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ARTIGOS

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ABSTRACT

Krause-Sakate, R.; Richard-Forget, F.; Redondo, E.; Pavan, M.A.; Zerbini, F.M.; Candresse, T.; Le Gall, O. Quantitative control of *Lettuce mosaic virus* fitness and host defence inhibition by P1-HCPro**.** ***Summa Phytopathologica***, v.33, n.2, p.119-123, 2007.

Two *Lettuce mosaic virus* isolates capable of overcoming the resistance afforded by the resistance gene *mo1*² in lettuce, LMV-AF199 from Brazil, and LMV-E, an European isolate, were evaluated for the rapidity and severity of symptoms induced on the lettuce variety Salinas 88 (*mo1*²). The mosaic symptoms on Salinas 88 plants inoculated with LMV-AF199 appeared 7 days post-inoculation (dpi) and 15 dpi for LMV-E. The symptoms induced by LMV-AF199 in this cultivar were also more severe than those induced by LMV-E. In order to identify the region of the viral genome responsible for this phenotype, recombinant viruses were constructed between these isolates and the phenotype of each recombinant was analysed. The region encoding proteins P1 and HcPro from LMV-AF199 was associated with the increased virulence in Salinas 88.

Additional keywords: Gene silencing, *Lactuca sativa*, infectious cDNA, *Potyvirus*.

RESUMO

Krause-Sakate, R.; Richard-Forget, F.; Redondo, E.; Pavan, M.A.; Zerbini, F.M.; Candresse, T.; Le Gall, O. P1-HC Pro do *Lettuce mosaic virus* atua de forma quantitativa na inibição da resposta de defesa do hospedeiro e adaptação viral. ***Summa Phytopathologica***, v.33, n.2, p.119-123, 2007.

Dois isolados de *Lettuce mosaic virus* capazes de contornar a resistência conferida pelo gene *mo1*² em alface, LMV-AF199 proveniente do Brasil e LMV-E um isolado europeu, foram avaliados quanto à rapidez e à severidade dos sintomas induzidos em alface variedade Salinas 88 (*mo1*²). Os sintomas de mosaico induzidos pelo isolado LMV-AF-199 em Salinas 88 são mais severos e aparecem aos 7 dias após a inoculação (dpi), enquanto que para o isolado LMV-E os sintomas são visíveis somente a partir dos 15 dpi. Com o intuito de identificar a região do genoma viral responsável por este fenótipo, vírus recombinantes foram construídos entre estes dois isolados, e o fenótipo avaliado quanto a rapidez e severidade dos sintomas em Salinas-88. A região codificadora para as proteínas P1 e Hc-Pro do LMV-AF199 foi associada com o aumento da virulência deste isolado em Salinas-88.

Palavras-chave adicionais: silenciamento gênico, *Lactuca sativa*, cDNA infeccioso, *Potyvirus*

Lettuce mosaic virus (LMV) is potentially the most destructive virus of lettuce (*Lactuca sativa*) crops. Transmitted by seeds and aphids, LMV is now distributed worldwide, probably due to the exchange of contaminated seed lots (5,7,9).

LMV is a member of the family *Potyviridae*. Viral particles are long, flexuous rods of approximately 750 nm x 13 nm (15). The LMV genome is composed of one molecule of a single-stranded, positive sense RNA with 10,080 nucleotides (14). The viral genomic RNA has a virus-encoded protein covalently linked at its 5' end and a poly-A tail at its 3' end. It contains a single open reading frame, which encodes a large polyprotein with 3,255 amino acids. This polyprotein undergoes self-cleavage as it is translated, generating 8-10 viral proteins (14, 15).

Three LMV resistance genes have been described in lettuce, two alleles (*mo1*^{1} and *mo1*^{2}) of the recessive gene *mo1*, which encodes the cap-binding protein eIF4E (10), and the dominant gene *Mo2* (2, 11, 12). Two main sub-groups of LMV isolates cause epidemics in the field, LMV-Common and LMV-Most (7). LMV-Most isolates (for *mo1*-breaking seed transmitted) overcome the resistance afforded by the *mo1*^{1}, *mo1*^{2} and *Mo2* genes and are seed-borne in cultivars carrying these genes. LMV-Common isolates are seed-borne but do not overcome the resistance associated with *mo1*^{1} and *mo1*² (7).

