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Molecular and biological characterization of two potyviruses infecting lettuce in south-eastern France

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Abstract

Several potyviruses affect lettuce (*Lactuca sativa*) and chicory (*Cichorium* spp.) crops worldwide and are important constraints for production because of the direct losses that they induce and/or because of their seed transmission. We describe the molecular and biological properties of two potyviruses that were recently isolated from lettuce plants showing mosaic or strong necrotic symptoms in an experimental field in south-eastern France. The first potyvirus belongs to the *Endive necrotic mosaic virus* species and is unable to infect lettuce cultivars with a resistance to *Turnip mosaic virus* that is present in many European cultivars and probably conferred by the *Tu* gene. The second potyvirus belongs to the tentative species lettuce Italian necrotic virus and infected all tested lettuce cultivars. Wild accessions of *Lactuca serriola*, *Lactuca saligna*, *Lactuca virosa* and *Lactuca perennis* were identified as resistant to one or the other potyvirus and could be used for resistance breeding in lettuce. No resistance against these two potyviruses was observed in the tested *Cichorium endivia* cultivars. In contrast, all tested *Cichorium intybus* cultivars or accessions were resistant.

Keywords: lettuce, *Lactuca* spp., Asteraceae, *Potyvirus*, resistance, host range, aphid transmission

Introduction

Lettuce (*Lactuca sativa*) is an important vegetable crop in many countries worldwide, with a global production of 25 million tons. In the Mediterranean basin, Spain, Italy, Turkey and France are the major lettuce-producing countries contributing 10% of the global production (FAOSTAT 2013). In France, a large part (~50%) of the production is localized in the south-eastern regions and especially in the Bouches du Rhône department (23% of the national production in 2014) (Agreste 2015). Lettuce is affected by several potyviruses among which *Lettuce mosaic virus* (LMV) is the most damaging. LMV induces mosaic and crinkling of the leaf lamina associated with plant stunting, and in some particular cultivars and/or for particular isolates, severe necrosis in older leaves and eventually death of the plant (Zink *et al.*, 1973; Moreno & Fereres 2012). Like other potyviruses, LMV is transmitted in a non-persistent manner by many aphid species, *Myzus persicae* and *Macrosiphum euphorbiae* being the most efficient vectors. The most widely used genes of resistance to LMV are *mo1*¹ and *mo1*², which have been identified in cultivated or wild *L. sativa* accessions (Bannerot *et al.*, 1969; Ryder, 1970). The lettuce genotypes carrying these genes are partly resistant to strain LMV-0, with no symptom although sometimes with virus accumulation. Other LMV resistance sources have been described in *Lactuca virosa*, *Lactuca saligna* and *Lactuca perennis*, notably the *Mo3* dominant resistance gene from *L. virosa* accession PIVT1398 (Dinant & Lot, 1992; Maisonneuve *et al.*, 1999; Pitrat, 2012).

Two additional potyviruses have been observed more occasionally and/or in more restricted areas in lettuce crops (Provvidenti & Hampton 1992; Blancard *et al.*, 2006): *Turnip mosaic virus* (TuMV) and *Bidens mottle virus* (BiMoV). TuMV induces leaf mottling, often with yellow and/or necrotic areas. Resistance to TuMV is controlled by the dominant gene *Tu*, which confers complete immunity and is present in a large majority of European cultivars (Zink & Duffus, 1973, 1975; Robinson & Provvidenti, 1993; Robbins *et al.*, 1994).

Interestingly, TuMV susceptibility in *Lactuca serriola* is associated with the presence of the *Dm5/8* gene conferring resistance to *Bremia lactucae*. The two traits have been introduced simultaneously in *L. sativa* cultivars during breeding programs aiming to improve lettuce resistance to *B. lactucae* in the USA (Robbins *et al.*, 1994; Zink & Duffus, 1969, 1970, 1973), mainly in American iceberg cultivars. TuMV also induces chlorotic spots on leaves and reduced plant growth on endive or escarole (*Cichorium endivia* var. *latifolium*) (Verhoyen 1983). BiMoV has been described only in Florida in the 1960s (Christie *et al.*, 1968), where it infected most lettuce and escarole cultivars in the field, with a prevalence reaching 100% (Purcifull *et al.*, 1971; Zitter & Guzman 1974). Symptoms were indistinguishable from those induced by LMV in lettuce but were more severe than LMV on escarole, with strong mottling patterns both in veins and interveinal areas. A recessive resistance gene with a narrow spectrum of action was identified in *L. sativa* cv. Valmaine and many *Cichorium intybus* accessions were also found resistant (Zitter & Guzman 1977).

In this article, we describe the molecular and biological properties of two other potyviruses that were isolated from lettuce plants showing either mosaic or strong necrotic symptoms in experimental fields in south-eastern France. We also investigated whether the LMV and TuMV resistances identified in *Lactuca* spp. were also efficient against these two potyviruses.

Materials and Methods

Virus isolates

Two virus isolates, named ‘7091’ and ‘7098’, were collected in November 2012 in an open-field experimental plot in INRA Montfavet (near Avignon, south-eastern France, 43°56’54N, 4°48’32E) from two different plants of *L. sativa* cv. Montemar showing mosaic and necrosis

symptoms typical of virus infections. Montemar is an iceberg lettuce cultivar selected from cv. Calmar (Mikel, 2007). The two infected plants reacted positively to a potyvirus-polyvalent diagnostic kit in ACP-ELISA (see below). Both isolates were then independently propagated by mechanical inoculation to seedlings of cv. Montemar and stored as dehydrated material for further molecular and biological characterization.

