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Molecular and biological characterization of two potyviruses infecting lettuce in southeastern France

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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. Here, the molecular and biological properties are described of two potyviruses that were recently isolated from lettuce plants showing mosaic or strong necrotic symptoms in an experimental field in southeastern France. The first potyvirus belongs to the species *Endive necrotic mosaic virus* and is present in a large number of wild plant species, especially *Tragopogon pratensis*. It 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 was not observed in wild plants. It 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: aphid transmission, Asteraceae, host range, *Lactuca* spp., *Potyvirus*, resistance

Introduction

Lettuce (*Lactuca sativa*) is an important vegetable crop in many countries worldwide, with a global production of 25 million tonnes. 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 (*c.* 50%) of the production is localized in the southeastern regions, 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 leading eventually to death of the plant (Zink *et al.*, 1973; Moreno & Fereres, 2012). Like other potyviruses, LMV is transmitted in a nonpersistent 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, showing no symptoms, although sometimes they show 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 the oomycete *Bremia lactucae*. The two traits have been introduced simultaneously into *L. sativa* cultivars during breeding programmes aiming to improve lettuce resistance to *B. lactucae* in the USA (Zink & Duffus, 1969,

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1970, 1973; Robbins *et al.*, 1994), 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 in both veins and interveinal areas. A recessive resistance gene with a narrow spectrum of action was identified in *L. sativa* 'Valmaine' and many *Cichorium intybus* accessions were also found to be resistant (Zitter & Guzman, 1977).

In this article, the molecular and biological properties are described of two other potyviruses that were isolated from lettuce plants showing either mosaic or strong necrotic symptoms in experimental fields in southeastern France. In addition, an investigation was made of 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, 7091 and 7098, were collected in November 2012 in an open-field experimental plot in INRA Montfavet (near Avignon, southeastern France, 43°56′54″N, 04°48′32″E) from two different plants of *L. sativa* 'Montemar' showing mosaic and necrosis symptoms typical of virus infections. Montemar is an iceberg lettuce cultivar selected from cultivar 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 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 Trireagent (Molecular Research Center) according to the manufacturer's recommendations. A region of *c.* 1600 nucleotides (nt) at the 3′ end of the virus genome was amplified with primers derived from Gibbs & 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′ ends of the primer sequences 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′-ATCAATGAGTACGTCACTGC-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 nt) 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 v. 7, with different word lengths (13–21 nt). For isolate 7091, a complete sequence of a closely related virus isolate (lettuce Italian necrotic virus; 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). 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 MEGA v. 6.0 (Tamura *et al.*, 2013). Details of the full-length or partial sequences included in the analysis are given in Figure 1. The best nucleotide substitution model was selected with MEGA v. 6.0. Neighbour-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 reverse transcription (RT)-PCR and partial sequencing of a virus isolate kept since 1999 in dehydrated leaf material, the two primers ENMV-Nib-fw (5′-GAGGAYYTRTGGTTCATGTC-3′) and ENMV-CP-rv (5′-C
GGAGCGTCACACAGYGAACGA-3′) were designed based on the sequence of isolate 7098 and closely related isolates collected from wild plants (Table S1).

Host range study

Different plant species and genotypes, mainly of the family Asteraceae (Tables 1 & 2), were mechanically inoculated at the seedling stage with 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 the infection status of the plants 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). Because isolate 7098 was found to be a member of the species *Endive necrotic mosaic virus* (ENMV; genus *Potyvirus*) 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 more than three times that of the healthy controls.

Aphid transmission

Aphids (*M. persicae*) were reared on virus-free pepper (*Capsicum annuum* 'Yolo Wonder') plants in growth cabinets with 16 h daylight, 21 °C night and day. Four weeks before experiments, aphid maintenance was shifted to synchronous rearing in order to manipulate 7- to 8-day-old apterous female cohorts. Virus-free

