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Molecular Plant Pathology

Biological and Molecular Variability of Lettuce Mosaic Virus Isolates

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ABSTRACT

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Lettuce mosaic potyvirus (LMV) causes severe disease of commercial lettuce crops. LMV isolates show wide biological variability, particularly in their ability to overcome the resistance genes described in *Lactuca sativa.* For a better understanding of the molecular interaction between lettuce and LMV, biological and molecular characterization of a collection of 10 LMV isolates known to differ in virulence or aggressiveness was performed. The ability of these isolates to overcome the resistance genes was reevaluated under standardized conditions. To study the molecular variability of LMV, an immunocapture-reverse transcription-polymerase chain reaction technique, coupled with direct sequencing, was used to obtain nucleotide sequence data from three short regions of the LMV genome. Clustering analysis was performed and compared to the biological properties of the 10 isolates. Three groups of LMV isolates were discriminated based on the molecular data. These groups appear to correlate with the geographic origin of the isolates rather than with their pathogenicity. Sequence comparison with California isolates clearly showed that the California isolates are related to the western European isolates, raising the possibility of past exchanges of LMV between western Europe and California.

Lettuce mosaic virus (LMV), a member of the genus *Potyvirus*, causes severe disease of commercial lettuce crops. LMV is a seed- and aphid-transmitted virus with worldwide distribution. Symptoms observed in infected plants include dwarfing, poor heading, mottling, and vein clearing (7).

The complete nucleotide sequences of the genomes of two LMV isolates (LMV-0 and LMV-E) have been determined ([27]; EMBL accession X97704 for LMV-0 and X97705 for LMV-E). The genome of LMV is a single, positive-sense 10,080-nt RNA molecule polyadenylated at its 3′ end. It contains a unique large open reading frame encoding a 3,255-amino acid polyprotein.

LMV isolates show a large array of biological variability, with variations in the severity of symptoms induced, seed transmissibility, and ability to overcome the three resistance genes described in lettuce (*Lactuca sativa*) cultivars (24,25). Two of these genes, *mo1*^{1} and *mo1*^{2}, are recessive and are believed to be either closely linked or allelic ([7,20,25]; B. Maisonneuve and H. Lot, *unpublished data*). These two genes have been introgressed into lettuce cultivars for LMV management. Depending on the particular genetic background and LMV isolate considered, the presence of *mo1*^{1} and *mo1*^{2} results in either the absence of virus multiplication or reduced virus multiplication and subsequent absence of symptoms (25). A third gene, *Mo2*, is a dominant gene that is not effective in practice for LMV control because it is overcome by most LMV isolates (25). Several pathotypic classifications of LMV isolates have been proposed based on the behavior of LMV isolates toward these resistance genes (4,7,25). The recent emergence in Europe of isolates that can overcome these resistance genes has caused serious lettuce yield losses (7) and indicates the clear possibility of increasing problems for LMV management.

Sequence analysis comparisons of coat protein (CP) genes can be used to identify and differentiate distinct potyviruses and their strains (33–35). Several studies have established phylogenetic relationships between strains of potyviruses based on CP gene sequences, e.g., sugarcane mosaic virus (11), potato virus Y (PVY) (36,37), plum pox virus (5), bean common mosaic virus (30), papaya ringspot virus (2,38), LMV (42), and zucchini yellow mosaic virus (6). The sequences of other regions of the potyvirus genome, such as the 3′ nontranslated region (3′NTR), also can be used to discriminate strains within a virus species (12,30,37). Several of these molecular analyses have shown a grouping of isolates correlated with either biological data (37) or geographic origin (2,38). Zerbini et al. (42) characterized 10 LMV isolates from California and demonstrated that variability existed in the CP N-terminal region.

However, in all of these studies, only one region of the potyvirus genome was analyzed and used to determine affinities between isolates. We recently have demonstrated that in some potyviruses the frequency of recombinant isolates is unexpectedly high (26). For example, almost one-third of PVY sequences present in the databases appears to result from recombination events located in the CP gene or the 3′NTR. Given such a high potential rate of recombination, determination of the phylogenetic affinities of virus isolates with a single, relatively short region of the genome may produce erroneous results. Because the comparison of entire genomic sequences currently is impractical, the use of several short regions scattered over the entire genome may be a simple and effective way to determine affinities or reconstruct phylogenies in potyviruses. Comparison of the results obtained with different short regions also may indicate the incidence of recombination within the set of isolates considered (26).

