ANALYSIS OF THE VARIABILITY OF TUNISIAN ISOLATES OF *LETTUCE MOSAIC VIRUS* USING BIOLOGICAL AND MOLECULAR PROPERTIES

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SUMMARY

Lettuce mosaic virus (LMV) can be very destructive on lettuce crops as, a very high proportion of infected plants may result from a low level of infected seeds. In addition, LMV isolates able to overcome the *mo1*[1] and *mo1*[2] resistance genes classically deployed by breeders to protect lettuce crops have now been reported from several countries. In order to gain an understanding of the variability present among LMV populations in Tunisia, nine LMV isolates from lettuce fields were submitted to biological and molecular analysis. Phenotypically, two isolates were able to overcome the resistance gene *mo1*[1] of lettuce cv. 'Mantilia'. At the molecular level, the RT-PCR technique, coupled with RFLP analysis or direct sequencing were used to study a short, hypervariable region of the LMV genome. Restriction patterns and sequence comparison with isolates representative of different pathotypes and/or geographic origins showed that Tunisian LMV isolates are related to other western European isolates, stressing the problem of exchanges of contaminated seed lots.

Key words: *Lettuce mosaic virus*, lettuce, resistance-breaking, RT-PCR-RFLP, sequence comparison.

INTRODUCTION

Lettuce mosaic virus (LMV) is potentially the most destructive virus of lettuce (*Lactuca sativa* L.) and has a worldwide distribution (Dinant and Lot, 1992; Zerbini *et al.,* 1995). It is transmitted through seed and by aphids in a non-persistent manner (Tomlinson, 1970; Dinant and Lot, 1992). Field symptoms include vein clearing, mosaic or mottling and/or leaf deformation, necrosis, and failure to form proper heads. Infection can severely damage the crop and considerably affect the yield. LMV can be controlled in areas where preventive measures such as seed certification, enforcement of crop-free periods and control of weed reservoirs of the virus can be applied, however it remains a damaging virus to lettuce in regions where such programs are lacking.

LMV is a member of the genus *Potyvirus* family *Potyviridae* with long flexuous particles measuring approximately 750 x 13 nm (Tomlinson, 1964). The single-stranded genomic RNA of potyviruses is typically about 10 kb, contains a single large open reading frame encoding a polyprotein, has a viral-encoded protein (VPg) linked to its 5' end and is polyadenylated at its 3' end (Shukla *et al.*, 1994). This polyprotein is proteolytically cleaved by the three virus-encoded proteinases P1-Pro, HC-Pro and NIa-Pro into ten mature proteins (Carrington and Dougherty, 1987; Carrington *et al.,* 1989, 1990; Riechman *et al.*, 1992). Specifically, the LMV genome has 10,080 nucleotides excluding polyA, and encodes a polyprotein of 3255 amino acids (Revers *et al.*, 1997b).

Resistance to LMV in lettuce was originally found linked to the recessive genes *g* (Bannerot *et al.,* 1969) derived from the Argentinean lettuce cultivar 'Gallega de Invierno' (Von der Pahlen and Crnko, 1965), and *mo* (Ryder, 1970) from an Egyptian cultivar (Ryder, 1976; Pink *et al.,* 1992). The genes *g* and *mo* are considered to be allelic or very closely linked (Pink *et al.,* 1992) and are now denoted *mo1*[1] and *mo1*[2]. A dominant gene, *Mo2,* found in the cv. 'Ithaca' and conferring resistance to a very limited number of LMV isolates, has also been described (Pink *et al.,* 1992). However, for practical purposes the *mo1*[1] and *mo1*[2] genes are the only available useful sources of resistance against LMV and have therefore been deployed worldwide by breeders (Dinant and Lot, 1992).

LMV is biologically variable and its isolates, according to their virulence to particular lettuce varieties, have been classified into four groups or pathotypes (Dinant and Lot, 1992; Pink *et al.*, 1992; Bos *et al.,* 1994; Revers *et al.*, 1997a). Of particular concern are the seed-transmissible isolates here referred to as MOST (*mo*-breaking, seed-transmitted), able to completely overcome *mo1*[1] and *mo1*[2] resistance genes. MOST isolates have the potential to severely affect resistant lettuce crops

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and to spread widely through distribution of contaminated seed (Dinant and Lot, 1992).