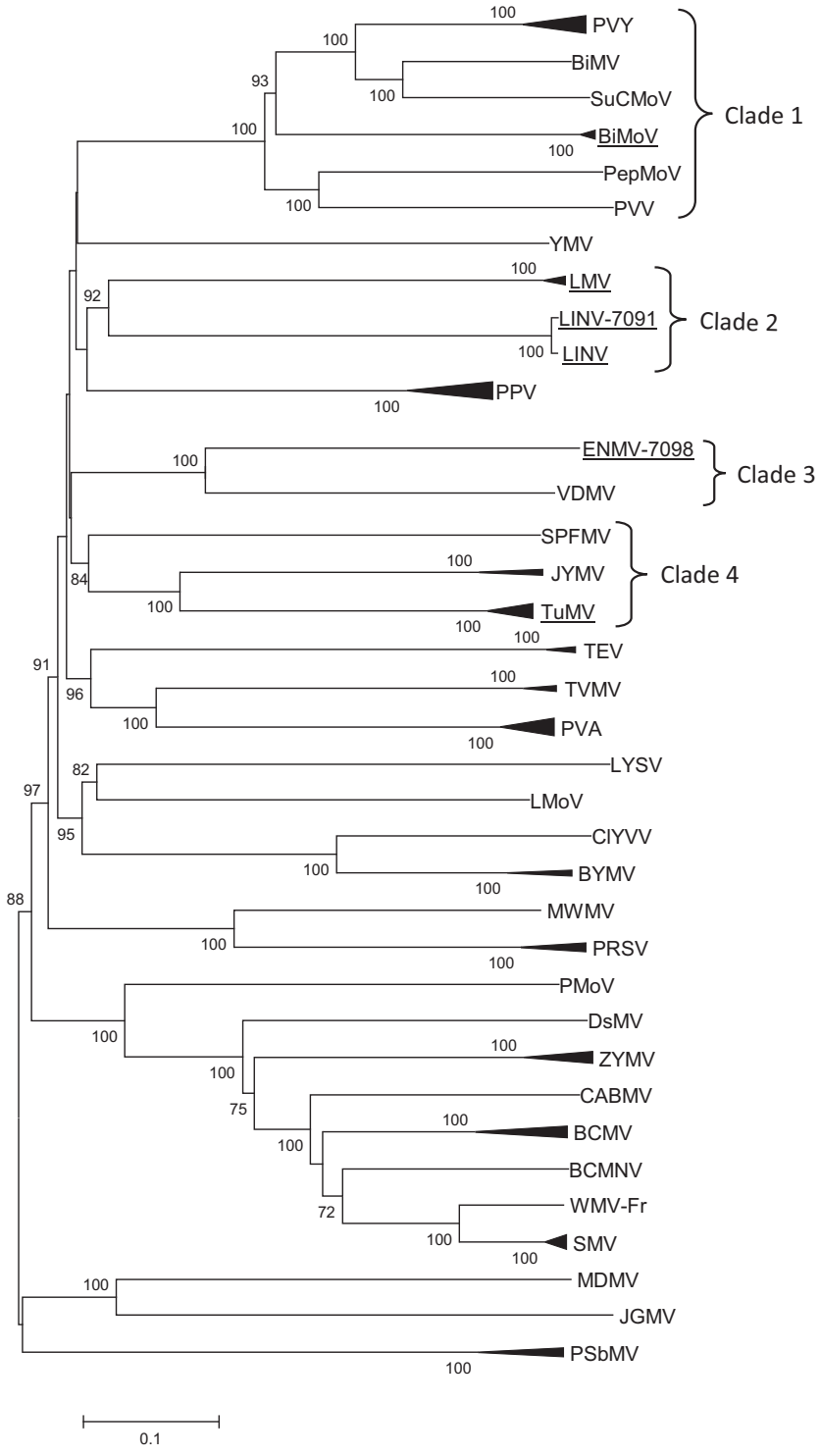

Figure 1 Neighbour-joining tree based on the complete amino acid sequences of virus isolates 7091 and 7098 from *Lactuca sativa* and 34 potyvirus species. Bootstrap support (n = 500) above 70% is indicated for each node. The scale bar represents the number of substitutions per amino acid. Lettuce-infecting potyvirus species are underlined on the tree and the clades to which they belong are indicated. BCMNV: *Bean common mosaic necrosis virus* (U19287); BCMV: *Bean common mosaic virus* (AJ312437, U05771); BiMoV: *Bidens mottle virus* (EU250212, EU250214, AF538686); BiMV: *Bidens mosaic virus* (KF649336); BYMV: *Bean yellow mosaic virus* (U47033, D83749); CABMV: *Cowpea aphid-borne mosaic virus* (AF348210); ClYVV: *Clover yellow vein virus* (AB011819); DsMV: *Dasheen mosaic virus* (AJ298033); ENMV: *Endive necrotic mosaic virus*; JGMV: *Johnsongrass mosaic virus* (Z26920); JYMV: *Japanese yam mosaic virus* (AB016500, AB027007); LINV: lettuce Italian necrotic virus (KP769852); LMoV: lily mottle virus (NC_005288); LMV: *Lettuce mosaic virus* (X97704, X97705, 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, AF394602, 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).

aphids were starved for 1 h before virus acquisition. To evaluate aphid transmission of isolates 7091 and 7098, plants of *L. sativa* '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 imidacloprid sprayings (0.1 g L^{-1}; NUPRID 200, Nufarm SAS). Test plants were checked for infection by ELISA 3 weeks after inoculation.

Table 1 Infections and symptoms induced by isolates of lettuce Italian necrotic virus (LINV)-Fr and *Endive necrotic mosaic virus* (ENMV)-Fr

Species	Accession or cultivar	Inoculation with LINV-Fr[a]	Inoculation with ENMV-Fr[a]
Asteraceae			
Calendula arvensis		mos (10/10)	mos (1/18)[b]
Calendula officinalis		Chlorotic and necrotic ringspots (10/10)	Negative (10/10)
Centaurea cyanus		Negative (10/10)	mos (10/10)