In this paper, we report the biological and molecular characterization of a collection of 10 LMV isolates selected to cover the biological and geographical diversity of LMV in Europe. The ability of these isolates to overcome the resistance genes present in a differential series of lettuce cultivars was evaluated in parallel experiments under standardized conditions. To study the molecular variability of these isolates, we used an immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) technique (40) coupled with direct sequencing to obtain nucleotide sequence data from three short regions of the LMV genome. Clus-

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tering analysis was performed with the sequence data, and the results of this analysis were compared to the behavior of the 10 isolates toward the lettuce resistance genes.

MATERIALS AND METHODS

Virus isolates. All 10 isolates, except 1, originated from Europe. The French isolates LMV-0, LMV-1, LMV-9, and LMV-13 and the Spanish isolate LMV-E, known to differ in their virulence and seed transmissibility, were described by Dinant and Lot (7). LMV-Aud was isolated in northern France in 1993 from lettuce cv. Audran, which contains the *mo1*[1] resistance gene. The isolate from Yemen Arab Republic (LMV-Yar) was provided by D. Walkey (HRI, Wellesbourne, England) and described by Pink et al. (24). LMV-Gr4 and LMV-Gr5, two isolates from Greece that were studied by Bos et al. (4), were provided by R. van der Vlugt (IPO, Wageningen, Netherlands), and another isolate (LMV-GrB) was provided by C. Varveri (Benaki Institute, Athens, Greece). LMV-0, LMV-Yar, LMV-Gr4, LMV-Gr5, and LMV-GrB were maintained in susceptible butterhead lettuce cv. Trocadero, and LMV-1, LMV-9, LMV-13, LMV-Aud, and LMV-E were maintained in butterhead lettuce cv. Mantilia, which contains the *mo1*[1] resistance gene.

Inoculation procedure and infectivity assays. Virus inoculum was prepared from leaves of infected lettuce plants 18 to 22 days after inoculation. The leaves were ground 1:4 (wt/vol) in a solution of 0.03 M Na_2HPO_4 containing 0.2% diethyldithiocarbamate (DIECA), and 100 mg of Carborundum and activated charcoal was added before rub-inoculation. Seeds of the differential lettuce cultivars were provided by B. Maisonneuve (Institut National de la Recherche Agronomique, Génétique et Amélioration des Plantes, Versailles, France). The five crisphead cultivars used were those described in Pink et al. (25). Six plants of each cultivar were inoculated with each isolate at the 4- to 6-leaf stage and maintained in insect-proof cages at 18 to 25°C. The experiment was repeated twice. Observations of symptoms were made at weekly intervals from 2 to 5 weeks after inoculation. The presence and concentration of virus were assessed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) 3 to 4 weeks after inoculation. Three leaves were sampled from each plant. A polyclonal antiserum raised against LMV-0 was used to test plants infected with all isolates, except LMV-Yar, for which a Yar-specific antiserum (provided by D. Walkey) was used because the LMV-0 reagents reacted very poorly with this specific isolate.

IC-RT-PCR. A protocol modified from Wetzel et al. (40) was used. Lettuce leaves were ground 1:3 (wt/vol) in PBS-Tween buffer (8 g of NaCl, 0.2 g of KH_2PO_4, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of KCl, 0.2 g of NaN_3, and 0.5 ml of Tween 20 per liter) containing 2% polyvinylpyrrolidone K25, 20 mM sodium DIECA, and 0.1% $MgCl_2$. The plant extracts were incubated overnight at 4°C in 0.5-ml tubes precoated with anti-LMV immunoglobulins. After washing the tubes once with PBS buffer, 100 µl of RT-PCR reaction mix (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.3% Triton ×100, 250 µM each of dNTPs, 1 µM each of the two primers, 0.25 units of AMV reverse transcriptase, and 0.5 units of *Taq* DNA polymerase) was added and overlaid by 100 µl of mineral oil. The tubes were incubated for 15 min at 42°C for reverse transcription, were incubated 5 min at 95°C for denaturation of RNA-DNA hybrids and reverse transcriptase, and were cycled as described for each primer pair in Table 1. Primer pairs P1 and P2, P3 and P4, and P5 and P6 were used to prime the amplification of the 5′ nontranslated region (5′NTR) together with the 5′ end of the P1 gene (region I), the 3′ end of the NIb gene together with the 5′ end of the CP gene (region II), and the 3′ end of the CP gene (region III), respectively (Fig. 1).