Genome sequencing and phylogenetic analyses

Total RNA of inoculated lettuce plants was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's recommendations. A region of *ca.* 1600 nucleotides at the 3' end of the virus genome was amplified with primers derived from Gibbs and Mackenzie (1997), designed to be polyvalent for members of the family Potyviridae. The only modification was that tails corresponding to the sequence of universal primers M13(-21) and M13rev derived from phage M13 were added to the 5' end of the primers' sequence in order to allow direct sequencing. Internal primers designed from the partial sequences made available were also used for sequencing. A 3.4 kb fragment encompassing the region from the CI to the NIb coding region of both viruses was amplified with the primer Poty-CI-5' of Colinet *et al.*, (1998) and specific primers 7091-NIb-3' (5'-ATCAATGAGTACGTCAGTGC-3') and 7098-NIb-3' (5'-GCAAAGTATTTGCAGTAGAGGTC-3'), designed from the partial sequences made available from isolates 7091 and 7098, respectively. In order to obtain complete sequences of isolates 7091 and 7098, next-generation sequencing (NGS) was performed on an Illumina MiSeq sequencer at Fasteris (Plan-les-Ouates, Switzerland). After total RNA extraction, small RNAs (21-24 nucleotide (nt) long) were purified from polyacrylamide gel and submitted to

sequencing. This method was shown to be highly versatile and sensitive for the diagnostic of single- or double-stranded RNA or DNA viruses and viroids (Kreuze et al. 2009).

The reads were assembled with the CLC Genomic Workbench version 7, with different word lengths (13 to 21 nt). For isolate 7091, a complete sequence of a closely related virus isolate (GenBank accession number KP769852) was used to perform realignments with the Illumina sequences using different stringencies with CLC. Using available sequences, new specific primers were designed in order to fill the few gaps remaining between sequence contigs. In addition, for isolate 7098, the sequence of the 5' extremity was obtained by 5' RACE according to the instructions of the manufacturer (Invitrogen, USA). The nucleotide and deduced amino acid (aa) sequences of isolates 7091 and 7098 were compared to GenBank sequences using Blast and PASC (<http://www.ncbi.nlm.nih.gov/sutils/pasc/>). Sequence alignments were performed using ClustalW as implemented in the software MEGA v. 6.0 (Tamura *et al.*, 2013). Characteristics of the full-length or partial sequences included in the analysis are detailed in the legend of Figure 1. The best nucleotide substitution model was selected with MEGA v. 6.0. Neighbor-joining and maximum likelihood trees were built using MEGA v. 6.0 on full-length aa sequences of 78 potyvirus isolates from 33 acknowledged or tentative species, as well as on 73 nucleotide sequences of the coat protein core (cCP) coding region from 35 species after codon-based alignment with ClustalW. Five hundred bootstrap resamplings were performed to assess tree branching significance.

For RT-PCR and partial sequencing of a virus isolate kept since 1999 in dehydrated leaf material, the two primers ENMV-Nib-fw (5'-GAGGAYYTRTGGTTCATGTC) and ENMV-CP-rv (5'-CGGAGCGTCACACAGYGAACGA) were defined based on the sequence of isolate 7098 and closely-related isolates collected from wild plants (Supplementary Table 1).

Host range study

Different plant species and genotypes, mainly of the Asteraceae family (Tables 1 and 2), were mechanically inoculated at the seedling stage by isolate 7091 or 7098 in insect-proof greenhouses, as described in Moury *et al.* (2004). Cultivated and wild *Lactuca* spp. accessions were chosen for their differential resistance to several potyviruses (Table 2). Symptoms were recorded regularly and infection status was confirmed by enzyme-linked immunosorbent assay (ELISA) on apical leaves, 4-6 weeks post inoculation. Isolate 7091 was detected by antigen-coated plate (ACP)-ELISA with a generic potyvirus-group kit (Agdia, Elkhart, USA). Since isolate 7098 was found to be a member of the ENMV (*Endive necrotic mosaic virus*; *Potyvirus* genus) species after the analysis of its genome sequence (see Results section), it was detected by double antibody sandwich (DAS)-ELISA with an ENMV-specific polyclonal antiserum obtained by H. Lot in 1999. Virus detection was considered positive when the absorbance at 405 nm (A_{405}) was above 3 times that of the healthy controls.

Aphid transmission

Aphids (*Myzus persicae*) were reared on virus-free pepper (*Capsicum annuum* cv. Yolo Wonder) plants in growth cabinets with 16h daylight, 21°C night and day. Four weeks before experiments, aphid maintenance was shifted to synchronous rearing in order to manipulate 7-8-day-old apterous female cohorts. Virus-free aphids were starved for 1 hr before virus acquisition. To evaluate aphid transmission of isolates 7091 and 7098, plants of *L. sativa* cv. Calmar were used as source and test plant. Groups of 15 aphids were placed on a detached source leaf for a 2-5 min acquisition access period and then transferred to virus-free test plants for an overnight inoculation access period (IAP). Each source leaf served only once to inoculate three test plants with five aphids per test plant. Ten source leaves detached from five source

plants were used per transmission experiment to inoculate 30 test plants. At the end of the IAP, aphids were killed by imidaclopride sprayings (0.1 g/L; NUPRID 200, Nufarm SAS). Test plants were checked for infection by ELISA three weeks after inoculation.

Search for virus reservoirs in wild plants

5284 weed or wild plants were sampled in the region of Avignon from 2009 to 2013 and tested for the presence of ENMV and LINV. Each plant was first individually tested by the potyvirus-group kit in ACP-ELISA. Positive samples were subjected to RT-PCR amplification with the “potyvirid” primers derived from Gibbs and Mackenzie (1997) as described above. The amplified fragments were directly sequenced with primers M13(-21) and M13rev to characterize the potyvirus(es) present in the samples.