LMV-AF199, an LMV-Most isolate collected in São Paulo, Brazil

(7), induces a severe mosaic and stunting on the lettuce cultivar Salinas 88, which carries the *mo1²* gene, 7 days post-inoculation (dpi). LMV-E, isolated from lettuce plants in Spain (5), has the ability to infect Salinas 88, but causes only mild symptoms in this cultivar, 15 dpi. These differences were only observed in lettuce cultivars carrying *mo1²*, such as Salinas 88 and Vanguard 75. In the cultivar Floribibb (*mo1¹*) both isolates induce similar symptoms at 8-10 dpi. This suggests that, although *mo1²* does not fully restrict the infection by LMV-E, it causes a delay in the appearance of the symptoms and also a decrease in their severity.

In order to identify the region of LMV-AF199 involved in the virulence of this isolate in Salinas 88, chimeric viruses between isolates LMV-AF199 and LMV-E were constructed and analysed.

MATERIALS AND METHODS

Construction of recombinant viruses between LMV-AF199 and LMV-E

A full-length infectious cDNA clone of LMV-E (19) was used as a basis to introduce various cDNA fragments from LMV-AF199. Fragments of cDNA covering, together, the entire genome of LMV-AF199 were amplified by RT-PCR using *Pfu* DNA Polymerase (Stratagene) or Dynazyme DNA Polymerase (Finnzymes) and cloned into the pZErO-2 (Invitrogen) or pGEM-T Easy (Promega) vectors. Fragments from LMV-AF199 and LMV-E that shared common restriction sites were exchanged to generate the set of recombinants as described (8). Each full-length recombinant cDNA was first inoculated using a biolistics device (Helios Gene Gun, BioRad) onto 'Trocadero' lettuce plants (no *mo1* resistance allele) at the four-leaf stage as described (13). Successfully infected plants showed typical vein-clearing symptoms from 8 to 12 dpi, and systemic mosaic within three weeks.

Samples of these plants were then ground using 0.05 M potassium phosphate and 0.02 M NaDieca buffer pH 7.5 and inoculated onto lettuce plantlets of the cultivars Trocadero (susceptible) and Salinas 88 (*mo1²*). Symptoms were evaluated visually and the presence and nature of the viral progeny was checked by RT-PCR amplification followed by digestion with specific restriction enzymes that discriminate between the sequences of LMV-AF199 and LMV-E.

GFP *Agrobacterium tumefaciens*-mediated transient expression

Transgenic *Nicotiana benthamiana* expressing the green fluorescent protein (GFP) from *Aequorea victoria* and homozygous for the nos-*nptII*-nos-35S-*gfp*-nos construct (lineage 16c, provided by Dr. David C. Baulcombe, Sainsbury Laboratory, Norwich, UK) were used in this experiment. Silencing of the *gfp* gene was mediated by agro-infiltration of a construct over-expressing *gfp* locally (Voinnet and Baulcombe, 1997; Voinnet et al., 1998). The *Agrobacterium tumefaciens* strain C58C1, carrying the pCH32 plasmid containing the *virE*, *virG* and tetracycline resistance genes, and a binary vector containing the nos-*nptII*-nos-35S-*gfp*-nos construct and a kanamycin resistance gene, were used.

Pre-cultures were grown overnight (28°C, 200 rpm) in 5 mL LB medium supplemented with 5 µg/mL of tetracyclin and 50 µg/mL of kanamycin, and used to cultivate 50 mL of LB supplemented with 5 µg/ml of tetracyclin, 50 µg/mL of kanamycin, 10 mM MES pH 6.3 and 20 µM acetosyringone during 12 hours at 28°C. Cells were harvested by centrifugation (15 min at 4000 rpm), washed twice with ice-cold water and resuspended in infiltration medium (10 mM MES pH 6.3, 10 mM $MgCl_2$ and 150 µM acetosyringone) to obtain a final absorbance of 0.5 at 600 nm. The bacterial suspension was incubated at room temperature for 2-3 hours before agroinfiltration. Suspension cultures in a blunt-tipped 1-mL plastic syringe were forced into intact leaves still attached to the plant by pressing the tip of the syringe against the lower surface of the leaf. Plants were kept in a greenhouse and symptoms were assessed visually.

