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

RENATE KRAUSE-SAKATE[1], RAQUEL N. MELLO[1], MARCELO A. PAVAN[2], EUNIZE M. ZAMBOLIM[1], MURILO G. CARVALHO[1], OLIVIER LE GALL[3] & F. MURILO ZERBINI[1***]**

[1]Departamento de Fitopatologia/BIOAGRO, Universidade Federal de Viçosa, CEP 36571-000, Viçosa, MG, e-mail: zerbini@ufv.br; [2]Departamento de Defesa Fitossanitária, UNESP/FCA, CEP 18603-000, Botucatu, SP; [3] Station 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

KRAUSE-SAKATE, R., MELLO, R.N., PAVAN, M.A., ZAMBOLIM, E.M., CARVALHO, M.G., LE GALL, O. & ZERBINI, F.M. Molecular characterization of two Brazilian isolates of *Lettuce mosaic virus* with distinct biological properties. Fitopatologia Brasileira 26:153-157. 2001.

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 $mo1^1$ or $mo1^2$) and IV (isolate AF199, which breaks the resistance conferred by $mo1^1$ 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 distinguish 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 $mo1^1$ or $mo1^2$.

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

RESUMO

Caracterização molecular de dois isolados brasileiros de *Lettuce mosaic virus* apresentando propriedades biológicas distintas

Efetuou-se a clonagem e seqüenciamento do gene que codifica a proteína capsidial de dois isolados do vírus do mosaico da alface (*Lettuce mosaic virus*, LMV) provenientes do estado de São Paulo, previamente caracterizados como pertencentes aos patótipos II (AF198, incapaz de infetar cultivares com os genes de resistência $mo1^1$ ou $mo1^2$) e IV (AF199, capaz de quebrar a resistência propiciada pelos genes $mo1^1$ e $mo1^2$), com base na virulência em cultivares diferenciadoras. Análise comparativa das seqüências de nucleotídeos de isolados provenientes da Europa, América do Norte, Oriente Médio e os dois isolados brasileiros não permitiu sua separação em estirpes, pois as porcentagens de homologia foram sempre superiores a 95%. Entretanto, análise filogenética dos isolados sugere uma origem comum entre o isolado AF-198 e os isolados LMV-R e LMV-0 (patótipo II, provenientes dos Estados Unidos e da França, respectivamente). O isolado AF199 apresentou uma alta homologia de seqüência com os isolados LMV-Aud e LMV-13, ambos provenientes da França. Esses isolados também são relacionados a isolados provenientes do Chile, embora uma origem comum não seja proposta. Eventos independentes de mutação podem estar ocorrendo em diferentes partes do mundo, propiciando o surgimento de novas estirpes de LMV capazes de quebrar a resistência conferida pelos genes $mo1^1$ e $mo1^2$.

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).

* Part of the D.S. thesis of the first author

** CNPq and CAPES-COFECUB bilateral cooperative program fellowship

*** CNPq fellowship

LMV particles are long, flexuous rods with approximately 750 × 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*^{1} (previously *g*) in the Argentine cultivar 'Gallega de Invierno' (Bannerot *et al.*, 1969), and the recessive gene *mo1*^{2} (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*^{1} gene, and cultivars grown in the United States contain the *mo1*^{2} 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*^{1} or *mo1*^{2}. Isolates classified in pathotype IV overcome the resistance genes *Mo2*, *mo1*^{1}, and *mo1*^{2} (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*^{1} and *mo1*^{2} 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 Preamplification System for First Strand cDNA Synthesis (GibcoBRL), according to the m anufacturer'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 $MgCl_2$, 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

(Invitrogen) and were transformed into *Escherichia coli* JM109 cells. Recombinant plasmids were identified by enzymatic cleavage using *Eco*R I, 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 *mo1*^{1} and *mo1*^{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-

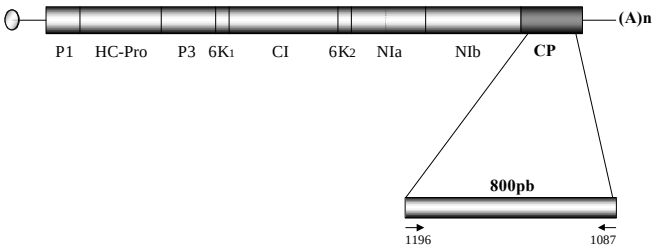

FIG. 1 - Schematic representation of the *Lettuce mosaic virus* genome indicating the capsid protein fragment PCR-amplified using primers 1196 and 1087.