Direct sequencing of amplified fragments. A protocol modified from Hultman et al. (15) was used. About 400 ng of biotinylated PCR products (obtained using one of the PCR primers in a 5′ biotinylated form) were immobilized with 0.2 mg of magnetic beads containing covalently coupled streptavidin (Dynabeads M-280 streptavidin, Dynal International, Oslo, Norway). The immobilized biotinylated double-stranded DNA was denatured by incubation at room temperature with 0.15 M NaOH for 5 min. The magnetic beads retaining the single-stranded biotinylated DNA were washed once in 0.15 M NaOH and twice in H_2O and resuspended with 2 pmol of the sequencing primer in a buffer containing 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl in a total volume of 10 µl. For sequencing of regions II and III, the sequencing primer was the primer used in its nonbiotinylated form in the PCR reaction. For region I, the sequencing primer was internal (5′GCCTAGCATCAGTTATGAAATC3′, positions 338 to 360) for LMV-1, LMV-9, LMV-13, LMV-Aud, LMV-Yar, and LMV-Gr5. For LMV-GrB and LMV-Gr4, the sequencing primer used to sequence region I was the nonbiotinylated PCR primer P2, because a shorter PCR product was obtained. The annealing mixture was heated to 65°C for 5 min and cooled to room temperature. Sequencing reactions were performed with a Sequenase, version 2.0, DNA sequencing kit (United States Biochemical Corporation, Cleveland). After extension, the supernatant was removed, and 4 µl of stop solution was added to the beads. Two microliters was loaded onto an 8 M urea, 6% polyacrylamide sequencing gel.

Cloning of PCR-generated fragments. The PCR fragments were electrophoresed in a 1% agarose gel in TAE buffer (40 mM Tris, 40 mM acetate, and 1 mM EDTA). Bands of the expected size were excised, and DNA was recovered with a Geneclean kit

TABLE 1. Primer pairs and cycling conditions used for amplification

Primer pair sequence[a]	Position[b]	Cycling conditions: 1 cycle	Cycling conditions: 35 cycles
P1 5′AAAATAAAACAACCCA-ACACAACTC3′	1	92°C 30 s	92°C 30 s
P2 5′GCAATAGTTGCAGTAG-TTCTGCCCTCCAACCTAGG3′	738	42°C 30 s +	54°C 30 s
		72°C 1 min	72°C 1 min
P3 5′ATTCGAAAATTYTAYAA-RTGGTG3′	8825	92°C 20 s	92°C 20 s
P4 5′GCGTTBATGTCGTCGT-CYTT3′	9171	42°C 20 s +	56°C 20 s
		72°C 40 s	72°C 40 s
P5 5′ACAAGAAGAAACCGTA-TATGCC3′	9588		92°C 20 s
P6 5′GCCAACACACGCCTTT-AGTG3′	9885		56°C 20 s
			72°C 40 s

[a] Y = C or T; R = A or G; and B = T, G, or A. All primers were synthesized in two forms, 5′ biotinylated or not.

[b] Position on the genome of the 5′ most nucleotide of the primer.

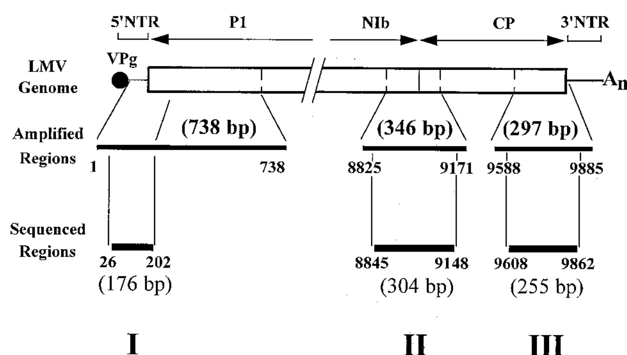

Fig. 1. Localization of regions I, II, and III along the lettuce mosaic virus (LMV) genome. The size in base pairs (bp) of the amplified (middle line) or sequenced (bottom line) regions are given in parentheses. The positions of the fragment along the LMV genome are given. The LMV genomic regions in which the studied fragments are included are labeled at the top (5′NTR [5′ nontranslated region], P1, NIb, CP [coat protein], and 3′NTR [3′ nontranslated region]).

II (Bio 101, Inc., La Jolla, CA). Purified DNA was blunted by the use of the Klenow fragment of *Escherichia coli* DNA polymerase I and was phosphorylated with T4 polynucleotide kinase. The DNA was ligated to *Hinc*II-linearized, dephosphorylated pBluescribe (Stratagene, La Jolla, CA) and used to transform competent *E. coli* XL1-blue cells (Stratagene). The recombinant plasmids were purified by alkaline lysis and digested with restriction enzymes. Plasmids containing fragments of the expected size were selected for sequencing. DNA templates were prepared as described by Zagurski et al. (41) and sequenced from both ends with Sequenase, version 2.0 (U.S. Biochemical).