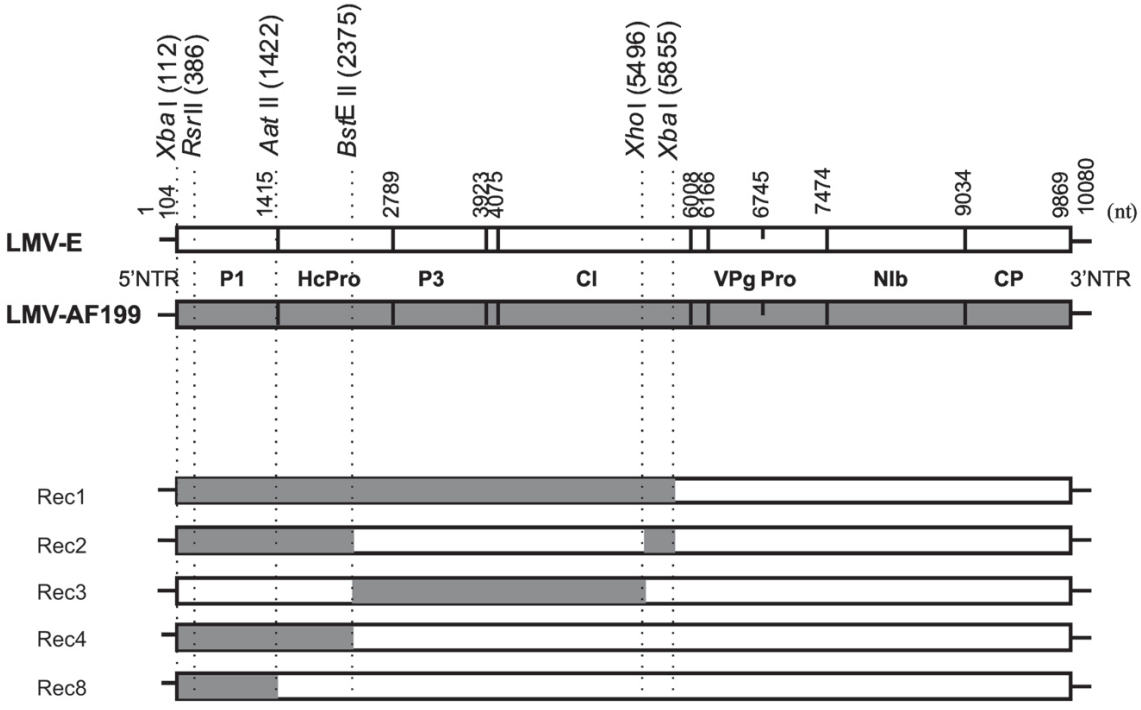

Figure 1. Schematic representation of *Lettuce mosaic virus* genome and structure of the recombinants constructed between LMV-AF199 and LMV-E. The restriction sites used are indicated at the top. Grey boxes represent coding sequences from LMV-AF199, and white boxes from LMV-E.