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

Isolate	Origin	Pathotype[a]	GenBank access number
0	France	II	X97704
13	France	IV	Z78223
Aud	France	IV	Z78224
E	Spain	IV	X97705
Gr4	Greece	*[b]	Z78229
Gr5	Greece	*	Z78228
GrB	Greece	IV	Z78230
YAR	Yemen Arab Republic	I	Z78227
R	California, USA	II	U24670
AF198	São Paulo, Brazil	II	AJ278855
AF199	São Paulo, Brazil	IV	AJ278854

[a] According to Pink *et al.* (1992b)

[b] Isolates Gr4 and Gr5 do not fit into any of the pathotypes proposed by Pink *et al.* (1992b).

TABLE 2 - Percent nucleotide sequence homology for the 800 bp fragment comprising the amino-terminal 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

	Isolate										
Isolate	198	199	0	E	Aud	13	R	Gr4	Gr5	GrB	Yar
198	-	93	97	89	92	92	90	61	63	63	71
199	97	-	93	91	98	98	90	61	61	61	71
0	98	98	-	89	93	93	92	61	63	63	73
E	97	97	98	-	91	91	85	64	66	66	73
Aud	97	99	98	97	-	100	90	61	61	61	71
13	96	99	98	98	100	-	90	61	61	61	71
R	96	97	98	98	97	97	-	61	63	63	70
Gr4	97	98	99	99	98	98	99	-	94	94	66
Gr5	97	98	99	99	98	98	99	100	-	100	69
GrB	97	97	98	98	98	97	98	99	99	-	69
Yar	94	93	95	95	93	93	95	95	95	95	-

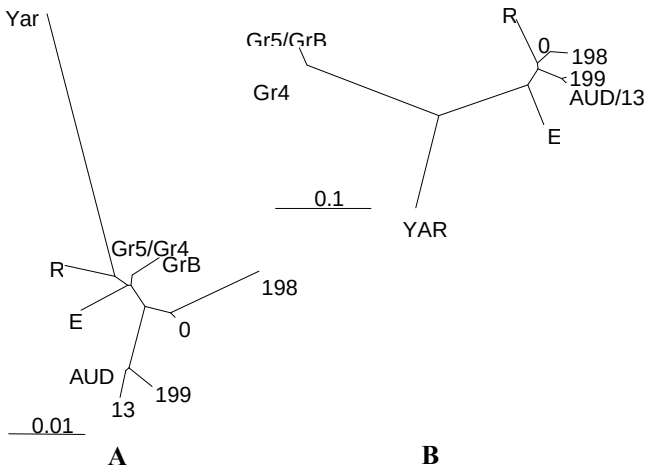

FIG. 2 - Phylogenetic tree prepared with Clustal W based on the amino acid sequence of the C-terminal (A) and N-terminal (B) regions of the capsid protein of *Lettuce mosaic virus* isolates from different geographical regions (0 e AF198, non resistance-breaking; E, resistance-breaking, not seed borne; 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 *mo1*[1] and *mo1*[2] 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 *mo1*[1] and *mo1*[2] 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 *mo1*[1] and *mo1*[2] genes. In North America, where the main strategy for controling 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 *mo1*[1] and *mo1*[2]-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.

```
0          GSKTDDKQKNSADPKDNIITEKGSGSGQMK
198        -X-------S--------------------
199        -X-------------------------V-
AUD        ----------------------------V-
13         ----------------------------V-
R          ------R--------N-T--------V-V-
E          ---ND----S---S---V----------VR
YAR        DN-A-N-L—-T--S—-SAV---------I-
GR5        DN-S-S---G—-ES--IAT-----S-V-P-
GRB        DN-S-S---G—-ES--IAT-----S-V-P-
GR4        DN-G-S---G—-ES--ITT-----S-V-P-
```

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

BANNEROT, H., BOULIDARD, L., MARROU, J. & DUTEIL, M. Etude de l'hérédité de la tólerance au virus de la mosaïque de la laitue chez la variété gallega de invierno. Annales de Phytopathologie 1:291-226. 1969.

