MOLECULAR CHARACTERIZATION OF TWO BRAZILIAN ISOLATES OF *Lettuce mosaic virus* WITH DISTINCT BIOLOGICAL PROPERTIES

RENATE KRAUSE-SAKATE*†, RAQUEL N. MELLO*2, MARCELO A. PAVAN2, EUNIZE M. ZAMBOLIM1, MURILO G. CARVALHO1, OLIVIER LE GALL3 & F. MURILO ZERBINI***

1Departamento de Fitopatologia/BIOAGRO, Universidade Federal de Viçosa, CEP 36571-000, Viçosa, MG, e-mail: zerbini@ufv.br; 2Departamento de Defesa Fitossanitária, UNESP/FCA, CEP 18603-000, Botucatu, SP; 3Station de Pathologie Végétale, INRA Bordeaux-Aquitaine, BP 81, 33881 Villenave d’Ornon Cedex, França

(Accepted for publication 25/01/2001)

Corresponding author: Francisco Murilo Zerbini


ABSTRACT

The coat protein genes of two field isolates of *Lettuce mosaic virus* (LMV) from São Paulo State, previously characterized based on their virulence on lettuce (*Lactuca sativa*) differential cultivars as belonging to pathotypes II (isolate AF198, unable to infect cultivars possessing the genes *mo1* or *mo1*2) and IV (isolate AF199, which breaks the resistance conferred by *mo1* or *mo1*2), were cloned and sequenced. Comparisons of the nucleotide sequences from European, Middle-Eastern, North American, and the two Brazilian isolates did not permit their separation in strains, because homologies were always greater than 95%. However, phylogenetic analysis indicated that the Brazilian isolate AF198 clusters with isolates LMV-R and LMV-0 (pathotype II, from the United States and France, respectively). Isolate AF199 clustered with two isolates (LMV-Aud and LMV-13) from France. These isolates are also closely related to isolates from Chile, although a common origin is not proposed. Independent mutation events may be occurring in different parts of the world, leading to the emergence of distinct LMV strains capable of overcoming the resistance genes *mo1* or *mo1*2.

**Key words:** *Lactuca*, lettuce, *Potyvirus*, LMV, capsid protein, cloning, resistance.

INTRODUCTION

*Lettuce* (*Lactuca sativa* L.) is one of the most economically important vegetable crops throughout the world. Lettuce mosaic is the main viral disease of this crop. It is caused by *Lettuce mosaic virus* (LMV), a member of the genus *Potyvirus* of the family *Potyviridae*, which is seed-borne in lettuce and disseminated by aphids. LMV may completely destroy lettuce fields if control measures are not taken, such as the elimination of weed hosts, the use of virus-free seed, and the use of resistant cultivars, when available (Dinant & Lot, 1992).
LMV particles are long, flexuous rods with approximately 750 x 13 nm. The viral genome is composed of one molecule of a single-stranded, positive sense RNA with 10,080 nucleotides (Revers et al., 1997b). The viral genomic RNA has a viral encoded protein covalently linked at the 5' end, a poly-A tail at the 3' end, and contains a single open reading frame (ORF) which encodes a large polyprotein with 3,255 amino acids. This polyprotein undergoes self-cleavage resulting in 8-9 viral proteins (Revers et al., 1997b).

In Brazil, and in most European countries, LMV has been controlled through the use of resistant cultivars. Two sources of resistance were identified in the late 1960's: the recessive gene mo1 (previously g) in the Argentine cultivar ‘Gallega de Invierno’ (Bannerot et al., 1969), and the recessive gene mo2 (previously mo) in a wild L. sativa access from Egypt (Ryder, 1968; Ryder, 1970). These genes were introgressed into commercial lettuce cultivars at the beginning of the 1970's. The vast majority of the lettuce cultivars now planted in Brazil and Europe contain the mo1 gene, and cultivars grown in the United States contain the mo2 gene (Dinant & Lot, 1992). LMV had been under control for over 20 years in the regions where they were used. However, in the early 1990's LMV isolates capable of infecting lettuce cultivars containing these genes were detected initially in Europe and the Middle East (Pink et al., 1992a, b), and more recently in Brazil (Stangarlin, 1995). Pink et al. (1992a, b) proposed the classification of LMV isolates in pathotypes (I to IV) based on their resistance-breaking phenotype. Pathotype II includes the typical strain, capable of infecting cultivars containing the dominant gene Mo2 but unable to infect those cultivars containing either one of the recessive genes, mo1 or mo2. Isolates classified in pathotype IV overcome the resistance genes Mo2, mo1, and mo2 (Pink et al., 1992b), and isolates of this pathotype have been detected in Europe and in Brazil (Pink et al., 1992a; Stangarlin, 1997). These isolates constitute a major threat to lettuce production in these areas since no resistance genes have been identified that effectively prevent virus multiplication in lettuce.