Phylogenetic analysis. Multiple alignments of the amplified regions were obtained by the Clustal V program (14). Phylogenetic relationships were determined by distance methods implemented in the PHYLIP package, version 3.5 (10). Distance matrices were calculated by DNADIST with the Kimura two-parameter option (18), and distance trees were constructed from these matrices by NEIGHBOR, which implemented the neighbor-joining method (29). The trees derived were displayed by DRAWTREE to optimize the fitting of branch lengths to Kimura's distances. A bootstrap value for each internal node was calculated by performing 100 random resamplings using SEQBOOT (9) and synthesizing the resulting set of trees using CONSENSE. To confirm the topologies of the neighbor-joining trees, the maximum likelihood method, using DNAML, the parsimony method, using DNAPARS, and the FITCH method also were used.

RESULTS

Isolate reactions on different lettuce genotypes. The symptoms of LMV infection observed in most lettuce genotypes were not typical mosaic symptoms but rather vein clearing, yellow spotting, and stunting. The results of the pathogenicity tests, including symptoms and virus multiplication (Table 2), provide evidence that the isolates differed in virulence and aggressiveness. All isolates multiplied and produced symptoms on cv. Salinas. LMV-0 did not induce any symptoms in three genotypes, cvs. Malika, Salinas 88, or Vanguard 75, that possess one of the recessive genes, *mo1*[1] or *mo1*[2], even in plants in which viral multiplication was detected by ELISA. LMV-1 and LMV-9 caused severe symptoms in cvs. Ithaca and Malika, which carry the *Mo2* and *mo1*[1] genes, respectively, but did not multiply in cvs. Salinas 88 and Vanguard 75, which contain the *mo1*[2] gene. LMV-E and LMV-13, as well as the newly characterized isolates LMV-Aud and LMV-GrB, were virulent on all genotypes, including those containing the *mo1*[2] gene. The interactions of the LMV-Yar isolate and the two other Greek isolates did not fit with those of the other groups. They did not infect cvs. Ithaca and Vanguard 75, which possess the *Mo2* gene, but were able to overcome the two recessive genes, *mo1*[1] and *mo1*[2]. Nevertheless, there were slight differences between the two Greek cultivars. In Malika, LMV-Gr4 infected fewer plants (8/11 versus 10/11 for LMV-Gr5 and 14/16 for LMV-Yar) and, like LMV-Yar, multiplied at a lower rate and was less aggressive when compared to LMV-Gr5. On Salinas 88, LMV-Gr4 induced milder symptoms than LMV-Yar and LMV-Gr5, which induced the most severe symptoms.

Amplification and sequencing of regions I, II, and III. To amplify the three short regions of the LMV genome, the IC-RT-PCR technique, developed by Wetzel et al. (40), was adapted, using LMV-infected lettuce leaves as the starting material. Capture of the virus particles, release of viral RNA, and RT-PCR were performed in a one-tube assay. Amplifications were performed twice, with either one of the primers in a biotinylated form, allowing sequence determination in both orientations. Single fragments of 738 bp (region I), 346 bp (region II), and 297 bp (region III) were generated successfully for nearly all the isolates tested (data not shown). However, RT-PCR products, in addition to the expected fragments, also were obtained in the case of the amplification of LMV-GrB and LMV-Gr4 region I and LMV-GrB, LMV-Gr4, and LMV-Gr5 region II.

A direct sequencing protocol was used to sequence the amplified fragments. However, because a mixture of amplified fragments was obtained after amplification of region II of LMV-GrB, LMV-Gr4, and LMV-Gr5, the product with the expected size was purified and cloned. For each of these three isolates, at least two independent clones were sequenced on both strands.

The three sequenced regions are presented in Figure 1. For region I, the size of the sequenced 5′NTR region was 87 nt for LMV-

```
  0 LEQAPYADLAKAGKAPYIAECALKRLYTSKEASEAELEKYMEAIRSLVN
  E -DE----------------------------------------------
  1 -------------------------------------------------
  9 ----------------------------------V--------------
 13 -------------------------------------------V-----
Aud -------------------------------------------V-----
Gr4 -----------E-----------------R----E---------HA-I-
Gr5 -----------E-----------------R----E-------V--A-I-
GrB -----------E-----------------R----E-------V--A-I-
Yar -----------S-----------------R-----------D-----IS

DEDDDDMDEVYHQ/VDAKLDAGQGSKTDDKQKNSADPKDNIITEKGSGSGQMK
-------------/--T---------N-----S---S---V----------VR
-------------/--T---------N-----S---S---V---R------V-
---N--T------/-ES----------G-----P--S---------I----V-
------T------/-------------------------------------V-
------T------/-------------------------------------V-
-D---GA------/--T------DN-G-S---G--ES--ITT-----S-V-P-
-D---GA------/--T------DN-S-S---G--ES--IAT-----S-V-P-
-D---GA------/--T------DN-S-S---G--ES--IAT-----S-V-P-
-D---E-------/--T------DN-A-N-L--T--S--SAV---------I-
```