Molecular variability analysis of LMV, based on limited sequence data, has identified three phylogenetic groups within ten chosen LMV isolates, that were correlated with geographical origin rather than with pathogenicity towards resistance genes (Revers *et al.*, 1997a). Sequencing of the coat protein genes of representative LMV isolates has since confirmed the clustering of LMV isolates in three phylogenetic/geographic groups, namely western-Europe/California (WE/C), Yemen, and Greece (Revers *et al.*, 1999). Molecular information was used to develop a PCR-RFLP assay allowing both the allocation of unknown LMV isolates to these phylogenetic groups and a finer analysis of isolate affinities within the large WE/C group (Revers *et al.*, 1999).

In Tunisia, during the last decade lettuce production has been developed through increased cultivated areas of field and greenhouse crops. Heavy losses caused by viral infections are observed mainly in field crops, but also in early greenhouse winter crops because seedlings are not raised under insect-proof conditions and are exposed to infection. The severity of losses also appears to be directly related to the lack of certified seed and to cultivation methods.

Several Tunisian lettuce-growing regions were surveyed in 1999, and serological and molecular techniques were used to detect LMV in the crops and to characterise the biological and molecular variability of a collection of LMV isolates selected so as to cover the geographical diversity of this virus in Tunisia. These experiments demonstrated, for the first time, the presence of MOST isolates in Tunisia and revealed a worrying situation for the future of lettuce cultivation in this country, if appropriate control measures are not taken.

MATERIALS AND METHODS

LMV sources. LMV isolates used in this work were collected from lettuce crops in three lettuce-growing regions of North Tunisia: Manouba, Mornag, and Cap Bon (Lebna). The isolates tested and their origin are listed in Table 1.

All LMV isolates were maintained and propagated on plants of susceptible butterhead lettuce (cv. 'Trocadéro') obtained from virus-free seed batches. The resistance-breaking properties of the LMV isolates was evaluated by inoculating plants of the butterhead cv. 'Mantilia', which contains the $mo1^1$ resistance gene. Inoculation of lettuce plants was done by inoculation of sap extracted by grinding infected leaf tissues (1/5, w/v) in a 0.03 M sodium phosphate (Na_2HPO_4) solution containing 0.25% (w/v) sodium-diethyldithiocarbamate (DIECA), and adding charcoal and carborundum to the mixture before rubbing plantlets at the 4- to 6-leaf stage. Two plants of each cultivar were inoculated with each isolate and maintained in insect-proof cages at 18 to 25°C under greenhouse conditions.

Two LMV isolates were used as reference for both biological indexing and RT-PCR-RFLP analysis. LMV-0, a non-resistance-breaking isolate representative of the WE/C phylogenetic group was isolated in France and described by Dinant and Lot (1992). LMV-Gr5, a resistance breaking isolate representative of the Greek phylogenetic group was isolated from Greece (Kyriakopoulou, 1985). This isolate was used in the pathotyping experiments of Bos *et al.* (1994) and generously provided by Dr R.A.A. van der Vlugt (IPO, Wageningen, Netherlands).

Tissue blotting. LMV in field samples was primarily detected by tissue blotting (Lin *et al.*, 1990). Leaf samples were directly printed on a nitrocellulose membrane (Protran, Schleicher et Schuell) by pressing a freshly cut section of the leaf mid-rib. The membrane was then air dried at room temperature and, if needed, stored at room temperature until processed. After saturation of the membrane for 30 min at room temperature in blocking buffer (0.35 M NaCl, 10 M Tris-HCl pH 7.4, 1% (w/v) gelatin), the membrane was incubated for 2 h at room temperature with alkaline phosphatase-conjugated LMV-specific IgG's derived from a polyclonal antiserum, diluted in RIA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS and 1% (w/v) Triton X-100) (conjugate diluted 1/1000, similar to the concentration used for a DAS-ELISA assay). After three 10 min washes in RIA buffer, the membrane was incubated in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris-HCl pH 9.5) supplemented with NBT and BCIP. Colour development was stopped by washing the membrane in tap water and drying. Tissue blots were individually scored by eye or by observing the membrane under a stereo-microscope at low magnification.