Cichorium endivia var. *crispum*	Grosse Pommant Seule	mos (10/10)	mos (10/10)
C. endivia var. *latifolium*	Géante maraîchère	mos+Nec (10/10)	cRS (10/10)
Cichorium intybus	Wild accession; Montfavet 2013 and 2015	Negative (7/7)	Chloro-necrotic local lesions; negative (10/10)
	Barba di Cappuccino	Negative (10/10)	Negative (10/10)
	Rossa di Verona	Negative (10/10)	Chloro-necrotic local lesions; negative (10/10)
	Chicorée de Bruxelles Zoom F_1	Negative (10/10)	Negative (10/10)
Cynara cardunculus subsp. *scolymus*		Not tested	Negative (10/10)
Helianthus annuus	Tournesols variés	cRS (8/10)	SL (3/18 ELISA positive)
Scorzonera hispanica	Géante noire de Russie	Negative (10/10)	Negative (10/10)
Tragopogon porrifolius	Mammouth	Negative (10/10)	mos (10/10)
Tragopogon pratensis	Wild accession; Montfavet, 2013	SL (2/10 ELISA positive)	mos (9/10)
Zinnia elegans		Mild mos (10/10)	mos (10/10)
Chenopodiaceae			
Chenopodium amaranticolor		NII; negative (10/10)	Negative (9/9)
Chenopodium quinoa		NII; negative (10/10)	Negative (9/9)
Cucurbitaceae			
Cucumis melo	Védrantais	Negative (10/10)	Negative (9/9)
Cucurbita pepo	Diamant F_1	Negative (10/10)	Negative (9/9)
Fabaceae			
Phaseolus vulgaris	BT2	Negative (10/10)	Negative (10/10)
Vigna unguiculata		Negative (10/10)	Negative (9/9)
Solanaceae			
Nicotiana benthamiana		cRS (8/10)	Negative (9/9)
Nicotiana tabacum	Xanthi NN	Negative (10/10)	Negative (9/9)
Solanum lycopersicum	Monalbo	Negative (10/10)	Negative (9/9)

[a]mos: mosaic; cRS: chlorotic ringspots; Nec: necrosis; SL: symptomless; NII: necrotic local lesions. Symptoms were associated with positive enzyme-linked immunosorbent assay (ELISA) on apical leaves. Negative: no symptoms and negative ELISA on apical leaves. For LINV-Fr and ENMV-Fr, antigen-coated plate (ACP)-ELISA was performed with the potyvirus-group antiserum or double-antibody sandwich (DAS)-ELISA was performed with an ENMV-specific polyclonal antiserum, respectively.

[b]The 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.