Fig. 2. Amino acid sequence alignment of region II of 10 lettuce mosaic virus (LMV) isolates. Amino acid differences are indicated with a single-letter code. Dashes indicate amino acids identical to the LMV-0 sequence. The DAG triplet is underlined. Slashes indicate the NIb/CP cleavage site.

TABLE 2. Interactions between lettuce mosaic virus isolates and lettuce differential cultivars

	Isolates							
	0		1 and 9		E, 13, Aud, and GrB		Yar, Gr4, and Gr5	
Cultivar[a]	Symptoms[b]	Vir. conc.[c]	Symptoms	Vir. conc.	Symptoms	Vir. conc.	Symptoms	Vir. conc.
Salinas	**	++++	**	++++	***	++++	* to ***	++++
Ithaca	**	++++	**	+++	***	++++	–	–
Malika	–	– to ±[d]	*	+++	* to **	++++	* to **	± to ++[d]
Salinas 88	–	++	–	–	* to **	++++	* to **	± to ++[d]
Vanguard 75	–	+	–	–	**	++++	–	–

[a] Ithaca and Vanguard 75 contain the *Mo2* gene (23). Malika contains the *mo1*[1] (formerly *g*) gene. Salinas 88 and Vanguard 75 contain the *mo1*[2] (formerly *mo*) gene (7).

[b] The number of asterisks (* to ***) scores symptom severity; symptoms included vein clearing, leaf deformation and stunting, and, occasionally, necrosis. – = no symptoms.

[c] The number of pluses indicates the relative virus concentration (vir. conc.) estimated by enzyme-linked immunosorbent assay: +, ++, and +++ indicate that the mean absorbance values were ~0.25, 0.5, or 0.75 of the absorbance on susceptible cv. Salinas (++++) respectively; ± indicates a low concentration of virus was detected; – indicates no virus was detected.

[d] In cases in which wide variability was observed in the virus content of individual plants, a concentration range is given.

Yar and 78 nt for all other isolates. The size of the sequenced P1 region was 98 nt for all isolates. The sequences obtained for regions II and III corresponded to the complete sequence of the amplified regions, excluding the primer sequences, and had the same number of nucleotides for all isolates. These nucleotide sequences have been submitted to the EMBL database and assigned the following accession numbers: Z78215 to Z78222 for region I, Z78223 to Z78230 for region II, and Z78231 to Z78238 for region III, respectively, for LMV-13, LMV-Aud, LMV-1, LMV-9, LMV-Yar, LMV-Gr5, LMV-Gr4, and LMV-GrB.

The amino acid sequences were deduced from the coding nucleotide sequences; for region I, they include the first 32 amino acids of the P1 protein; for region II, they include the last 62 amino acids of the NIb protein and the first 39 amino acids of the CP; and for region III, they include the last 85 amino acids of the CP (excluding the very last one). All LMV isolates tested contained in