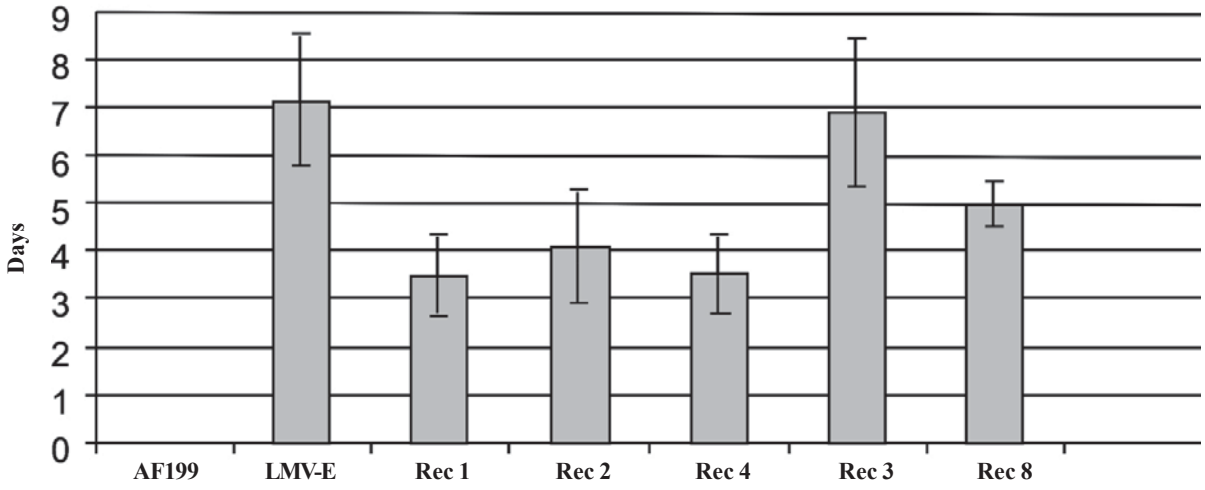

Figure 2. Delay in the appearance of symptoms after inoculation of LMV-E and the recombinant viruses in Salinas 88, in comparison to LMV-AF199. The average and standard deviation of five replications containing three plants each are shown.

Dosage of total protein and fluorescence emitted by GFP

The total protein content of *Nicotiana benthamiana* extracts was estimated using the Bradford method (3). Samples were ground (1:5 p/v) in 50 mM sodium phosphate, 10 mM ascorbic acid, pH 7.5, with the aid of a Ribolyzer Cell Disrupter (Hybaid). The plant extract was centrifuged during 10 minutes at 13.000 rpm, and 100 μL were added to 1 mL of Bradford buffer (0.01% Coomassie Blue G-250, 4.7% ethanol, 8.5% phosphoric acid). After 2 minutes the absorbance was measured at 595 nm. A standard curve using bovine serum albumin (BSA) at several concentrations was used for the estimation of the total protein concentration.

The fluorescence of samples prepared by the Bradford method was measured to estimate the expression of the *gfp* gene in the leaves. The excitation wavelength for GFP is 395 nm (UV) or 473 nm (blue), and the maximum emission occurs at 512 nm. A sample (600 μL) was excited at 395 nm, and the fluorescence measured at 512 nm using a spectrophotometer (model F-2000, Hitachi). This data was compared to the value obtained for total protein concentration and the fluorescence was measured by mg of total proteins.

The visual detection of GFP fluorescence in the whole plant was performed using a 100-W hand-held long wave ultraviolet lamp (UV Products). Plants were photographed with a digital camera.

RESULTS

The recombinant viruses Rec1, Rec2, Rec3, Rec4 (8) and Rec8 were constructed by introducing various segments of LMV-AF199 cDNA into a cloned infectious LMV-E cDNA (Figure 1). All recombinant viruses were infectious and accumulated systemically in the cultivar Trocadero (no *mo1* resistance allele) and in Salinas 88 (*mo1*^{2}).

The incubation time (measured as dpi) when symptoms became apparent was measured for LMV-AF199, LMV-E and the recombinant viruses after sap-inoculation on Salinas 88. Symptoms in Trocadero appeared at the same time for LMV-E and LMV-AF199 (data not shown), but a 7-day delay was observed after inoculation of LMV-E in Salinas 88 when compared to LMV-AF199 (Figure 2). Symptoms for LMV-AF199 appeared at 7 dpi. The chimeric viruses containing the region coding for the P1 and HcPro proteins from LMV-AF199 (Rec1, Rec2 and Rec4) had an intermediate phenotype, with symptoms appearing with a 3 to 4-days delay compared to LMV-AF-199 (Figure 2). Symptoms induced by Rec8, which only contains part of the P1 coding region from LMV-AF199, appeared with a 5-day delay compared to LMV-AF199. These results indicate that the P1-HcPro coding region (or the proteins themselves) is involved in the difference in kinetics of symptom development observed between LMV-AF199 and LMV-E in Salinas 88. This region might be important for the fitness of LMV-AF199 on lettuce cultivars with the *mo1*^{2} gene, such as Salinas 88. LMV-AF199 and the recombinant Rec1 had higher accumulation rate on these plants when compared with LMV-E (dot blot assays, data not shown).