Results

Genome sequencing of isolates 7091 and 7098

Two weeks after mechanical inoculation to lettuce plants in a greenhouse, isolate 7098 induced mosaic symptoms, whereas isolate 7091 induced mosaic and necrosis symptoms (Supplementary Fig. 1). Observation of crude sap preparations of the plants inoculated either with isolate 7091 or 7098 in electron microscopy revealed numerous flexuous particles *ca.* 700 to 730 nm long and 11 nm wide associated with “pinwheel”-like cytoplasmic inclusions, typical of the family Potyviridae. In addition, the two isolates reacted positively to a potyvirus-polyvalent ELISA kit. There was no evidence of the presence of other virus types in these extracts. For both isolates, RT-PCR amplification of a *ca.* 1600 nucleotide genome fragment was performed with primers polyvalent for the family Potyviridae, followed by

direct sequencing. There was no evidence of mixtures between virus species or variants in each of the isolates according to the sequence chromatograms. A BLASTn search in GenBank performed in September 2014 revealed a highest score with ENMV accession number AJ223827 (74% nucleotide identity) for isolate 7098 and with *Angelica virus Y* (genus *Potyvirus*) accession number EF488741 (71% nucleotide identity) for isolate 7091. These preliminary results suggested that the two isolates corresponded to two different potyvirus species.

The full-length sequence of isolates 7091 and 7098 was obtained by classical (Sanger) sequencing, NGS and/or 5' RACE. Again, there was no evidence for the presence of other potyviruses in the NGS contigs (data not shown). The genomes were 9829 and 9993 nucleotide long, respectively, excluding the poly(A) tail. Sequences are available in GenBank under accession numbers KU941945 and KU941946, respectively. Both viruses had a typical potyvirus genetic organization, with a large open reading frame (ORF) translated as a polyprotein of 3173 and 3236 amino acids for isolates 7091 and 7098, respectively, including 9 autocatalytic cleavage sites and a putative smaller protein P3N-PIPO expressed through translational frameshift.

Isolate 7091 shared 98% nt identity (99% aa identity) with a potyvirus recently described as lettuce Italian necrotic virus (LINV; accession KP769852; Ciuffo *et al.*, 2016). For isolate 7098, the closest virus identified from whole genome sequence analysis was vanilla distortion mosaic virus (VDMV; 55% nt identity, 62% aa identity). However, isolate 7098 also shared 79% nt (82% aa) identity in the coat protein (CP)-coding region with ENMV (accession AJ223827) and 75% nt (80% aa) identity with ornamental onion stripe mosaic virus (OOSMV, accession EU042750), for which only partial sequences are available.

Phylogenetic analyses based on complete aa sequences or cCP coding sequences of isolates 7091 and 7098 and 33 (35 for cCP sequences) additional potyviruses confirmed the clustering

of isolate 7098 with ENMV, OOSMV and VDMV and the very close relationship between isolate 7091 and LINV (Fig. 1 and 2). Isolate 7091 and LINV clustered with LMV (Fig. 1), although they unambiguously belong to a distinct species. Isolates 7091 and 7098 did not cluster together or with TuMV and BiMoV, the other lettuce-infecting potyviruses, within the potyvirus phylogenetic tree. Similar results were obtained from the neighbor-joining and maximum-likelihood analyses (data not shown). These results suggest that isolate 7091 is an isolate of LINV that we will refer to as LINV-Fr for clarity and isolate 7098 an isolate of ENMV that we will refer to as ENMV-Fr

Host range

LINV-Fr and ENMV-Fr were used to inoculate mechanically a range of plant species and genotypes (Tables 1 and 2). Given the coat protein sequence similarity between ENMV-Fr and ENMV, the serological reactivity of ENMV-Fr with an ENMV-specific antiserum prepared in 1999 was checked by DAS-ELISA. A strong reactivity was observed, with a >30 times difference in A_{405} between positive and negative samples after two hours of substrate reaction. Moreover, partial sequencing of the NIb and CP coding regions of the virus isolate that was used to prepare the antiserum revealed that it was closely related to ENMV-Fr, with 92% nucleotide identity. Consequently, the infection status of the plants inoculated with ENMV-Fr was checked by DAS-ELISA with this antiserum. Plants inoculated with LINV-Fr were tested by ACP-ELISA with the potyvirus-generic detection kit. This was justified by the lack of evidence of mixture of different potyviruses in LINV-Fr by direct sequencing of RT-PCR products obtained with potyvirus-polyvalent primers and, above all, the absence of virus or viroid sequence contigs other than LINV detected using a highly versatile and sensitive diagnostic method based on NGS of small RNAs.

The occurrence of symptoms correlated with ELISA results, except in *Helianthus annuus* for ENMV-Fr and in *Tragopogon pratensis* for LINV-Fr, which were asymptomatic. In both cases, a low percentage of infected plants ($\leq 20\%$) was observed. ENMV-Fr infected only plants of the Asteraceae family (11/16 species) at the systemic level, including *Centaurea cyanus*, *C. endivia*, *Tragopogon porrifolius*, *Tragopogon pratensis*, *Zinnia elegans*, *L. sativa*, *L. serriola*, *L. saligna* and *L. virosa*. A low percentage ($< 17\%$) of inoculated plants of *Calendula arvensis* and *Helianthus annuus* were infected at the systemic level. In addition, two of four *C. intybus* cultivars (or accessions) showed infections limited to chloro-necrotic local lesions on inoculated leaves.

LINV-Fr also mostly infected plants in the Asteraceae family (11/15 species) at the systemic level. These included *C. arvensis*, *Calendula officinalis*, *C. endivia*, *H. annuus*, *L. saligna*, *L. sativa*, *L. serriola*, *L. virosa*, *L. perennis* and *Z. elegans* and a lower percentage (20%) of *T. pratensis*. *Nicotiana benthamiana*, belonging to the Solanaceae family, was also readily infected. In addition, necrotic local lesions were observed in inoculated leaves of *Chenopodium amaranticolor* and *Chenopodium quinoa* (Chenopodiaceae family).

In the *Cichorium* genus, different reactions were observed. The different types of curly endive (*C. endivia* var. *crispum*) or escarole/endive (*C. endivia* var. *latifolium*) were susceptible to LINV-Fr and ENMV-Fr (Table 1). In chicory, radicchio or witloof (*C. intybus*), all plants were resistant to both isolates. However, necrotic local lesions typical of a hypersensitive reaction were observed for ENMV-Fr only in two of the four tested cultivars/accessions: a wild accession collected in Montfavet, France, and the cultivar Rossa di Verona. This suggests the occurrence of different resistance mechanisms in these plant genotypes against these two viruses.