Search for virus reservoirs in wild plants

A total of 5284 weed or wild plants, belonging to 40 different botanical families, were sampled in the region of Avignon from 2009 to 2013 and tested for the presence of ENMV and LINV. First, each plant was 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 & 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 of lettuce plants in a greenhouse, isolate 7098 induced mosaic symptoms, whereas isolate 7091 induced mosaic and necrosis symptoms (Fig. S1). Observation by electron microscopy of crude sap preparations of the plants inoculated either with isolate 7091 or 7098 revealed numerous flexuous particles, *c*. 700–730 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 *c*. 1600 nt 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

Table 2 Infections and symptoms induced on cultivars or accessions of *Lactuca* spp. by isolates of lettuce Italian necrotic virus (LINV)-Fr and *Endive necrotic mosaic virus* (ENMV)-Fr and comparison with resistance to other potyviruses

Accession or cultivar	Type or origin	Resistance to LMV[b]	Resistance to TuMV[c]	Inoculation with LINV-Fr[a]	Inoculation with ENMV-Fr[a]
Lactuca perennis					
LS293	Cervières, France, 1985	Heterogeneous for resistance to LMV-E	No data	Mild mos (1/3); negative (2/3)	Negative (3/3)
LS297	Nancy, France	No data	No data	Negative (2/2)	Negative (2/2)
L. saligna					
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
L. sativa					
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 *mo1*^{2}	Nearly isogenic line of Girelle	Resistant (*mo1*^{2})	Resistant	Severe wilt	Negative
Mariska	Dutch butterhead	Susceptible	Resistant	VC	Negative
Mariska *mo1*^{1}	Nearly isogenic line of Mariska	Resistant (*mo1*^{1})	Resistant	VC	Negative
Mariska *mo1*^{2}	Nearly isogenic line of Mariska	Resistant (*mo1*^{2})	Resistant	VC	Negative
Kinemontepas	French butterhead	Susceptible	Resistant	VC+Nec	Negative
Mantilia	French butterhead	Resistant (*mo1*^{1})	Resistant	Severe wilt	Negative
Salinas 88	American iceberg	Resistant (*mo1*^{2})	Susceptible	VC	mos
L. serriola					
LS436	Velorgues, France, 2006	No data	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+crinkling (9/9)	mos+Nec+stunting (9/9)
L. virosa					
PIVT1398	Unknown	Resistant to LMV-0, LMV-E and LMV-13 (*Mo3*)	No data	Negative (9/9)	Negative
LS238	Sézanne, France, 1982	Susceptible to LMV-E	No data	VC+mos (9/9)	mos+Nec

[a]VC: vein clearing; mos: mosaic; Nec: necrosis. Symptoms were associated with positive enzyme-linked immunosorbent assay (ELISA) on apical leaves. Negative: no symptoms and negative ELISA on apical leaves. For LINV-Fr and ENMV-Fr, antigen-coated plate (ACP)-ELISA was performed with the potyvirus-group antiserum or double-antibody sandwich (DAS)-ELISA was performed with an ENMV-specific polyclonal antiserum, respectively. For each plant accession or cultivar and for each virus isolate, at least 10 plants were inoculated, except when specified.

[b]After Ryder (1970), Wehner & Barrett (1996), Candresse *et al.* (2002), Pelet (1964), Dinant & Lot (1992), Mazier *et al.* (1999), Maisonneuve *et al.* (1999) and B. Maisonneuve, Y. Bellec, S. Souche, H. Lot, data not shown. *Lettuce mosaic virus* (LMV) strains are described in Dinant & Lot (1992).

[c]B. Maisonneuve, E. Verdin, P. Gognalons, B. Moury, data not shown.

[d]Progenitor of the *Tu* resistance gene in the genetic analysis of Montesclaros *et al.* (1997).

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 sequences of isolates 7091 and 7098 were 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 of 7091 and 7098 were 9829 and 9993 nucleotides 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 nine 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 (Figs 1 & 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 neighbour-joining and maximum-likelihood analyses (data not shown). These results suggest that isolate 7091 is an isolate of LINV that will be referred to here as LINV-Fr for clarity, and isolate 7098 is an isolate of ENMV that will be referred to as ENMV-Fr.