Nucleic acid extraction. Lettuce leaves were ground (1/5, w/v) in PBS-Tween buffer (8 g of NaCl, 0.2 g of KH_2PO_4, 2.9 g of Na_2HPO_4 (12 H_2O) and 0.5 ml of Tween 20 per litre) containing 2% (w/v) polyvinylpyrrolidone K25 and 20 M DIECA. After centrifugation at 13,000 rpm for 10 min, 200 µl of supernatant were transferred to a microfuge tube, mixed with SDS to 1% (w/v) final concentration and incubated at 55°C for 15 min. One hundred microliters of 3 M potassium acetate were added, the mixture vigorously vortexed

Table 1. Origin, field symptoms and LMV content of the samples examined in this study.

Area of origin	Cultivar of origin[a]	Symptoms[b]	Tissue blot	Type of LMV[c]	Isolate[d]
Manouba	Butterhead #15	none (40 plants)	–	n.p.	Tn1B
	Cos	mosaic (16 plants)	+ (14/16)	WE/C	Tn2R
	Cos	mosaic (2 plants)	+ (2/2)	WE/C	Tn3R
	Butterhead #15	mosaic ? (1 plant)	–	WE/C	Tn3B
	Butterhead #15	stunting, mosaic (1 plant)	+ (1/1)	WE/C-Most	Tn4B
	Butterhead Augusta	chlorosis of old leaves (5 plants)	–	n.p.	Tn7B
	Crisphead	stunting, chlorosis (3 plants)	–	n.p.	Tn8F
	Cos	mosaic (4 plants)	+ (3/4)	WE/C	Tn9R
	Butterhead	mosaic ? (1 plant)	–	n.p.	Tn9B
Mornag	Butterhead Audran	? (1 plant)	–	n.p.	Tn10B
	Iceberg	stunting ? (3 plants)	–	n.p.	Tn10R
	Butterhead Augusta	none (40 plants)	–	n.p.	Tn11B
	Butterhead Vista	? (5 plants)	–	n.p.	Tn12B
	Butterhead	mosaic (5 plants)	+ (5/5)	WE/C-Most	Tn13B
	Butterhead and Cos	none (13 plants)	–	n.p.	Tn14BR
Lebna	Cos	none (7 plants)	–	n.p.	Tn15R
	Cos	none (9 plants)	–	n.p.	Tn16R
	Butterhead [e]	mosaic (1 plant)	–	n.p.	Tn16B
	Cos and Iceberg	none (19 plants)	–	n.p.	Tn17R
	Cos	mosaic (3 plants)	–	n.p.	Tn17R2
	Iceberg	none (46 plants)	–	n.p.	Tn18I
	Cos	mosaic (2 plants)	+ (2/2)	WE/C	Tn19R
	Butterhead	mosaic (1 plant)	+ (1/1)	WE/C	Tn19B1
	Butterhead	? (1 plant)	–	n.p.	Tn19B2
	Cos Marvel	stunting ? (1 plant)	–	n.p.	Tn20R
	Cos Parris island	mosaic (10 plants)	+ (5/10)	WE/C	Tn21R
	Cos Parris island	mosaic (1 plant)	+ (1/1)	WE/C	Tn21.4R
		total asymptomatic plants (174 plants)	0/174		
		total symptomatic plants (67 plants)	34/67		

[a] The type of lettuce cultivar, and the cultivar name when indicated orally by the growers, are shown.
[b] Symptoms observed in the field.
[c] n.p.: LMV not present.
[d] A single plant representative of the cultivar/location combination was selected to constitute the local isolate. When both positive and negative results were obtained, this plant was selected among the LMV positive ones.
[e] Tn16B was sampled from a butterhead-type lettuce growing at the edge of a field planted with a cos-type cultivar, and probably the result of an escape from the previous crop cycle.

and incubated on ice for 5 min. After centrifugation (5 min, 13,000 rpm, 4°C), the supernatant was adjusted to 4.2 M NaI. Five microliters of a suspension of silica particles (Sigma, France) were added, carefully mixed by low speed vortexing and the mixture incubated at room temperature for 5 min. After a brief centrifugation (1 min, 5000 rpm at room temperature) the supernatant was discarded and the pellet gently resuspended in 500 µl of washing buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 50% (v/v) ethanol). The centrifugation was repeated and the pellet of silica particles similarly washed twice more, before being resuspended in 400 µl sterile water. After incubation at 55°C for 5 min and centrifugation at 13,000 rpm for 2 min, 300 µl of the supernatant were transferred to a new tube. Total nucleic acid extracts were then used directly for RT-PCR amplification or stored at -20°C until use.