Most host species in the Asteraceae were common to both viruses, except *C. cyanus* and *T. porrifolius*, which are hosts for ENMV-Fr but not for LINV-Fr and *C. officinalis*, which is a

host for LINV-Fr but not for ENMV-Fr. *L. perennis* was also infected by LINV-Fr only, though only one plant from one accession was infected (Table 1).

Symptoms observed at the systemic level were rather mild for both viruses, with mostly chlorotic spots on leaves, except on *C. endivia* infected by LINV-Fr and some *Lactuca* spp. cultivars or accessions that displayed severe mosaics and/or necrosis and wilt for both isolates (Suppl. Fig. 1).

Identification of resistant genotypes in *Lactuca* spp.

All *L. sativa* cultivars displaying resistance to TuMV (*i.e.* Avoncrisp, Girelle, Mariska, Kinemontepas and Mantilia; data not shown), and therefore probably carrying the *Tu* resistance gene, were also resistant to ENMV-Fr (Table 2; Suppl. Fig. 1). In contrast, cultivars Calmar, Montemar and Salinas 88, which do not carry the *Tu* resistance gene, were susceptible to ENMV-Fr (Suppl. Fig. 1). The correlation between susceptibility to ENMV and TuMV in iceberg lettuce cultivars was previously mentioned (Blancard *et al.*, 2006). Cultivar Salinas 88, which does not carry the *Tu* resistance gene but is homozygous for the *mol*² gene, was susceptible to ENMV-Fr (Table 2). This shows that the *mol*² allele does not protect lettuce against infection by this virus. The question remains open if the *mol*¹ allele could confer a resistance to ENMV-Fr. All *L. sativa* cultivars tested, either resistant or susceptible to TuMV, were susceptible to LINV-Fr (Table 2; Suppl. Fig. 1). Consequently, the *Tu* gene does not confer any resistance to this isolate. In addition, the nearly-isogenic lines of Mariska and Girelle with the *mol*¹ or *mol*² resistance alleles, as well as Salinas 88 carrying *mol*², were susceptible to LINV-Fr, showing that *mol*¹ and *mol*² do not confer resistance to LINV-Fr.

Overall, five of the nine wild accessions of *Lactuca* spp. were resistant to ENMV-Fr. They belonged to the four tested wild species of *Lactuca*: *L. serriola*, *L. saligna*, *L. virosa* and *L. perennis*. Three of these resistant accessions were also resistant to LMV-E, whereas *L. perennis* ‘LS293’, showed a heterogeneous response to both ENMV-Fr and LMV-E (Lot and Maisonneuve, data not shown). The fifth accession, *L. perennis* ‘LS297’, has not been tested for LMV resistance. In contrast, only two of these nine wild accessions were resistant to LINV-Fr: *L. virosa* ‘PIVT1398’, which is resistant to LMV-13 (Maisonneuve *et al* 1999), and *L. perennis* ‘LS297’.

Aphid transmission of LINV-Fr and ENMV-Fr

Under controlled conditions, LINV-Fr and ENMV-Fr were efficiently transmitted by *M. persicae* from lettuce to lettuce. Indeed, using five aphids per plant for transmission, 30 of 30 (100%) and 26 of 30 (87%) plants inoculated by LINV-Fr and ENMV-Fr, respectively, were infected.

Occurrence of EMDV and LINV in wild plants or weeds

Among the 5284 individual wild plants or weeds collected in the surroundings of Avignon in 2009-2011, ENMV-Fr was frequently detected in wild salsify (*T. pratensis*) (40.0% infected plants) and exceptionally in *Scorzonera laciniata*, *Sonchus asper*, *L. serriola* (all belonging to Asteraceae family) and *Diplotaxis erucoides* (Brassicaceae family) (Supplementary Table 1). In contrast, LINV was not detected in the sampled plants.

Discussion

Molecular studies showed that isolates 7091 and 7098, collected on symptomatic lettuce plants in south-eastern France in 2012, belong to two different potyvirus species. Isolate 7091 shared 98% nt sequence identity over the whole genome with a potyvirus isolate collected in Italy in July 2014 in *L. sativa* cv. Romana (Ciuffo *et al.*, 2016) and described as a member of the tentative new species LINV. The Italian isolate induces necrosis and leaf distortion on lettuce similar to those observed for isolate 7091. Therefore, isolate 7091 is unambiguously an isolate of the same species that we named consequently LINV-Fr. The low molecular variability between the French and Italian isolates suggests a short time of divergence and thus a recent introduction in at least one of these two countries.

Isolate 7098 shared less than 70% nt identity in the whole genome sequence with potyvirus species available in GenBank, but it shares up to 79% nt (82% aa) identity in the CP-coding region with ENMV. The identity threshold for species demarcation in the *Potyvirus* genus is 76% at the nt level and 80% at the aa level in the CP or the whole genome (Adams *et al.*, 2005), so isolate 7098 could be considered as an isolate of ENMV. However, CP identity between isolate 7098 and OOSMV (75% at the nt level, 80% at the aa level), as well as between ENMV and OOSMV (76% nt, 84% aa) are very close to the species demarcation threshold. In situations where strain demarcation between viruses in a cluster is not clear-cut based on CP sequence, the use of whole sequences or comparison of the CI-coding region may help distinguishing closely related species (Romay *et al.*, 2014). Since only partial sequences of ENMV and OOSMV are available, the taxonomic position of these viruses remains unclear. However, isolate 7098, ENMV, OOSMV and, to a lower extent, VDMV obviously belong to a cluster of related viruses. Considering that isolate 7098 belongs to a divergent strain of ENMV, we suggest to name it ENMV-Fr. Its status may change when the complete sequence of the original ENMV isolate will be available.

A striking result of the molecular characterization of ENMV-Fr and LINV and the comparison with other lettuce-infecting potyviruses (LMV, TuMV and BiMoV; Fig. 1) is the fact that lettuce potyviruses belong to a quite large number of independent clades. Since LINV clusters with LMV, lettuce potyviruses are present in four different clades (clades 1 to 4 in Fig. 1). This suggests that the jump to *Lactuca* spp. occurred multiple times during potyvirus evolution.