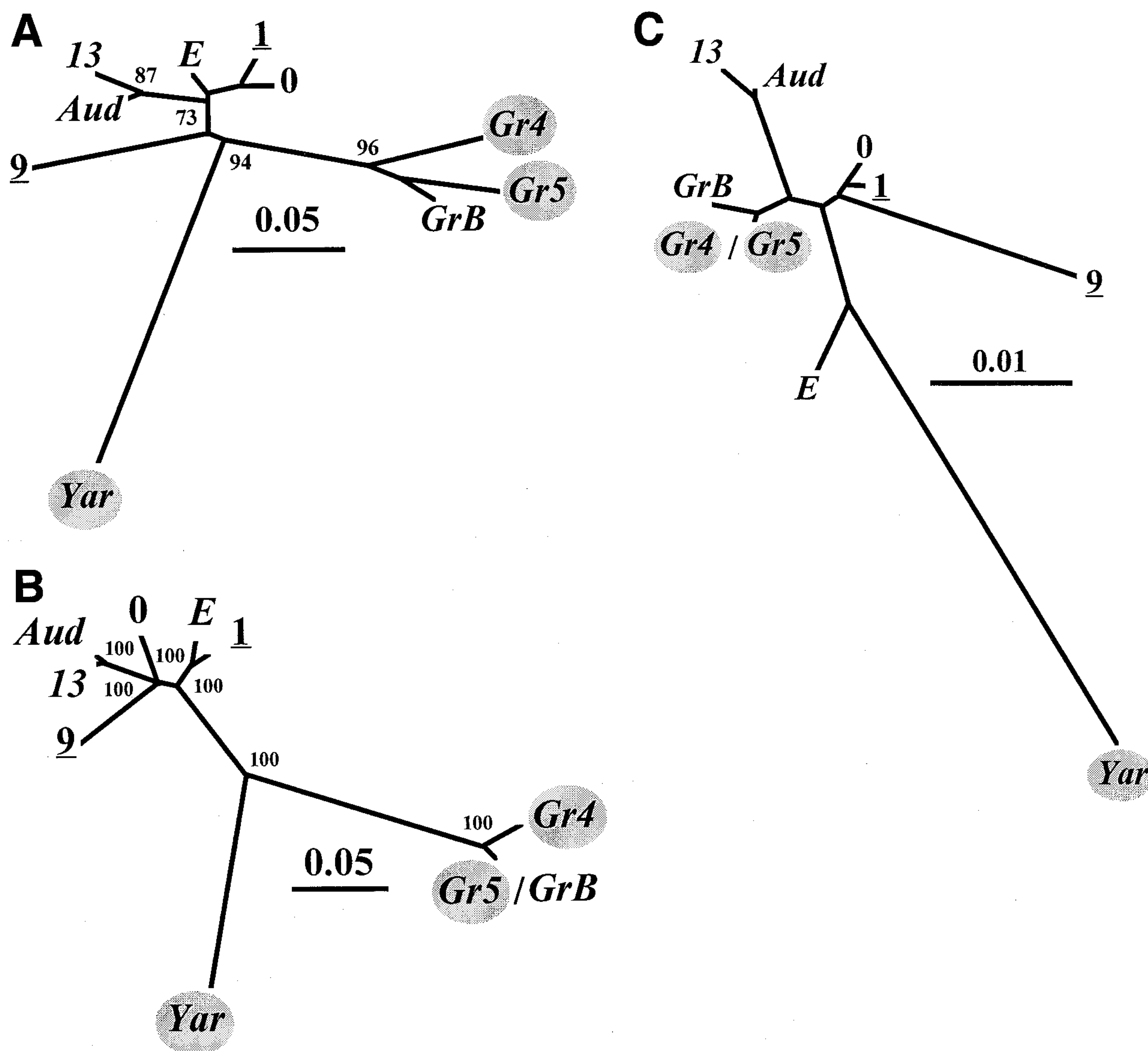

Fig. 3. Clustering neighbor-joining trees derived from regions **A,** I, **B,** II, and **C,** III of the lettuce mosaic virus (LMV) genome, as defined in text. The isolates that overcome only *mo1*[1] are underlined; the isolates that overcome both *mo1*[1] and *mo1*[2] are italic; the isolates that do not overcome *Mo2* are shaded. The percentage of bootstrap replicates in which each node was recovered is given when above 70%. The scale bar represents, for the branch lengths, Kimura distances of 0.05 (**A and B**) and 0.01 (**C**).

TABLE 3. Percentage of nucleotide sequence identity of regions I (above the diagonal) and II (below the diagonal) among the 10 lettuce mosaic virus isolates tested

	Isolate									
Isolate	0	E	1	9	13	Aud	Gr4	Gr5	GrB	Yar[a]
0		96.6	97.2	90.9	94.9	96.6	88.6	89.8	88.6	78.5
E	92.1		95.5	92.0	94.9	96.6	89.2	88.6	86.4	78.5
1	93.1	97.0		90.9	93.8	94.3	88.6	89.8	88.6	79.0
9	90.8	89.1	89.5		90.3	92.0	88.6	89.2	87.5	78.0
13	93.4	92.4	92.8	90.1		98.3	88.6	89.8	87.5	79.0
Aud	92.8	92.4	92.4	89.5	99.0		89.2	90.3	88.1	78.5
Gr4	74.3	75.0	75.3	73.4	73.4	74.3		92.6	94.3	77.3
Gr5	75.0	76.6	77.3	74.7	74.3	75.3	96.7		96.0	78.5
GrB	75.0	76.6	77.3	74.7	74.3	75.3	96.7	100.0		78.0
Yar	78.6	78.9	79.3	76.3	77.0	76.6	74.0	74.7	74.7	

[a] The nine positions at which insertions occur in the Yar sequence in the region I alignment were omitted to calculate the identity percentage.

their CP N-terminus sequence the DAG triplet involved in aphid transmission (1) and the NIb/CP cleavage dipeptide Q/V described for LMV-0 by Dinant et al. (8) (Fig. 2).