RT-PCR and direct sequencing of amplified fragments. LMV-specific primers used in this work were the sense primer NIb corresponding to nucleotides 8894-8915 of the LMV genome (Revers *et al.*, 1997a, 1999) and the antisense primers P4 (Revers *et al.*, 1999) and P4Gr, both complementary to nucleotides 9151-9171 of the LMV genome but with different isolate specificity. The sequence of P4Gr is 5'-GCGTTGAT-

GTCCTCATCYTT-3’, (Y=C or T).

RT-PCR was performed using the one buffer, one tube format described by Revers *et al.* (1997a). Briefly, 3 µl of total RNA extract were submitted to amplification in a 50 µl RT-PCR reaction mix [10 M Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.3% (v/v) Triton X100, 250 µM of each dNTPs, 1 µM of each primer pair (NIb/P4 or NIb/P4Gr), 2.5 µl of formamide, 0.25 units of AMV reverse transcriptase (Stratagene, France) and 0.5 units of *Taq* DNA polymerase (Appligen-Oncor, France)]. The mix was overlaid by 50 µl of mineral oil and the tubes were incubated for 45 min at 42°C for reverse transcription, followed by incubation for 5 min at 95°C for denaturation of RNA-DNA hybrids and reverse transcriptase. Forty cycles of amplification with the following thermal profile were then performed: 20 s at 92°C, 20 s at 56°C and 40 s at 72°C. PCR products were finally analyzed by electrophoresis in 1.2 % agarose gels in TBE buffer and visualized under UV light following ethidium bromide staining.

RFLP analysis of PCR products. For RFLP analysis of the amplified material, an aliquot (2 µl) from the RT-PCR reaction was cut with one of the restriction enzymes *Alu*I, *Acc*I, *Bam*HI, *Dde*I, *Eco*RI, *Hae*III, *Rsa*I, *Sac*I or *Taq*I using the conditions described by the manufacturer (Gibco-BRL). The digestion products were analyzed by electrophoresis on a 12% polyacrylamide vertical gel. The DNA was stained after electrophoresis with SYBR Green I (Molecular Probes, USA) and visualized under UV light.

Phylogenetic analysis. PCR-amplified material was directly sequenced using primer NIb and an automated sequencer (Genome Express, Grenoble, France). Reference LMV sequences from Revers *et al.* (1997a, 1999) for multiple alignments were retrieved from the EMBL database. Multiple alignments of the region comprised between positions 8936 and 9151 of LMV-0, or the corresponding region of other isolates, were obtained using the program ClustalX, a Windows version of ClustalW (Thompson *et al.*, 1994). Phylogenetic relationships were determined by the neighbor-joining method (Saitou and Nei, 1987), implemented in ClustalX.

RESULTS

LMV identification and biological characterization of Tunisian isolates. Lettuce collected from fields showed mosaic, vein clearing, chlorosis, yellow spotting, leaf deformation and growth retardation. In addition, a number of samples were also collected at random from symptomless plants since the *mo1*[1] and *mo1*[2] resistance genes are known to afford either resistance or tolerance (virus multiplication but absence of symptoms) depending on the particular LMV isolates they are confronted with (Dinant and Lot, 1992; Pink *et al.*, 1992; Bos *et al.*, 1994). All the samples were tissue-printed on nitrocellulose membranes and tested for the presence of LMV using a polyclonal antiserum coupled with alkaline phosphatase. In a first experiment the processing of membranes on which symptomless samples had been spotted demonstrated the absence of LMV in all symptomless samples tested, representing a total of 7 cultivar/location combinations and 174 plants (Table 1).

In a second experiment, membranes on which symptomatic and doubtful samples had been printed was processed. A total of 19 cultivar/location combinations were tested in this way, each combination representing a variable number of plants. LMV was unambiguously detected in 9 of these symptomatic cultivar/location combinations (Table 1, Fig. 1). In addition, the likely presence of other viruses was suggested by the observation that no LMV was detected on 6 cultivar/location combinations even though they showed severe viral symptoms (Table 1). No specific efforts were made to try to identify the virus(es) responsible for these disorders. It can be concluded from these experiments that LMV alone was responsible for about half (34/67) of symptomatic viral infections in the regions surveyed and at the time of the survey (November). LMV was also detected in a single pea (*Pisum sativum*) plant with mosaic and deformation of the leaves, collected in a field neighbouring a lettuce field in the area of Lebna.