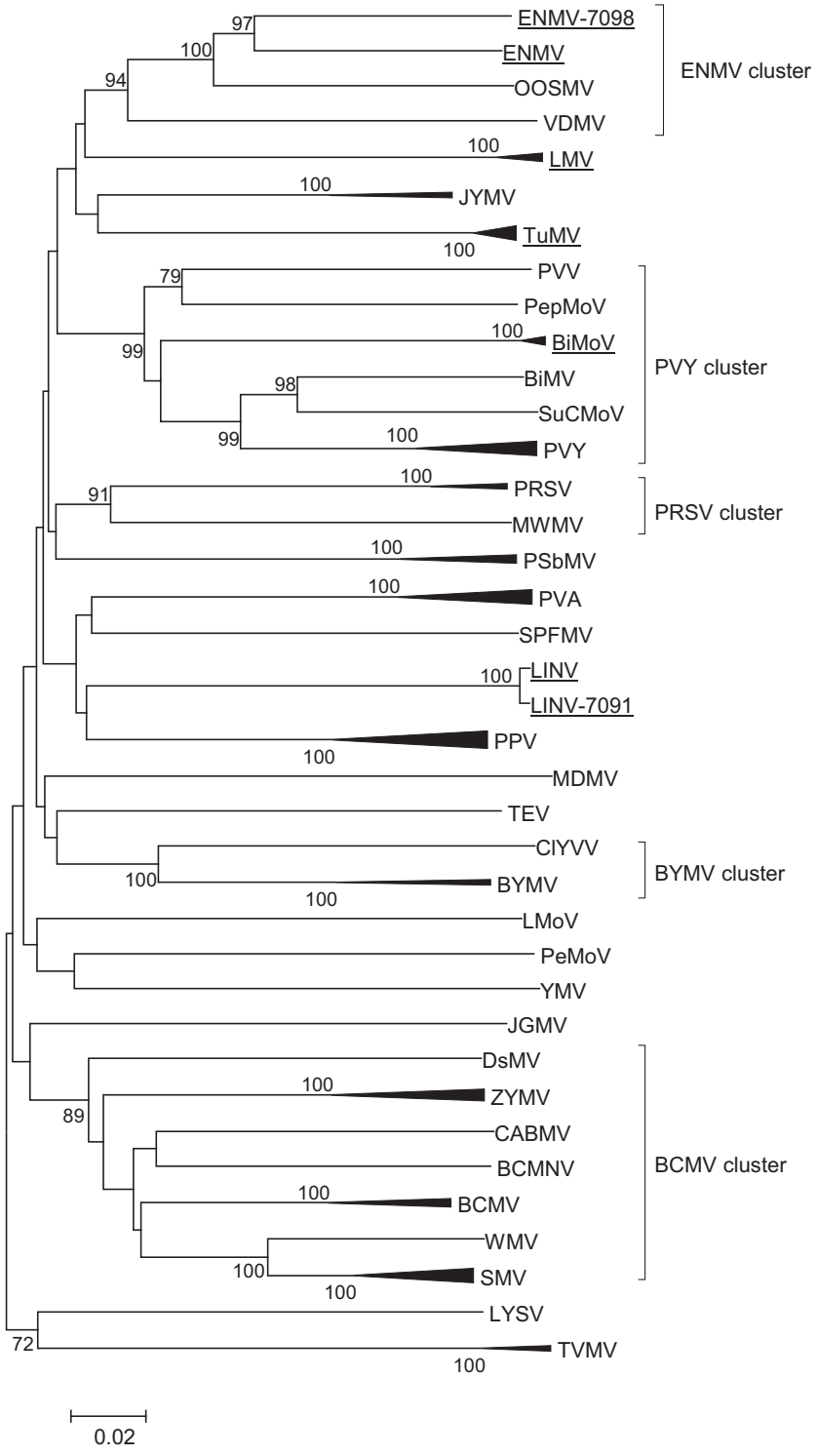

Figure 2 Neighbour-joining tree based on core coat protein coding sequences of virus isolates 7091 and 7098 from *Lactuca sativa* and 35 potyviruses. The best-fit general time reversible nucleotide substitution model with gamma-distributed substitution rates was used on a 705 nt sequence alignment. Bootstrap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

Host range

LINV-Fr and ENMV-Fr were used to mechanically inoculate a range of plant species and genotypes (Tables 1 & 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 2 h of substrate reaction. Moreover, partial sequencing of the NIb and CP coding regions of the ENMV isolate that was used to prepare the antiserum revealed that it was closely related to ENMV-Fr, with 92% nt identity. Consequently, the infection status of 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 for mixing of LINV-Fr with different potyviruses after 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 when the highly versatile and sensitive diagnostic method based on NGS of small RNAs was used.