Sequence analysis. Multiple alignments of the nucleotide sequences were produced by the program Clustal V for each of the three analyzed regions. For LMV-0 and LMV-E, the corresponding regions derived from the complete sequence (27) were used. These three alignments have been submitted to the EMBL database and assigned the accession numbers DS26767 for region I, DS26768 for region II, and DS26770 for region III. The phylogenetic trees reconstructed for each of the three regions based on these data show the relationships among the various LMV isolates more clearly (Fig. 3). The general topologies of the various trees, obtained by the neighbor-joining method and supported by bootstrap analysis, are similar and show three subgroups of isolates, except in region III, for which only two subgroups can be distinguished. One subgroup included the western European isolates (LMV-0, LMV-1, LMV-9, LMV-13, LMV-Aud, and LMV-E), the second subgroup included the isolates from Greece (LMV-GrB, LMV-Gr4, and LMV-Gr5), and LMV-Yar (from Yemen Arab Republic) was the sole member of the third subgroup. The same subgroups were obtained by the maximum likelihood, parsimony, and FITCH methods (data not shown). Sequence variability was greater in regions I and II, with 77 to 98% and 73 to 100% identity, respectively, among the isolates (Table 3), whereas region III was much more conserved (94 to 100% identity). This range of variability agrees with what is known about other potyviruses (32). No specific nucleotide changes were related to any resistance-breaking properties of the LMV isolates.

The variability of the amino acid sequences was similar to that of the nucleotide sequences: identity levels in regions I and II of the 10 isolates ranged between 72 to 100% and 74 to 100% (Fig. 2), respectively, and the topologies of the phylogenetic trees constructed with amino acid sequences were similar to those obtained with the nucleotide sequence data (data not shown). All isolates had exactly the same predicted amino acid sequence in their region III (CP C terminus), except the last amino acid of LMV-Yar, which is a valine instead of a leucine in all other isolates. It is now well established that distinct potyviruses have CP amino acid identities <70%, whereas strains of a same potyvirus species have identities >90% (33,34). Our results, therefore, confirm that all of the isolates studied here are isolates of LMV. As for the nucleotide sequences, no specific amino acid change could be associated with any of the LMV pathotypes analyzed.

The CP sequences of the 10 LMV isolates used in this study were compared with those of the 10 California isolates determined by Zerbini et al. (42). Because the sequences determined by these authors began at nucleotide 26 of the CP gene and the N-terminal sequences determined in this work terminated at nucleotide 117, only the common 92 nt were available for comparison. The phylogenetic tree derived from a multiple alignment of this short region is presented in Figure 4. The California isolates clearly cluster with the western European group.

DISCUSSION

The emergence in Europe of new LMV isolates causing severe symptoms in commercial lettuce cultivars containing the *mo1*[1] resistance gene was reported recently by several authors (4,7,25). Because such cultivars are extensively grown in open fields, especially in France, resistance breaking was evoked (25). These data and the report in California of very aggressive isolates (42) have aroused interest in establishing a better understanding of the molecular interactions between lettuce (*L. sativa*) and LMV. The molecular basis for resistance breaking are known only for a few plant viruses. Resistance-breaking determinants have been located in the CP of tobacco mosaic virus (19,28), pepper mild mottle virus (3), and potato virus X (17,31) and in the replicase (21,23) or movement proteins (22,39) of the tobacco and tomato mosaic viruses. In potyviruses, such determinants are thought to be found in the 5′ end of the genome (13,16), as well as the VPg domain of the NIa protein in the pea seedborne mosaic potyvirus (E. Johansen, *personal communication*).

In an attempt to link phylogenetic affinities with LMV resistance-breaking properties, we carried out an analysis of the molecular diversity of 10 LMV isolates and compared the results to the virulence of these isolates on various LMV-resistance genes. We chose isolates that previously differed in virulence or aggressiveness. All 10 isolates originated from Europe, except LMV-Yar. Greek isolates were included because they had been reported to be very aggressive and had been studied by other authors (4).