The simultaneous appearance in distant geographical regions of new LMV strains infecting cultivars with the recessive genes mo1 and mo2 indicates the possible occurrence of multiple, independent mutation events in the viral genome. Alternatively, a single isolate could have emerged and spread throughout the world via infected seed lots. Several LMV isolates from distinct geographical regions have been characterized as belonging to distinct pathotypes. Phylogenetic analysis based on the nucleotide sequence of their capsid protein genes indicated the clustering of isolates based on geographical origin rather than the ability to overcome specific resistance genes (Revers et al., 1997a). Such analysis has not yet been carried out for LMV isolates from Brazil. Therefore, the objective of this work was to clone and sequence the capsid protein gene of two Brazilian isolates of LMV previously characterized as belonging to pathotypes II (AF198) and IV (AF199) by Stangarlin (1997) in order to determine their taxonomical relationship with LMV isolates from Europe, the Middle East, and the United States. Preliminary reports have been published (Mello et al., 1998; Krause-Sakate et al., 1999).

**MATERIALS AND METHODS**

**Viral isolates**

The isolates used in this study originated from a collection of isolates from fields surrounding the city of Campinas, São Paulo State. This collection of isolates was subjected to single local lesion passages in Chenopodium quinoa Willd., and some of the local lesion isolates were selected based on their ability to infect lettuce cultivars containing resistance genes (Stangarlin, 1997). Further inoculations on the six lettuce differential cultivars used by Pink et al. (1992b) determined that two of the local lesion isolates, AF198 and AF199, belonged to pathotypes II and IV, respectively (Stangarlin, 1997). The isolates were maintained in Nicotiana benthamiana Domin. plants through successive sap-inoculations using 0.2 M potassium phosphate buffer, pH 7.2, containing 0.1% (w/v) sodium sulfite. Plants were inoculated at the 4-6 leaf-stage using Carborundum (600 mesh) as an abrasive and kept in a greenhouse.

**Virus concentration, RNA extraction, and RT-PCR**

Virus particles were concentrated from infected N. benthamiana plants according to Lane (1992) and were resuspended in 500 µl of 0.01 M potassium phosphate buffer, pH 7.0. Viral RNA was extracted from 200 µl of viral concentrate, with the addition of 50 µl of RNA extraction buffer (0.2 M glycine, 0.2 M NaCl, 0.02 M EDTA, pH 9.5). This mixture was adjusted to 1.5% (w/v) SDS and 100 µg/ml Proteinase K (GibcoBRL) and then kept for 1 h at 37 °C. The RNA was extracted with one volume of phenol/chloroform (1:1) and precipitated with 2.5 vols of 100% ethanol. The pellet was washed with 70% (v/v) ethanol and resuspended in 20 µl of DEPC-treated, sterile water. cDNA was synthesized from viral RNA using the SuperScript Pre amplification System for First Strand cDNA Synthesis (GibcoBRL), according to the manufacturer’s instructions. Viral fragments corresponding to a portion of the capsid protein gene were amplified by PCR using 5 µl of cDNA, 5 µl of enzyme buffer, 5 µl of 25 mM MgCl2, 1.0 µl of the deoxynucleotide mixture (0.01 M), 20 pmol of each primer and 1 unit of Taq DNA polymerase. PCR was carried out using LMV-specific primers 1196 (5’-A-A-G-G-C-A-G-T-A-A-A-A-C-T-G-A-T-G-3’) and 1087 (5’-T-T-T-A-T-A-C-T-A-C-A-G-T-C-T-T-T-A-3’) (Zerbini et al., 1995), which direct the amplification of a fragment of approximately 800 base pairs comprising almost the entire capsid protein gene (Figure 1). PCR reactions consisted of 35 cycles of denaturing at 94 °C for 1 min, primer annealing at 55 °C for 2 min and primer extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Amplified fragments were directly cloned into the pCR2.1 plasmid vector using the TA Cloning System.
Molecular characterization of two Brazilian isolates of *Lettuce mosaic virus*...