ENMV was first observed in escarole and curly endive plants (*C. endivia* var. *latifolium* and *C. endivia* var. *crispum*) in Frankfurt (Germany) in 1995 (GenBank accession number AJ223827) and had probably been present in this area for at least 10 years (Blancard *et al.*, 2006; Vetten, H., Lesemann, D.E. and Schoenfelder, M., unpublished). It was then observed in iceberg lettuce cultivars in several regions of Germany and in *L. sativa* cv. Commander in South Africa in 2011 (accession numbers JQ437576 and JQ437577; Jooste, A.E.C., Kasdorf, G.G.F. and Van der Merwe, M., unpublished). Isolates from these two countries are molecularly much more related to each other than to ENMV-Fr, indicating that the presence of ENMV in France is not the result of a recent exchange between France and one of these countries, which contrasts with the situation of French and Italian isolates of LINV. In accordance with this hypothesis, sequencing of a 413-nucleotide-long genome part at the junction of the NIb and CP coding regions of the ENMV isolate collected in France in 1999 and used to produce the antiserum showed that this isolate was closer to ENMV-Fr (92% nucleotide identity) than to the German isolate (73% nucleotide identity) (data not shown).

How long ENMV-Fr has been present in south-eastern France is not known. It was first detected by ELISA in butterhead lettuce in 1999 in Provence (Blancard *et al.*, 2006). Its frequent occurrence in wild salsify and occasional detection in other weed species suggest that this virus is well established in the agricultural environment. Therefore, the presence of ENMV-Fr in lettuce crops probably results from a “spillover” from wild reservoirs rather than from the

cultivated compartment, considering that most lettuce cultivars grown in France have the *Tu* gene and are thus probably resistant to ENMV-Fr.

The situation is different for LINV, since it has only been found in cultivated lettuce. No natural wild plant or weed has been found to host this virus in the same surveys, which is consistent with a recent introduction. On the other hand, its efficient aphid transmission and ability to infect most lettuce cultivars provide an opportunity to move from a lettuce crop to another, since lettuces are grown all year round. Results from host range experiments (Tables 1 and 2) also give some clues to identify putative LINV alternative hosts (especially *L. serriola*, sunflower and other ornamental or vegetable species in the Asteraceae family). The presence of closely related isolates in France and Italy is also compatible with putative seed transmission.

The experimental host range of the two viruses seems mostly, but not exclusively for LINV-Fr, restricted to species of the Asteraceae family, including approximately two thirds of the tested species in that family.

Possible sources of resistance to ENMV and/or LINV have been identified in the *Cichorium* and *Lactuca* genera and may be used for breeding purposes (Tables 1 and 2). None of the *C. endivia* cultivars was resistant, but the four *C. intybus* cultivars were resistant to both viruses. Two different kinds of reactions to ENMV-Fr were observed among the four *C. intybus* genotypes, with or without necrotic local lesions reminiscent of a hypersensitive reaction, suggesting differences in the resistance mechanisms and/or genes. Similar results were previously observed for resistance to TuMV and/or BiMoV. All *C. endivia* tested (23/23) were susceptible to TuMV, while most *C. intybus* (35/36) were resistant to both TuMV and BiMoV (Provvidenti *et al*, 1979; Zitter & Guzman 1977).

Interestingly, in *Lactuca* spp., most resistant genotypes showed resistance to several viral species (Table 2). Most of the time, in the wild *Lactuca* species where resistance to a given

potyvirus was identified, there were also other accessions susceptible to that potyvirus. This indicates that the resistances are not non-host resistances distributed at the plant species level, but are specific to particular accessions within these species.

This situation was also observed in lettuce cultivars. All *L. sativa* accessions that were resistant to TuMV, probably because of the presence of the *Tu* gene, were also resistant to ENMV-Fr, while all the accessions devoid of *Tu* were susceptible to ENMV-Fr. In *L. serriola*, *L. saligna*, *L. virosa* and *L. perennis*, accessions that were resistant to LMV-E (at least for some of the plants) were also resistant to ENMV-Fr. In the case of *L. perennis* 'LS293', plants were either resistant or susceptible to LMV-E and to LINV-Fr, suggesting that this accession is not fixed for the genetic factors involved in the resistance. *L. virosa* accession 'PIVT1398', which carries the *Mo3* gene conferring resistance to all tested LMV isolates was also resistant to ENMV-Fr and LINV. Finally, *L. perennis* accession 'LS297' was also resistant to both ENMV-Fr and LINV. An important prospect of this study would therefore be to study the inheritance of these characters and to evaluate if the same gene(s) is (are) involved in resistance to these different potyviruses.

In conclusion, we have described two potyviruses that have been overlooked in lettuce production. Their rare occurrence and/or low prevalence in lettuce crops seem to be due to different reasons. ENMV-Fr is frequent in wild reservoirs including biennial or perennial species like *T. pratensis* but is controlled by the *Tu* resistance gene that is present in almost all European lettuce cultivars. In contrast, no resistance against LINV has been evidenced in *L. sativa* cultivars and its low prevalence could be due to a recent emergence in France, also suggesting a still limited distribution.