Symptoms induced in Salinas 88 by Rec1, Rec2 and Rec4 were more severe than those induced by Rec3 or Rec8. For LMV-E as well as for Rec3 and Rec8, symptoms were difficult to distinguish at 15 and 22 dpi, and even at 36 dpi for LMV-E (Figure 3). Together, these results indicate that the P1-HcPro coding region from LMV-AF199, when introduced in a chimeric virus originating from LMV-E, influences the symptom development and also the severity of the symptoms induced in Salinas 88.

In potyviruses, HcPro acts as a suppressor of RNA silencing, a natural defence mechanism that limits the accumulation of viral RNAs in plants (1, 4, 6). In this context, our data suggest that the P1-HcPro region originating from different isolates of the same virus could have different abilities to suppress gene silencing, which could be related to differences in fitness between natural isolates. To test this hypothesis, LMV-AF199, LMV-E and the chimeric virus Rec4 (part of the P1-HcPro from LMV-AF199 in an LMV-E background) were inoculated onto transgenic *Nicotiana benthamiana* plants carrying the *gfp* gene, but previously silenced for its expression by infiltration of an *Agrobacterium tumefaciens* strain carrying a construct to direct local GFP over-expression (18).

Transgenic *N. benthamiana* plants expressing the GFP protein emit a green fluorescence that can be visually observed with an ultraviolet (UV) lamp, in contrast to the transgenic silenced GFP lines where only the red colour caused by auto-fluorescence of the chlorophyll is observed (Figure 4). At 20 dpi, LMV-AF199 was able to suppress GFP silencing (i.e. restore GFP expression) in the new leaves (1000 GFP arbitrary units per grame of leaf tissue), while

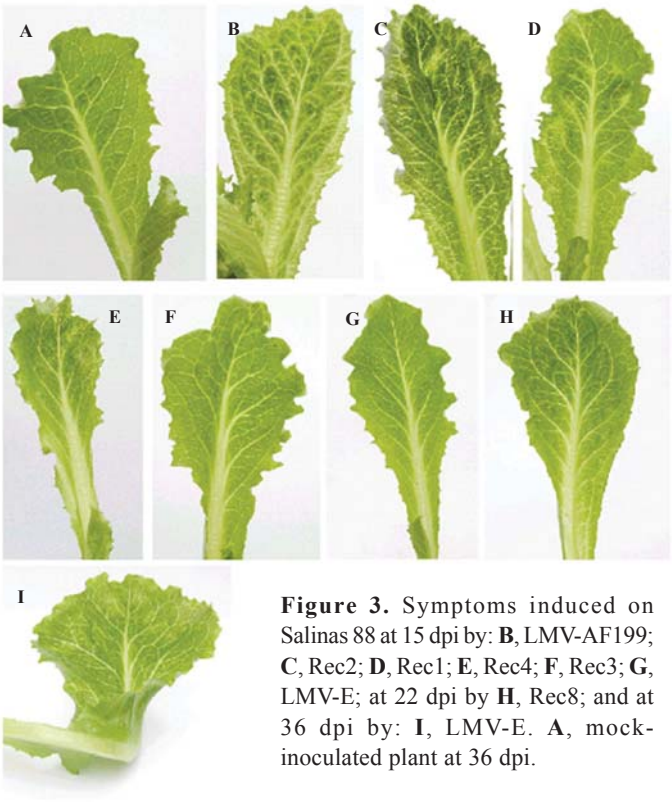

Figure 3. Symptoms induced on Salinas 88 at 15 dpi by: **B**, LMV-AF199; **C**, Rec2; **D**, Rec1; **E**, Rec4; **F**, Rec3; **G**, LMV-E; at 22 dpi by **H**, Rec8; and at 36 dpi by: **I**, LMV-E. **A**, mock-inoculated plant at 36 dpi.