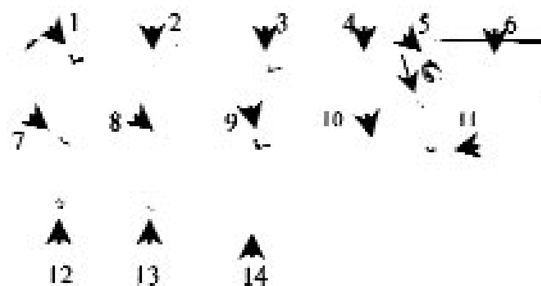

Fig. 1. Results of the tissue blotting test of fourteen LMV isolates investigated in this work. Presence of LMV infection was revealed using a polyclonal antibody coupled with alkaline phosphatase. 1: Tn9R; 2: Tn17R; 3: Tn13B; 4: Tn16B; 5: Tn3R; 6: Tn3B; 7: Tn4B; 8: Tn8F; 9: Tn19R; 10: Tn19B2; 11: Tn19B1; 12: Tn21.4R; 13: Tn21R; 14: Tn2R. Arrows indicate the blotting sites.

Table 2. Distribution of the restriction sites along the RT-PCR fragment obtained from different LMV isolates. The nucleotide positions refer to the full-length LMV-0 sequence, as in Revers *et al.* (1999). +: denotes the presence of a restriction site.

Origin	Isolates	*Acc*I		*Alu*I					*Bam*HI	*Dde*I	*Eco*RI	*Hae*III	*Rsa*I		*Sac*I	*Taq*I
		8927	9036	8942	8956	8975	9046	9135	9097	9091	9088	9057	8927	8979	8958	8958
Tunisian	Tn2R		+			+	+		+	+		+				
	Tn3R		+			+	+		+	+		+				
	Tn4B					+	+		+		+	+	+	+		
	Tn9R		+			+	+		+	+		+				
	Tn13B					+	+		+		+	+	+	+		
	Tn19R		+			+	+		+	+		+				
	Tn19B1		+			+	+		+	+		+				
	Tn21R		+			+	+		+	+		+				
	Tn21.4R		+			+	+			+		+				
Greek	Gr5	+		+	+		+	+		+					+	+
WE/C	0		+			+	+		+	+		+				
	13					+	+		+		+	+	+	+		

In order to evaluate the severity of the LMV isolates involved and their ability to overcome lettuce resistance genes, isolates representative of the nine cultivar/location combinations in which LMV had been detected by tissue blotting were mechanically inoculated to plants of cv. 'Trocadéro' and the resistant ($mo1^1$) cv. 'Mantilia'. Two weeks later, all isolates had induced typical vein clearing and mosaic symptoms on cv. 'Trocadéro'. Only isolates Tn4B and Tn13B induced symptoms on cv. 'Mantilia', indicating that both are able to overcome the $mo1^1$ resistance gene. The ability to overcome $mo1^2$ resistance gene was not tested since this allele is not predominant among the lettuce cultivars grown in Tunisia.

RT-PCR amplification and RFLP analysis of Tunisian LMV isolates. For the isolates representative of the nine cultivar/locations in which LMV was detected, total nucleic acids were used in a one-tube assay for RT-PCR amplification using LMV-specific primers. The primers used are able to hybridize to all known isolates and to promote the amplification of the 3' end of the polymerase (NIb) gene together with the 5'end of the coat protein gene (Revers *et al.*, 1999). This amplified region is highly discriminative since it contains the hypervariable N-terminal end of the coat protein (Shukla *et al.*, 1994; Revers *et al.*, 1997a). Using the NIb primer in combination with either the P4 or the P4Gr primers, a single PCR fragment of 278 base pairs was successfully generated for the nine Tunisian LMV isolates. Positive controls included the amplification of a comparable fragment from the LMV-0 and LMV-Gr5 isolates, representative respectively of the WE/C and Greek phylogenetic groups of LMV isolates (Revers *et al.*, 1997a). The PCR product generated from each isolate was cut with each of nine restriction enzymes: *Acc*I, *Alu*I, *BamH*I, *Dde*I, *EcoR*I, *Hae*III, *Rsa*I, *Sac*I and *Taq*I. The *Hae*III, *Taq*I, *Acc*I, *Alu*I and *Sac*I profiles allow discrimination between isolates belonging to the three known LMV phylogenetic groups (Revers *et al.*, 1999). As a reference, the products generated from LMV-0 and LMV-Gr5 were similarly analysed.