(Invitrogen) and were transformed into *Escherichia coli* JM109 cells. Recombinant plasmids were identified by enzymatic cleavage using EcoRI, and the viral insert was sequenced using the Thermocycle Sequencing Dye Terminator Kit (Perkin-Elmer) and an ABI 310 automated sequencer (Applied Biosystems).

**Phylogenetic analysis**

Sequences were compared with those from previously characterized LMV isolates, and phylogenetic trees were prepared using the programs Clustal W Interactive and TreeView (Thompson *et al.*, 1994). The LMV isolates used in the analyses are listed in Table 1.

RESULTS AND DISCUSSION

Variability at the molecular level among LMV isolates was initially determined by pairwise comparisons of the nucleotide sequence of the capsid protein gene. The percent similarity of this genomic region of members of the *Potyvirus* genus is a taxonomic criterion used for the characterization and identification of new species, although it does not usually allow for strain differentiation (Shukla *et al.*, 1994; Mayo & Pringle, 1997; Revers *et al.*, 1997a). This analysis confirmed that isolates AF198 and AF199 are LMV isolates, since their capsid protein gene sequences (deposited into the GenBank under the accession numbers AJ278855 and AJ278854, respectively) have greater than 95% similarity with previously characterized LMV isolates (Table 2). However, because their sequences are also greater than 95% similar, pairwise comparisons do not indicate any obvious differences between them (Table 2). To distinguish between the two isolates it is necessary to inoculate differential lettuce cultivars containing different resistance genes, since AF199 can overcome the resistance provided by the *mol*1 and *mol*2 genes, while AF198 cannot (Stangarlin, 1997).

Phylogenetic trees are used to propose the evolutionary and taxonomical relationships among different isolates, strains of a given species, or different species in a given genus. Two distinct regions of the capsid protein, the variable amino (N)-terminal region and the conserved carboxy (C)-terminal region were used to construct phylogenetic trees of LMV isolates (Figure 2).

The phylogenetic tree constructed based on the C-terminal region did not allow a clear separation of isolates into clusters. The tree constructed based on the N-terminal region clearly separated the isolates into three clusters on the basis of their geographical origin. The first cluster contains isolates from Western Europe and the United States, the second cluster contains isolates from Eastern Europe, and the third cluster contains the isolate from the Middle East. These are the same results reported by Revers *et al.* (1997a). The two Brazilian isolates, AF198 and AF199, cluster with the Western European/North American isolates (Figure 2). Isolate AF198 is very closely related to isolates LMV-0 (originated from France) and LMV-R (from the United States), which suggests that AF198 might have been introduced into Brazil through infected seed lots imported from Europe and/or the United States. Isolate AF199 is closely related to two other French isolates, LMV-Aud and LMV-

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**TABLE 1 - *Lettuce mosaic virus* isolates used for sequence comparisons and phylogenetic analysis**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Pathotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GenBank access number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>France</td>
<td>II</td>
<td>X97704</td>
</tr>
<tr>
<td>13</td>
<td>France</td>
<td>IV</td>
<td>Z78223</td>
</tr>
<tr>
<td>Aud</td>
<td>France</td>
<td>IV</td>
<td>Z78224</td>
</tr>
<tr>
<td>E</td>
<td>Spain</td>
<td>IV</td>
<td>X97705</td>
</tr>
<tr>
<td>Gr4</td>
<td>Greece</td>
<td>a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Z78229</td>
</tr>
<tr>
<td>Gr5</td>
<td>Greece</td>
<td>*</td>
<td>Z78228</td>
</tr>
<tr>
<td>GrB</td>
<td>Greece</td>
<td>IV</td>
<td>Z78230</td>
</tr>
<tr>
<td>YAR</td>
<td>Yemen Arab Republic</td>
<td>I</td>
<td>Z78227</td>
</tr>
<tr>
<td>R</td>
<td>California, USA</td>
<td>II</td>
<td>U24670</td>
</tr>
<tr>
<td>AF198</td>
<td>São Paulo, Brazil</td>
<td>II</td>
<td>AJ278855</td>
</tr>
<tr>
<td>AF199</td>
<td>São Paulo, Brazil</td>
<td>IV</td>
<td>AJ278854</td>
</tr>
</tbody>
</table>

<sup>a</sup>According to Pink *et al.* (1992b)

<sup>b</sup>Isolates Gr4 and Gr5 do not fit into any of the pathotypes proposed by Pink *et al.* (1992b).

