. ACA49239 and ABK92203, respectively); *It1* and *It2* represent the Italian isolates of PeLV (accession Nos. NP_835241 and AAO92354, respectively); *Le* represents the Lebanese isolate of EMCV (accession No. AAT00612); *Is* represents the Israeli isolate of EMCV (accession No. ACP21314); *Ja* represent the Japanese isolate of TBSV (accession No. BAF37071) that served as an outgrouping control. (**B**). Phylogenetic analysis of the P33 gene of EMCV-Is (accession No. ACR49216) compared with EMCV-Le (accession No. AAZ08583), PeLV-It1 (accession No. NP_835239), LNV-Ta (accession No. CAM98052) and TBSV-Ja (accession No. AAT67235). Bootstrap values in 1000 replicates are shown at the trees nodes (**A**, **B**). The *scale bar* represents a distance of 0.02 substitutions per site

(Fisher Scientific, Epsom, UK). cDNA encoding for the CP was amplified in PCR reaction using Taq polymerase (DreamTaqTM, Fermentas, Lithuania) with specific primers flanking the CP coding region: forward primer (F-CP 5′ AAGTGAACACAAGCGA CATAG 3′) (position 1–23) and the reverse primer (R-CP 5′ TATCCATGAACTGGTCTGTTC 3′) (position 1207–1230) were synthesized according to the published sequence of EMCV CP (accession No. AY500890). The viral replicase P33 was amplified using the forward primer (F-Rep 5′ AGAAATTCCTC AGATTTCTCCCTG 3′) (position 1–23) and the reverse primer (R-Rep 5′ gttgtggagtgcgagtgaga 3′) (position 1296–1316), both synthesized according to the published sequence of the P33 kDa RNA polymerase pre-readthrough protein from *Pear latent virus* (PeLV) (accession No. AY100482). The PCR amplification step was programmed for the following reaction conditions: 2 min 95°C, 1 min 95°C, 1 min 55°C, 2 min 72°C. The reaction was programmed to a total of 38 cycles followed by 5 min at 72°C and kept at 20°C after termination.

Cloning and sequence analysis PCR products were cloned into a pGEM-T-easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA plasmids were extracted with a plasmid extraction kit (BioNeer, Daejeon, Korea). Nucleotide sequencing was done on both strands for each of the cloned genes by the Sequencing Service of the Molecular Biology Center (HyLab) in Rehovot, Israel. Nucleotide sequence analysis was carried out using DNAMAN (Lynnon BioSoft, Montreal, Canada) and software from the National Center for Biotechnology Information (NCBI). Sequence homology was compared using the Basic Local Alignment Search Tools (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi. Multiple sequence alignments were done with BioEdit and ClustalX software. Phylogenetic tree prediction was carried out using the TreeTop software http://www.genebee.msu.su/services/phtree_reduced.html. The viral nucleotide sequences encoding for both the coat protein and the replicase (P33) genes of the Israeli isolate of EMCV were deposited in GenBank (accession numbers FJ872112 and FJ977166, respectively).

The deduced amino acid (aa) sequence of the CP (ACP21314) and of the P33 (ACR49216) were used for BLAST analysis against GenBank data and for phylogenetic analysis.

Results and discussion

Host range Table 1 summarizes the symptoms induced by mechanical inoculation of test plants. The experimental host range of the Israeli isolate is similar to the previously described host range of the Lebanese (Makkouk et al. 1981) and Iranian (Rasoulpour and Izadpanah 2008) isolates of EMCV. However, in contrast to the local reaction caused by the Lebanese and the Iranian isolates in *D. stramonium*, infection with the Israeli virus resulted in diffuse local lesions on the inoculated leaves followed by systemic spread and induction of leaf distortion accompanied by severe mosaic leading finally to plant collapse.

Virus purification and particle morphology The purification procedure was found to be highly efficient and the resultant preparation contained large amounts of isometric viral particles 37–40 nm in diameter with an icosahedral structure (Fig. 1). The average virus yield was 0.27 mg g^{-1} of *D. stramonium* leaf tissue calculated using the TBSV extinction coefficient=4.5 (Martelli et al. 2001). Electron microscopy analysis (Fig. 1) and CP separation by SDS-PAGE (Fig. 2) indicated a high purity of the preparation.

Protein characterization Performing SDS-PAGE separation of the purified viral particles indicated that the MW of a single protein subunit is ~38–39 kDa (Fig. 2), which is smaller than the 41 kDa value calculated for the Lebanese isolate (Makkouk et al. 1981) and the Indian isolates (Raj et al. 1989).

Serology No serological cross reaction was found when an antiserum against TBSV was reacted with crude sap from infected plants or purified preparations of the Israeli isolate of EMCV (data not shown). Earlier studies (Makkouk et al. 1981) have shown a distant serological relationship between the Lebanese isolate of EMCV and TBSV-BS-3.