The results obtained (Fig. 2, Table 2) unambiguously showed that all the Tunisian isolates tested belong to the WE/C phylogenetic group. Within this group, which is the one showing the largest variability, isolates can be discriminated on the basis of the restriction patterns obtained with *Acc*I, *Alu*I, *Bam*HI, *Dde*I, *EcoR*I, *Rsa*I and *Sac*I (Revers *et al.*, 1999). Using the restriction patterns obtained with these enzymes, the nine Tunisian isolates were allocated to three restriction groups. One group, composed of isolates Tn2R, Tn3R, Tn9R, Tn19R, Tn19B1 and Tn21R, had a restriction profile identical to that of the reference isolate LMV-0,

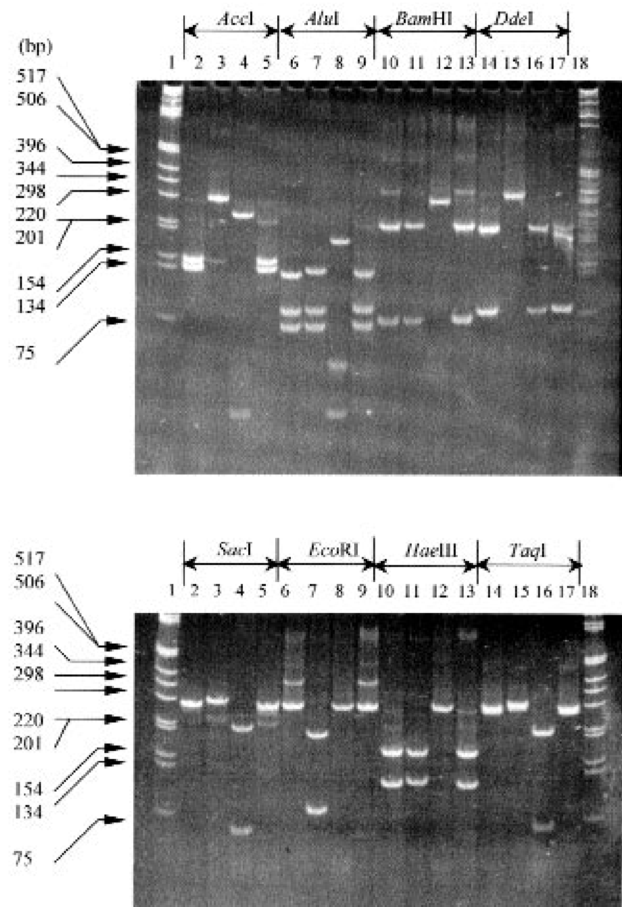

Fig. 2. RFLP analysis of NIb-P4 RT-PCR products obtained from four LMV isolates, on a 12% polyacrylamide gel, stained after electrophoresis with SYBR-Green I nucleic acid gel stain (Molecular Probes). The isolates analysed are Tn3R (2, 6, 10, 14), Tn13B (3, 7, 11,15), LMV-Gr5 (4, 8, 12, 16) and LMV-0 (5, 9, 13, 17). Lanes 1 and 18: 1 kb DNA ladder (Gibco BRL).

the prototypical seed-transmissible, non resistance-breaking member of the WE/C phylogenetic group (Pink *et al.*, 1992; Revers *et al.*, 1997a, 1999). The second group was composed of isolate Tn21.4R, which differed from LMV-0 as it lacks the *BamH*I restriction site. The last group, composed of isolates Tn4B and Tn13B, had a restriction profile identical to that of isolates LMV-13 and LMV-Aud, two known resistance-breaking (MOST) isolates (Pink *et al.*, 1992; Revers *et al.*, 1997a, 1999).