DINANT, S. & LOT, H. Lettuce mosaic virus. Plant Pathology 41:528-542. 1992.

GROGAN, R. G. Control of lettuce mosaic with virus-free seed. Plant Disease 64:446-449. 1980.

KRAUSE-SAKATE, R., LE GALL, O., PAVAN, M.A., MACIEL-ZAMBOLIM, E., CARVALHO, M.G. & ZERBINI, F.M. Phylogenetic analysis of the capsid protein of two Brazilian isolates of *Lettuce mosaic virus* (LMV) belonging to distinct pathotypes. Virus Reviews and Research 4(Supplement):153. 1999 (Abstract)

LANE, L. A general method for detecting plant viruses. In: Maramorosch, K. (Ed.) Plant diseases of viral, viroid, mycoplasma and uncertain origin. New Delhi. Oxford

& IBH Publishing. 1992. pp.1-15.

MAYO, M.A. & PRINGLE, C.R. Virus taxonomy - 1997. Journal of General Virology 79:649-657. 1997

MELLO, R.N., KRAUSE-SAKATE, R., CARVALHO, M.G., MACIEL-ZAMBOLIM, E., PAVAN, M.A. & ZERBINI, F.M. Cloning and partial sequencing of the capsid protein gene of two brazilian isolates of lettuce mosaic potyvirus (LMV) with distinct biological properties. Virus Reviews and Research 3(Supplement):145. 1998 (Abstract)

PINK, D.A.C., KOSTOVA, K. & WALKEY, D.G.A. Differentiation of pathotypes of lettuce mosaic virus. Plant Pathology 41:5-12. 1992a.

PINK, D.A.C., LOT, H. & JOHNSON, R. Novel pathotypes of lettuce mosaic virus: breakdown of a durable resistaence ?. Euphytica 63:169-174. 1992b.

REVERS, F., LOT, H, SOUCHE, S., LE GALL, O., CANDRESSE, T. & DUNEZ J. Biological and molecular variability of lettuce mosaic virus isolates. Phytopathology 87:397-403. 1997a.

REVERS, F., YANG, S.J., WALTER, J., SOUCHE, S., LOT, H., LE GALL, O., CANDRESSE, T. & DUNEZ, J. Comparison of the complete nucleotide sequences of two isolates of lettuce mosaic virus differing in their biological properties. Virus Research 47:167-177. 1997b.

RYDER, E.J. Evaluation of lettuce varieties and breeding lines for resistance to common lettuce mosaic. USDA Technical Bulletin 1391. 8p. 1968.

RYDER, E.J. Inheritance of resistance to common lettuce mosaic. Journal of the American Society for Horticultural Science 95:378-379. 1970.

SHUKLA, D.D., WARD, C.W. & BRUNT, A.A. The Potyviridae. Cambridge, Academic Press, 1994.

STANGARLIN, O. S. Identificação dos vírus causadores de mosaico em cultivares de alface (*Lactuca sativa* L.) resistentes ao vírus do mosaico da alface nas regiões produtoras do Estado de São Paulo (Dissertação de Mestrado). Botucatu. Faculdade de Ciências Agronômicas. 1995.

STANGARLIN, O.S. Variabilidade do vírus do mosaico da alface e comportamento de cultivares tolerantes de alface (*Lactuca sativa* L.) (Tese de Doutorado). Botucatu. Faculdade de Ciências Agronômicas. 1997.

THOMPSON, J.D, HIGGINS, D.G. & GIBSON, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence aligment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680. 1994.

ZERBINI, F.M., KOIKE, S.T. & GILBERTSON, R.L. Biological and molecular characterization of lettuce mosaic potyvirus isolates from the Salinas Valley of California. Phytopathology 85:746-752. 1995.

00022