Genome characterization RNA extracted from the purified virus preparation was separated on 1% agarose MOPS gel containing ethidium bromide. The viral genomic RNA displayed two bands: a major fraction of 4.2 kb representing the genomic viral RNA and a second band of ~2 kb which is the putative sub-genomic RNA1 found in viruses in the genus *Tombusvirus* (Fig. 3). BLAST search analysis of the cloned CP of EMCV-Is at the nucleotide level indicated a high sequence identity with the other compared viruses in the genus *Tombusvirus*: 97% identity with the Lebanese isolate of EMCV and with PeLV, and 95% identity with *Lisianthus necrosis virus* (LNV) and the Iranian isolates of EMCV from pelargonium. BLAST search analysis at the nucleotide level of the P33 of EMCV-Is revealed 95% nucleotide sequence identity with the Lebanese isolate of EMCV (the only available EMCV P33 sequence), and 94% identity when compared with the LNV P33 gene. Furthermore, similar values of identity were calculated for the amino acid sequences of the coat proteins of the above mentioned viruses: 96% identity among the Lebanese isolate of EMCV, PeLV and LNV, and 95% identity calculated when compared with the Iranian isolate of EMCV from pelargonium.

Comparison of the deduced aa sequence of the CP of the Israeli isolate with that of the Lebanese isolate showed 13 mismatches (aa No. 10, 21, 141, 190, 219, 279, 285, 302, 304, 305, 308, 317, 368) (Fig. 4). The majority of these mismatches were found to be located upstream to the C-terminus of the CP (aa 279–317) (Fig. 4).

Comparison of the CP deduced aa sequence of EMCV-Is with the related LNV isolates (accession numbers CAM98054 and YP_588432) indicates 12 unique differences at the following positions: 61, 119, 141, 285, 302, 304, 305, 308, 317, 322, 329 and 368 (Fig. 4). One additional modification was specific for each of the two LNV isolates (Fig. 4). However, only ten aa mismatches were identified between EMCV-Is CP and the CP genes of PeLV isolates (accession numbers NP_835241 and AAO92354): Arg141, Val 190, Asn 279, Val285, Ile302, Val304, Ala305, Ala308, Ala317, Asp368) (Fig. 4). Two of the aa changes were located in the central part of the CP coding region where Leu was changed to Arg (aa 141) and Glu to Val (aa 285). The rest of the aa changes were limited to the region between positions 302 to 317, and one additional mismatch was located at the C-terminal region of the CP where Gly replaced Asp (aa 368) (Fig. 4).

Comparison of the deduced aa sequence of the P33 of the Israeli isolate with that of the Lebanese isolate showed 13 mismatches (aa No. 12, 14, 21, 50, 56, 80, 91, 101, 105, 120, 147, 194, 220) (Table 2). Surprisingly the aa sequence of the P33 of the Lebanese

isolate was found to be identical to the Italian PeLV P33 gene (Table 2).

The sequence identities found among the compared CPs of viruses in the genus *Tombusvirus* are over 87%, the value which was decided by the ICTV (Lommel et al. 2000) as the threshold for the demarcation of *Tombusvirus* species. It seems, therefore, that EMCV, PeLV and LNV are strains of EMCV rather than distinct viruses, as had been proposed by Koenig et al. (2004). This suggestion was further supported by our biological assays. Inoculation of purified EMCV-Is preparations to lisianthus (*Eustoma russellianum*) resulted in systemic infection and symptoms identical to those described for LNV in this host plant (Iwaki et al. 1987). However, for an unknown reason purified preparations of EMCV-Is failed to infect pelargonium plants. It is possible that EMCV is able to infect certain pelargonium genotypes whereas others are immune.

A phylogenetic analysis of the EMCV strains was carried out using the CP published sequences of nine viruses of the genus *Tombusvirus*. A P33-based phylogenetic analysis was performed by using available sequences of the P33 gene of five viruses in the genus *Tombusvirus*. In both cases relevant sequences of TBSV served as an outgroup control (Fig. 5A–B). The phylogenetic tree constructed on the basis of the CP sequences indicated high bootstrap values for the Israeli and Lebanese isolates of EMCV and the Italian isolate of PeLV (PeLV-It1), whereas the Iranian isolates of EMCV and the Taiwanese isolate of LNV were grouped in a separate, more distant cluster (Fig. 5A). On the other hand, when the P33 gene sequence served for the phylogenetic analysis, the Lebanese isolate of EMCV was grouped together with the Italian PeLV and the Taiwanese isolate of LNV, whereas the Israeli isolate formed a distinct branch—indicating a distant phylogenetic relationship with the other compared isolates (Fig. 5B). Based on the CP phylogenetic analysis, we suggest a geographical clustering of EMCV isolates. Therefore, according to this hypothesis the Lebanese isolate of EMCV has evolved from the Israeli isolate of the virus and both isolates share a high sequence identity with the PeLV-It1, thus forming a regional 'Mediterranean cluster'. Nevertheless, the two Iranian isolates of EMCV (accession Nos. ACA49239 and ABK92203, respectively) (Rasoulpour and Izadpanah 2008) share a relatively high sequence identity with the two Taiwanese isolates of LNV, indicating again a positive correlation between geographical distances and sequence identities.

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