Partial sequence characterization and phylogenetic analysis of Tunisian LMV isolates. PCR products from eight of the nine Tunisian isolates (isolate Tn21R was not further processed), were sequenced; sequences were aligned and compared with those already determined in

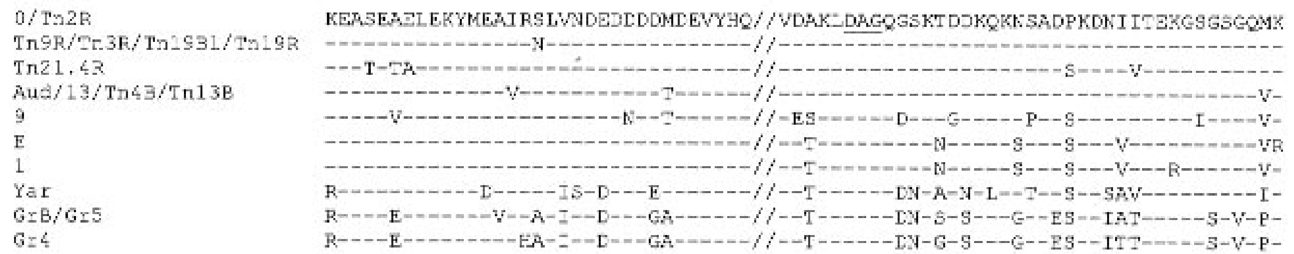

Fig. 3. Amino-acid sequence alignment of the region analysed for the Tunisian LMV isolates and the 10 LMV reference isolates described in Revers *et al.* (1997a). The cleavage site between NIb and CP is indicated by parallel bars, and the DAG triplet involved in aphid transmission of potyviruses is underlined. The amino-acids identical to those found in LMV-0 at the same position are indicated with dashes.

the same region for other LMV isolates (Revers *et al.*, 1997a, 1997b, 1999) using the ClustalX program (Thompson *et al.*, 1994). The region analyzed (Fig. 3) comprises the last 33 amino acids of the NIb protein and the first 39 amino acids of the coat protein (CP). As expected for the CP of an aphid-transmissible potyvirus, the DAG triplet was present in the amino terminus of the CP of all Tunisian isolates of LMV. All isolates had NIb/CP cleavage dipeptide identical to the Q/V cleavage site described for LMV-0 by Dinant *et al.* (1991).

The phylogenetic tree was consistant with the results of the RFLP analysis presented above and showed that Tunisian isolates cluster in two subgroups (Fig. 4).

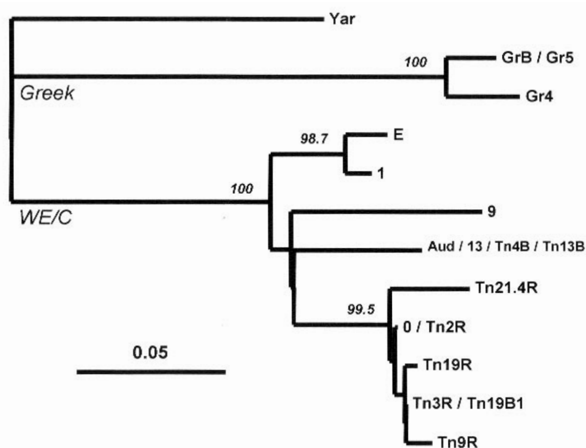

Fig. 4. Phylogenetic analysis of the LMV isolates found in Tunisia, compared with the 10 reference isolates described in Revers *et al.* (1997a). The Neighbor-Joining method described by Saitou and Nei (1987) was used as implemented in ClustalX (Thomson *et al.*, 1994). The scale bar length represents 0.05 substitutions per position. The tree, rooted using LMV-Yar as an outgroup, was finally drawn using Tree View (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).
The bootstrap values (1000 replicates) are indicated at the nodes when higher than 70%.

In the region analysed, isolates Tn4B and Tn13B were identical in sequence with MOST isolates LMV-13 and LMV-Aud (Revers *et al.*, 1997a). The second subgroup included the rest of the isolates and tightly clustered around LMV-0. Isolate Tn2R was identical in sequence to LMV-0 in this region, as well as, on the other hand, Tn3R and Tn19B1. Other isolates differed from LMV-0 by one nucleotide (Tn3R and Tn19B1) to six nucleotides (Tn21.4R). The most important divergence within this group was observed between Tn9R and Tn21.4R, with nine nucleotide differences. The overall higher divergence of Tn21.4R confirmed its slight shift also detected by the RFLP analysis and amino-acid sequence comparison (Fig. 3).