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Table 1 Infections and symptoms induced by LINV-Fr and ENMV-Fr ^a

Species	Accession or cultivar	Inoculation with LINV-Fr	Inoculation with ENMV-Fr
Asteraceae			
<i>Calendula arvensis</i>		mos (10/10)	mos (1/18) ^b
<i>Calendula officinalis</i>		Chlorotic and necrotic ringspots (10/10)	negative (10/10)
<i>Centaurea cyanus</i>		negative (10/10)	mos (10/10)
<i>Cichorium endivia</i> var. crispum	Grosse Pommant Seule	mos (10/10)	mos (10/10)
<i>Cichorium endivia</i> var. latifolium	Géante maraîchère	mos + Nec (10/10)	cRS (10/10)
<i>Cichorium intybus</i>	wild accession; Montfavet 2013 and 2015	negative (7/7)	Chloro-necrotic local lesions; negative (10/10)
<i>Cichorium intybus</i>	Barba di Cappuccino	negative (10/10)	negative (10/10)
<i>Cichorium intybus</i>	Rossa di Verona	negative (10/10)	Chloro-necrotic local lesions; negative (10/10)
<i>Cichorium intybus</i>	Chicorée de Bruxelles Zoom F ₁	negative (10/10)	negative (10/10)
<i>Cynara cardunculus</i> subsp. scolymus		Not tested	negative (10/10)
<i>Helianthus annuus</i>	Tournesols variés	cRS (8/10)	AS (3/18 ELISA positive)
<i>Scorzonera hispanica</i>	Géante noire de Russie	negative (10/10)	negative (10/10)
<i>Tragopogon porrifolius</i>	Mammouth	negative (10/10)	mos (10/10)
<i>Tragopogon pratensis</i>	wild accession; Montfavet, 2013	AS (2/10 ELISA positive)	mos (9/10)
<i>Zinnia elegans</i>		Mild mos (10/10)	mos (10/10)
Chenopodiaceae			
<i>Chenopodium amaranticolor</i>		Nll; negative (10/10)	negative (9/9)
<i>Chenopodium quinoa</i>		Nll; negative (10/10)	negative (9/9)
Cucurbitaceae			
<i>Cucumis melo</i>	Védrantais	negative (10/10)	negative (9/9)
<i>Cucurbita pepo</i>	Diamant F ₁	negative (10/10)	negative (9/9)
Fabaceae			
<i>Phaseolus vulgaris</i>	BT2	negative (10/10)	negative (10/10)
<i>Vigna unguiculata</i>		negative (10/10)	negative (9/9)
Solanaceae			
<i>Nicotiana benthamiana</i>		cRS (8/10)	negative (9/9)
<i>Nicotiana tabacum</i>	Xanthi NN	negative (10/10)	negative (9/9)
<i>Solanum lycopersicum</i>	Monalbo	negative (10/10)	negative (9/9)

^amos: mosaic; cRS: chlorotic ringspots; Nec: necrosis; AS: asymptomatic; Nll: necrotic local lesions. Symptoms were associated with positive ELISA on apical leaves. Negative: no

symptoms and negative ELISA on apical leaves. For ENMV-Fr and LINV-Fr, ACP-ELISA was performed with the Potyvirus-Group antiserum or DAS-ELISA was performed with an ENMV-specific polyclonal antiserum, respectively.

^bThe plant showing mosaic symptoms was used to back-inoculate nine healthy *C. arvensis* plants. Of these, eight plants were infected at the systemic level showing mosaic symptoms.

Table 2 Infections and symptoms induced on cultivars or accessions of *Lactuca* spp. by LINV-Fr and ENMV-Fr) and comparison with resistance to other potyviruses^a

Accession or cultivar	Type or origin	Resistance to LMV ^b	Resistance to TuMV ^c	Inoculation with LINV-Fr	Inoculation with ENMV-Fr
<i>L. sativa</i>					
Calmar	American iceberg	Susceptible	Susceptible	mild VC	mos+Nec+stunting
Montemar	American iceberg	Susceptible	Susceptible	mild VC	mos+Nec+stunting
Avoncrisp	English crisphead	No data	Resistant (Tu ^d)	mos+Nec+severe wilt	negative
Girelle	French butterhead	Susceptible	Resistant	severe wilt	negative
Girelle <i>moI</i> ²	Nearly-isogenic line of Girelle	Resistant (<i>moI</i> ²)	Resistant	severe wilt	negative
Mariska	Dutch butterhead	Susceptible	Resistant	VC	negative
Mariska <i>moI</i> ¹	Nearly-isogenic line of Mariska	Resistant (<i>moI</i> ¹)	Resistant	VC	negative
Mariska <i>moI</i> ²	Nearly-isogenic line of Mariska	Resistant (<i>moI</i> ²)	Resistant	VC	negative
Kinemontepas	French butterhead	Susceptible	Resistant	VC+Nec	negative
Mantilia	French butterhead	Resistant (<i>moI</i> ¹)	Resistant	severe wilt	negative
Salinas 88	American iceberg	Resistant (<i>moI</i> ²)	Susceptible	VC	mos
<i>L. serriola</i>					
LS436	Velorgues, France; 2006		No data	vein necrosis (8/8)	mos+Nec
LS190	Voisin le Bretonneux, France; 1992	Susceptible to LMV-0 and LMV-13; resistant to LMV-E	No data	mos+wilt, dead (9/9)	negative (9/9)
LS300	Montpellier, France; 1985	Susceptible to LMV-E	No data	mos+crinckling (9/9)	mos+Nec+stunting (9/9)
<i>L. saligna</i>					
CR11	UK, before 1980	Resistant to LMV-E	No data	mos+Nec (9/9)	negative
LS326	Spain; before 1987	Susceptible to LMV-E	No data	mos (9/9)	mos+Nec+stunting
<i>L. virosa</i>					
PIVT1398	Unknown	Resistant to LMV-0, LMV-	No data	negative (9/9)	negative

		E and LMV-13 (<i>Mo3</i>)			
LS238	Sézanne, France; 1982	Susceptible to LMV-E	No data	VC+ mos (9/9)	mos +Nec
<hr/>					
<i>L. perennis</i>					
LS293	Cervièrès, France; 1985	Heterogeneous for resistance to LMV-E	No data	mild mos (1/3)	negative (3/3)
LS297	Nancy, France	No data	No data	negative (2/2)	negative (2/2)
<hr/>					

^aVC: vein clearing; mos: mosaic; Nec: necrosis. Symptoms were associated with positive ELISA on apical leaves. Negative: no symptoms and negative ELISA on apical leaves. See Table 1 for ELISA description. For each plant accession or cultivar and for each virus isolate, at least ten plants were inoculated, except when specified.