LMV-E was much less efficient at that (300 units). Rec4 suppressed partially GFP silencing (560 units), as could be seen by the faint green fluorescent areas in the new leaves (Figure 4). The expression of GFP in plants inoculated with LMV-AF199 was similar to that of non-silenced control plants (1265 units), suggesting that LMV-AF199 was able to fully restore GFP expression after silencing. The expression of GFP on control transgenic *N. benthamiana* plants silenced was 121 units and on nontransgenic 98 units.The results confirmed that LMV-AF199 is more efficient than LMV-E to suppress silencing of the *gfp* gene in *N. benthamiana*. Rec4 had an intermediate ability to do so.

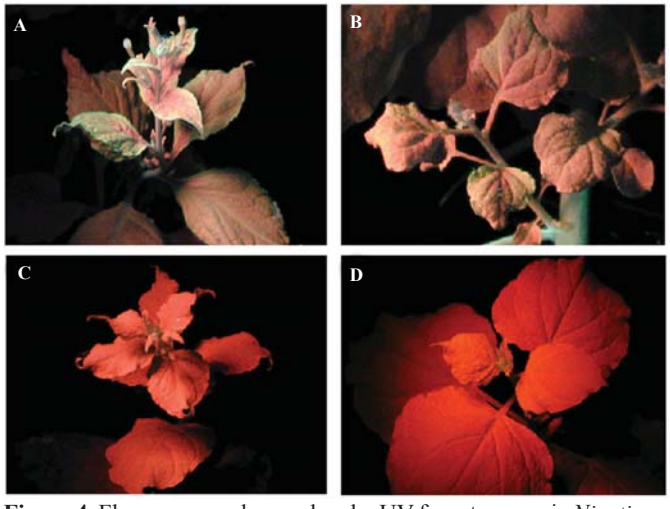

Figure 4. Fluorescence observed under UV from transgenic *Nicotiana benthamiana* plants previously silenced for the expression of *gfp* and inoculated (20 dpi) with: **A**, LMV-AF199; **B**, Rec4; **C**, LMV-E and **D**, healthy plant (totally silenced).

DISCUSSION

Our data are the first indication that different isolates from the same virus can differ quantitatively in their abilities to suppress RNA silencing. These isolates also differ in their fitness, as evaluated by the kinetics of symptom development in a lettuce cultivar carrying the *mo1*² resistance gene, which they can both overcome. This raises the possibility that the fitness assessment, speed of infection in a *mo1*² background, is related to suppression of the silencing-based host defence.

The fitness measurements were carried out in lettuce, while silencing suppression was evaluated in *N. benthamiana*. This was done because an assay for silencing suppression is currently not available in lettuce, while a difference in fitness was only measurable between LMV-E and LMV-AF199 in some lettuce cultivars.

The involvement of the P1-HcPro region in both phenotypes was demonstrated by the effect of inserting this region from LMV-AF199 into an LMV-E background in the Rec4 construct. The reverse construct could not be assayed because an infectious cDNA is not available for LMV-AF199, but the difference observed between Rec4 and Rec8 confirms a direct role for HcPro.

In both types of assays, all recombinants behaved in an intermediate fashion between LMV-E and LMV-AF199. This suggests that an additional region of the viral genome might be involved, downstream of the CI coding region which is present in Rec1. Alternatively, the recombinant nature of the chimeric viruses *per se* could affect their cycle in Salinas 88, and result in a global decrease of fitness.

The involvement of P1-HcPro and other viral suppressors of gene silencing in virulence has been described several years ago (17). The present study gives evidence that the ability to suppress this type of host defence mechanism can differ not only qualitatively, but also quantitatively, between virus isolates. This provides obvious grounds for virus evolution and possibly for virus-host co-evolution.

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