DISCUSSION

The recent world-wide emergence of highly pathogenic and resistance-breaking isolates of LMV is alarming (Dinant and Lot, 1992). These isolates not only cause severe damage to previously resistant cultivars, but are also seed-transmitted in genotypes containing the resistance genes $mo1^1$ or $mo1^2$. These MOST isolates, have therefore a clear potential to be spread to new areas through the distribution of contaminated seed lots. This new situation has increased interest in developing tools for the study of the epidemiological properties of LMV isolates and for a better understanding of correlations between biological properties such as resistance-breaking and seed-transmissibility, and molecular characteristics.

In the present work we have tested a large number of samples collected in different lettuce growing regions of Tunisia for the presence of LMV. The tissue blotting assay proved simple, fast, reliable and very economical. As previously reported (Lin *et al.*, 1990) this simple technique offers numerous advantages

when a large number of samples need to be processed rapidly. In this respect, this technique should prove, in the future, very useful for epidemiological studies of LMV. The only key parameter seems to be the quality of the antiserum, which causes interpretation problems if it gives a significant cross-reaction to healthy plant components.

The sanitary status of lettuce crops in Tunisia seems satisfactory as none of 174 random samples of symptomless lettuce was infected with LMV. This result confirms the overall visual impression gained during field visits. Analysis of the symptomatic samples indicated that at least during our survey season (November 1999) LMV was the major virus infecting lettuce in Tunisia since it was observed in half of the symptomatic samples. Comparison of the prevalence of symptoms in fields planted with susceptible or LMV-resistant varieties indicate that the $mo1^1$ and $mo1^2$ resistance genes still afford a significant degree of protection against LMV infection in Tunisia. Among the nine representative LMV isolates finally analysed, two of them, Tn4B and Tn13B, induced very severe symptoms on the susceptible cv. 'Trocadéro' and were able to overcome the $mo1^1$ resistance gene of cv. 'Mantilia'.

The nine Tunisian LMV isolates selected were further analysed using a combination of RT-PCR coupled with either RFLP analysis (Revers *et al.*, 1999) or direct sequencing (Revers *et al.*, 1997a). Both techniques proved equally effective for defining molecular affinities between LMV isolates. In particular, both identified all isolates as belonging to the WE/C group, and, within this group, both showed that isolates Tn4B and Tn13B were close to LMV-13 and LMV-Aud, whereas all other isolates were close to LMV-0, the prototypical seed transmissible, non resistance-breaking isolate (Dinant and Lot, 1992), with Tn21.4R slightly more divergent. However, only RFLP fully detected the slight but real variability in sequence between isolates, corroborating those found by molecular characterization.

Seven out of nine of the isolates have close affinities with LMV-0. These isolates can efficiently be controlled by the deployment of varieties carrying either form of the *mo1* resistance gene because: (*i*) we showed that this resistance is directly effective against them; (*ii*) these genes also provide a protection against seed transmission even when symptomless viral multiplication takes place (Dinant and Lot, 1992; H. Lot, personal communication). There are two likely sources for these isolates: contaminated seed lots of susceptible varieties and/or persistence in the environment in weeds or through continuous cultivation of lettuce. The sequence variability between isolates, collected in a single location, *e.g.* Tn19B and Tn19R or Tn21R and Tn21.4R, tends to favour the first hypothesis.

Analysis of the phylogenetic affinities of the $mo1^1$-overcoming isolates Tn4B and Tn13B shows that they are very closely related to known MOST isolates such as LMV-13 or LMV-Aud (Revers *et al.*, 1997a). Although we have not shown that the two Tunisian isolates are seed-transmitted, the very close molecular relationships observed (sequence identity in the short region analysed) makes this very likely. The presence of such isolates clearly represents a threat to the Tunisian lettuce industry. The observation that isolates very closely related at the molecular level, such as Tn4B, Tn13B, LMV-13, LMV-Aud and others (O. Le Gall and T. Candresse, unpublished observations), are observed in widely separated countries is a clear indication that their geographical distribution results primarily from the distribution of contaminated seed lots. Improvement of the sanitary situation of lettuce crops in Tunisia clearly calls for the more widespread use of certified lettuce seed if further entry of similar resistance-breaking isolates is to be avoided.

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