The results obtained for LMV-0, LMV-1, LMV-9, LMV-E, and LMV-13 confirmed our previous work (7,20). We also confirmed that these five isolates overcame the dominant resistance gene *Mo2* identified by Pink et al. (24). According to these data, these isolates were grouped into three distinct pathotypes. The two previously uncharacterized isolates, LMV-Aud and LMV-GrB, had virulence characteristics similar to LMV-E and LMV-13. The results obtained for LMV-Yar, LMV-Gr4, and LMV-Gr5 on cvs. Ithaca and Vanguard 75 agreed with results presented by Pink et al. (24) and Bos et al. (4): they are unable to infect cultivars containing the *Mo2* gene. However, Pink et al. (24) described LMV-Yar as avirulent on the other resistant cultivars because it did not induce discernible symptoms, although it multiplied. We also found a low concentration of LMV-Yar in cvs. Malika and Salinas 88, as estimated with a homologous antiserum, but under our conditions, Malika and Salinas 88 exhibited symptoms in all cases when infected with LMV-Yar. The status of LMV-Gr4 and LMV-Gr5 in terms of virulence on differential cultivars was more difficult to interpret. The rate of infection was never 100%, and the virus concentration was rather low in the infected plants. This situation probably is not an experimental artifact caused by the low efficiency of LMV-0 antiserum in detecting these isolates, because our results fit with those of Bos et al. (4), who used an antiserum raised against LMV-Gr4.

Based on the results of our molecular analyses, at least three groups of LMV isolates can be discriminated. The largest group in our sample contains isolates from western Europe (France and Spain) that are closely related to isolates from California. This analysis does not seem to show evidence of any clearly recombinant LMV isolate. One possible recombination event might have occurred for

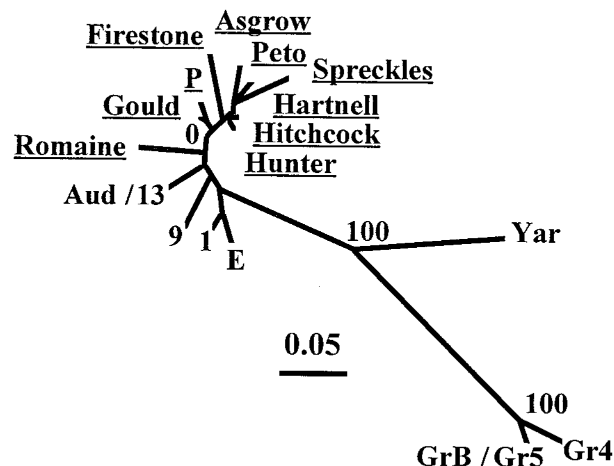

Fig. 4. Clustering neighbor-joining tree derived from the region of the N terminal of the coat protein gene discussed in text for California isolates of lettuce mosaic virus and the isolates studied here. The California isolates are underlined. The percentage of bootstrap replicates in which each node was recovered is given when above 70%. The scale bar represents, for the branch lengths, Kimura distances of 0.05.

the Greek isolates, with a recombination break point located between regions II and III (based on comparison of the position of the Greek isolates in the trees of regions I and II with that of region III). An alternate possibility is that the low intrinsic variability of region III simply may not allow discrimination of these Greek isolates from the pool of western European isolates. Indeed, the tree derived for region III is not statistically supported by significant boostrap values (Fig. 3). Sequencing of the entire CP gene of the Greek isolates is now underway and should allow us to settle this aspect of LMV phylogeny.

The three subgroups of LMV isolates correlate with the geographic origin of the isolates rather than with their virulence on differential cultivars. For example, the ability to overcome the resistance gene $mo1^2$ is shared by LMV-Yar, the three Greek isolates, and LMV-E, LMV-13, and LMV-Aud in the western European subgroup. However, these last three isolates cluster closer to LMV-0, LMV-1, and LMV-9, which are not virulent on $mo1^2$ (Fig. 3). Overcoming the $mo1^2$ gene is probably a relatively recent event (7). Therefore, it is reasonable to conclude that the common ancestor of the LMV isolates was unable to overcome this gene. A similar conclusion could be made about the $mo1^1$ and *Mo2* genes. Absence of evidence of recombination and failure to correlate differences in virulence properties and molecular variability among the LMV isolates suggest that the ability of these isolates to overcome the resistance genes may have appeared independently several times subsequent to the differentiation of the three LMV subgroups. Therefore, it would be interesting to determine the genomic determinants of resistance breaking and, particularly, to observe whether they are identical among isolates belonging to different subgroups.

Comparison of the isolates examined in this study with the partially sequenced California isolates (42) clearly showed that they are closely related to the isolates in the western European cluster. Of particular interest is the case of LMV-0: although it was originally isolated in France, it is more closely related to the California isolates than to the other western European isolates. In their study, Zerbini et al. (42) already showed that the LMV-0 sequence was 95 to 99% identical to those of the California isolates. These observations clearly indicate the possibility that LMV-0 may have been exchanged between the two continents in the recent past, perhaps via contaminated seed lots, because LMV-0 is seed transmissible.

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