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**TABLE 2 - Percent nucleotide sequence homology for the 800 bp fragment comprising the aminoterminal region (above the diagonal) and the carboxy-terminal region (below the diagonal) of the capsid protein gene of *Lettuce mosaic virus* isolates from different geographical regions**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>1998</th>
<th>1999</th>
<th>0</th>
<th>E</th>
<th>Aud</th>
<th>13</th>
<th>R</th>
<th>Gr4</th>
<th>Gr5</th>
<th>GrB</th>
<th>Yar</th>
</tr>
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<td>-</td>
<td>93</td>
<td>97</td>
<td>89</td>
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<td>63</td>
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<td>71</td>
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<tr>
<td>199</td>
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<td>-</td>
<td>93</td>
<td>91</td>
<td>98</td>
<td>98</td>
<td>90</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>71</td>
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<td>100</td>
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<td>100</td>
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<td>69</td>
</tr>
<tr>
<td>YAR</td>
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<td>93</td>
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<td>95</td>
<td>93</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>-</td>
</tr>
</tbody>
</table>

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*Fitopatol. bras.* 26(2), junho 2001
FIG. 2 - Phylogenetic tree prepared with Clustal W based on the amino acid sequence of the C-terminal (A) and N-terminal (B) regions of the capsid protein of *Lettuce mosaic virus* isolates from different geographical regions (0 e AF198, non resistance-breaking; E, resistance-breaking, not seed borne; AF199, Aud, 13 e GrB, resistance-breaking, seed borne). The scale bar represents Kimura’s genetic distance.

13. However, these isolates are distinct from other Western European isolates, and have a higher degree of similarity with partially characterized LMV isolates from Chile (O. LeGall, unpublished results). These isolates have the common capability of overcoming the *mol1* and *mol2* resistance genes and being seed-borne, whereas other resistance-breaking isolates are not seed-borne (eg., LMV-E, originated from Spain). Therefore, considering the recent detection of AF199 relative to LMV-Aud or LMV-13, isolate AF199 might have been introduced into Brazil through infected seed lots originating from Chile and/or Europe.

The phylogenetic analysis confirms the clustering of isolates based on their geographical origin rather than resistance-breaking phenotype, also reported by Revers et al. (1997a). The Western European/American cluster, for example, which includes the two Brazilian isolates, contains isolates capable of overcoming the resistance provided by the *mol1* and *mol2* genes (LMV-E, LMV-Aud, LMV-13, and LMV-AF199) as well as isolates which are incapable of overcoming this resistance (LMV-0, LMV-R, and LMV-AF198). The same is true for the Eastern European cluster. This may indicate the simultaneous emergence of resistance-breaking isolates in several regions of the world, such as Spain, Greece, Chile, and France. Not surprisingly, these isolates have emerged in areas where lettuce mosaic is controlled primarily through the use of resistant cultivars containing the *mol1* and *mol2* genes. In North America, where the main strategy for controlling lettuce mosaic is the use of virus-free seed (Grogan, 1980), resistance-breaking isolates have not yet been detected (Zerbini et al. 1995; F.M. Zerbini and R.L. Gilbertson, unpublished results). Thus, selection pressure due to the use of resistant cultivars may play a role in the emergence and selection of new viral strains.

These results suggest that isolates AF198 and AF199 could have been introduced into Brazil through infected seed lots, pointing out the need for better surveillance for early detection and eradication of plant material containing exotic pathogens, and new variants (eg, with greater virulence) of pathogens already occurring in Brazil. Currently, there are no sources of resistance to *mol1* and *mol2*-breaking LMV isolates. The cloned capsid protein gene of isolate AF199 could be used for the generation of resistant, transgenic lettuce plants. This work is currently being conducted at the Universidade Federal de Viçosa.

![Phylogenetic tree](image)

**FIG. 3 - Multiple sequence alignment of the amino acid sequences from the N-terminal region of the capsid protein of *Lettuce mosaic virus* isolates from different geographical regions used for construction of the phylogenetic tree shown on Figure 2B. This region corresponds to part of region II from Revers et al. (1997a). “X” indicates the absence of an amino acid in that position.**

**LITERATURE CITED**


Molecular characterization of two Brazilian isolates of *Lettuce mosaic virus*...