^bafter Ryder (1973), Wehner & Barrett (1996), Candresse *et al.* (2002), Pelet (1964), Dinant & Lot (1992), Mazier *et al.* (1999), Maisonneuve *et al.* (1999) and Maisonneuve B, Bellec Y, Souche S, Lot H, data not shown. LMV strains are described in Dinant & Lot (1992).

^cMaisonneuve B, Verdin E, Gognalons P, Moury B, data not shown.

^dProgenitor of the *Tu* resistance gene in the genetic analysis of Montesclaros *et al.* (1997).

Figure 1 Neighbor-joining tree based on the complete amino-acid sequences of isolates 7091 and 7098 and 34 potyvirus species. Bootstrap support (n=500) above 70% is indicated for each node. The scale bar represents the number of substitutions per nucleotide. BiMoV: *Bidens mottle virus* (EU250212, EU250214, AF538686); BiMV: *Bidens mosaic virus* (KF649336); CIYVV: *Clover yellow vein virus* (AB011819); BCMNV: *Bean common mosaic necrosis virus* (U19287); BCMV: *Bean common mosaic virus* (AJ312437-437, U05771); BYMV: *Bean yellow mosaic virus* (U47033, D83749); CABMV: *Cowpea aphid-borne mosaic virus* (AF348210); DsMV: *Dasheen mosaic virus* (AJ298033); JGMV: *Johnsongrass mosaic virus* (Z26920); JYMV: *Japanese yam mosaic virus* (AB016500, AB027007); LINV: Lettuce Italian necrosis virus (KP769852); LMoV: *Lily mottle virus* (NC_005288); LMV: *Lettuce mosaic virus* (X97704-705, AJ278854); LYSV: *Leek yellow stripe virus* (AJ307057); MDMV: *Maize dwarf mosaic virus* (AJ001691); MWMV: *Moroccan watermelon mosaic virus* (EF579955); PeMoV: *Peanut mottle virus* (AF023848); PepMoV: *Pepper mottle virus* (M96425); PRSV: *Papaya ringspot virus* (X97251, S46722, AY010722); PSbMV: *Pea seed-borne mosaic virus* (D10930, AJ252242); PVA: *Potato virus A* (AJ131400-403, AJ296311, Z21670); PVV: *Potato virus V* (AJ243776); PVY: *Potato virus Y* (X12456, D00441, A08776, U09509, M95491, X97895); SMV: *Soybean mosaic virus* (D00507, S42280, AJ312439, AB100443, AF241739); SPFMV: *Sweet potato feathery mottle virus* (D86371); SuCMoV: *Sunflower chlorotic mottle virus* (GU181199); TEV: *Tobacco etch virus* (M15239, L38714); TuMV: *Turnip mosaic virus* (D83184, AF394601-602, AF169561, D10927); VDMV: *Vanilla distortion mosaic virus* (KF906523); WMV: *Watermelon mosaic virus* (AY437609); YMV: *Yam mosaic virus* (U42596); ZYMV: *Zucchini yellow mosaic virus* (L31350, L29569, AF014811, AF127929). Lettuce-infecting potyvirus species are underlined on the tree and the clades to which they belong are indicated.

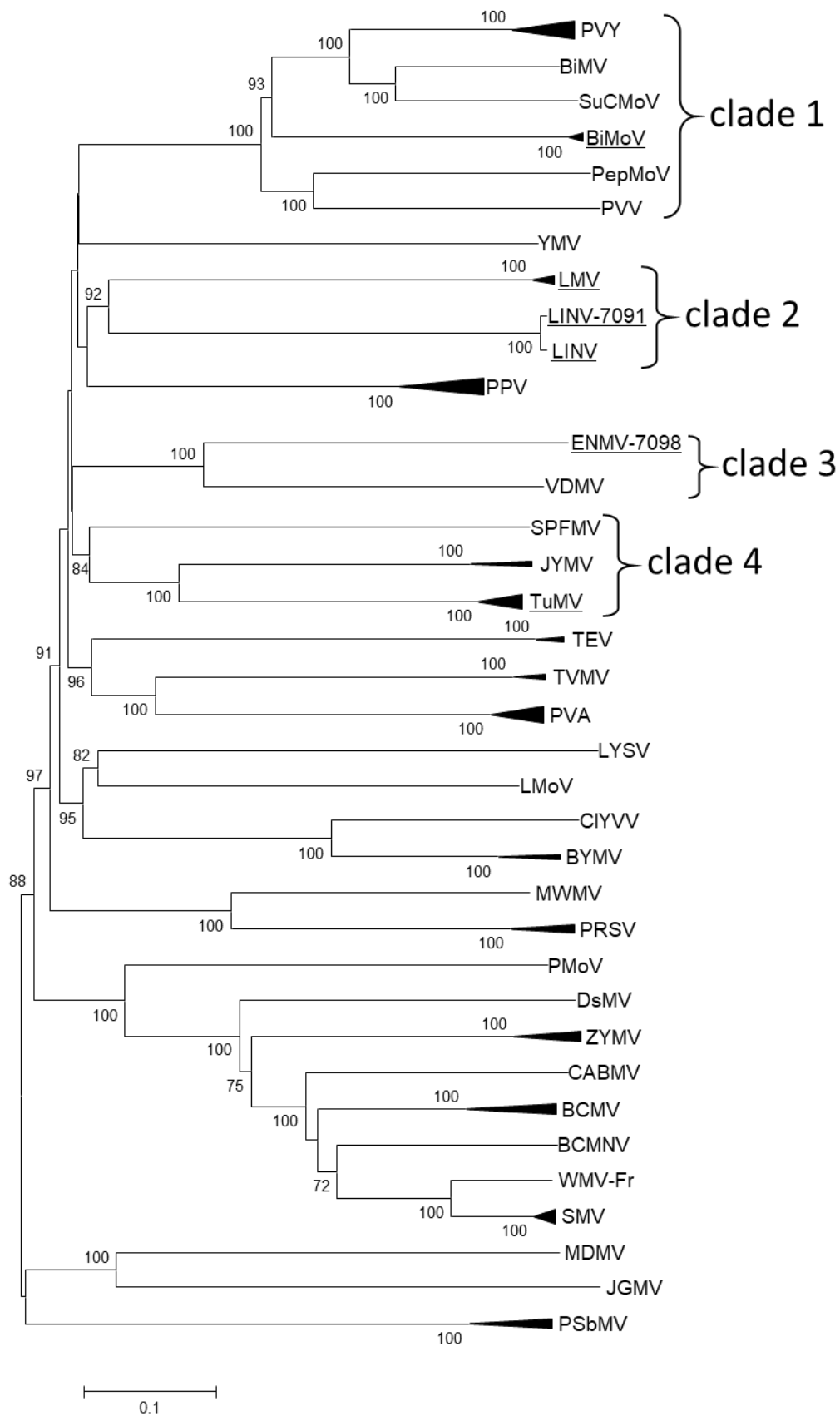
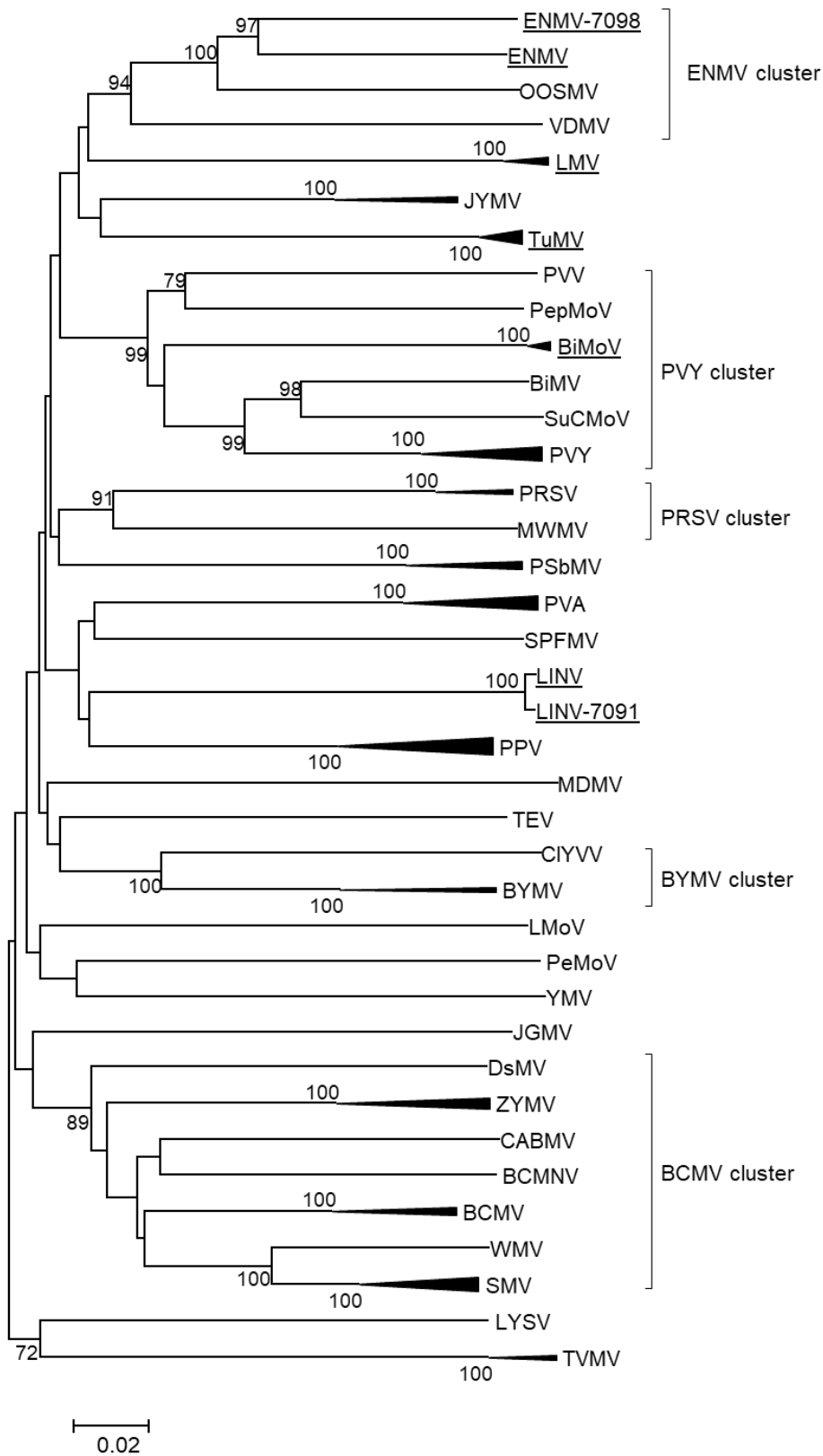


Figure 2 Neighbor-joining tree based on core coat protein coding sequences of isolates 7091 and 7098 and 35 potyviruses. The best-fit general time reversible nucleotide substitution model with gamma-distributed substitution rates was used on a 705-nucleotide long sequence alignment. Bootstrap supports (n=500) above 70% are indicated for each node. ENMV: *Endive necrotic mosaic virus* (accession AJ223827); OOSMV: ornamental onion stripe mosaic virus (accession EU042750). Other virus names are as in Figure 1. Lettuce-infecting potyvirus species are underlined on the tree. The scale bar represents the number of substitutions per nucleotide.



Supplementary Figure 1 Symptoms induced by isolates 7091 or 7098 on mechanically-inoculated *Lactuca sativa* plants. Plants of cultivars Montemar (a, b, d, e), devoid of the resistance gene *Tu*, or Avoncrisp (c, f), carrying the gene *Tu*, were inoculated with isolates 7091 (a, b, c) or 7098 (d, e, f). a. vein clearing. b. close-up of a. c. necrosis and wilt. d. vein clearing and necrosis. e: close-up of d. f. non infected plant. Pictures were taken one month post-inoculation.