The occurrence of symptoms correlated with ELISA results, except in *Helianthus annuus* for ENMV-Fr and in *Tragopogon pratensis* for LINV-Fr, which were symptomless despite a low percentage (≤20%) of positive ELISA results. ENMV-Fr infected only plants of the family Asteraceae (11/16 species) at the systemic level, including *Centaurea cyanus*, *C. endivia*, *Tragopogon porrifolius*, *T. pratensis*, *Zinnia elegans*, *L. sativa*, *L. serriola*, *L. saligna* and *L. virosa*. A low percentage (<17%) of inoculated plants of *Calendula arvensis* and *H. 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.

Similarly, LINV-Fr infected mostly plants in the family Asteraceae (11/15 species) at the systemic level, including *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 family Solanaceae, was also readily infected. In addition, necrotic local lesions were observed in inoculated leaves of *Chenopodium amaranticolor* and *Chenopodium quinoa* (family Chenopodiaceae).

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. *Lactuca perennis* was also infected by LINV-Fr only, although only one plant from one accession was infected (Table 2).

Symptoms observed at the systemic level were rather mild for both viruses, with mostly chlorotic spots on leaves; exceptions were *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 (Fig. S1).

Identification of resistant genotypes in *Lactuca* spp.

All *L. sativa* cultivars displaying resistance to TuMV (i.e. Avoncrisp, Girelle, Kinemontepas, Mantilia and Mariska; data not shown), and therefore probably carrying the *Tu* resistance gene, were also resistant to ENMV-Fr (Table 2; Fig. S1). In contrast, cultivars Calmar, Montemar and Salinas 88, which do not carry the *Tu* resistance gene, were susceptible to ENMV-Fr (Fig. S1). The correlation between susceptibility to ENMV and TuMV in iceberg lettuce cultivars was previously mentioned (Blancard *et al.*, 2006). Salinas 88, which does not carry the *Tu* resistance gene but is homozygous for the $mo1^2$ gene, was susceptible to ENMV-Fr (Table 2), indicating that the $mo1^2$ allele did not protect lettuce against infection by this virus. All *L. sativa* cultivars tested, either resistant or susceptible to TuMV, were susceptible to LINV-Fr (Table 2; Fig. S1), demonstrating that the *Tu* gene did not confer any resistance to this isolate. In addition, the nearly isogenic lines of Mariska and Girelle with the $mo1^1$ or $mo1^2$ resistance alleles, as well as Salinas 88 carrying $mo1^2$, were susceptible to LINV-Fr, showing that $mo1^1$ and $mo1^2$ 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. perennis*, *L. saligna*, *L. serriola* and *L. virosa*. Three of these resistant accessions were also resistant to LMV-E, whereas *L. perennis* 'LS293', showed a heterogeneous response to both LINV-Fr and LMV-E (Lot and Maisonneuve, data not shown). The fifth accession, *L. perennis* 'LS297', was not tested for LMV resistance. In contrast, only two of these nine wild accessions were resistant to LINV-Fr: *L. virosa* 'PIVT1398', which is also resistant to LMV (Maisonneuve *et al.*, 1999) and ENMV-Fr, and *L. perennis* 'LS297', also resistant to ENMV-Fr.

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 2009–2013, 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 the family Asteraceae) and *Diplotaxis erucoides* (family Brassicaceae; Table S1). In contrast, LINV was not detected in the sampled plants.

Discussion

The molecular studies showed that isolates 7091 and 7098, collected on lettuce plants with symptoms in southeastern France in 2012, belonged 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* '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 the symptoms observed for isolate 7091. Therefore, isolate 7091 is unambiguously an isolate of the same species that is consequently named 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 shared up to 79% nt (82% aa) identity in the CP-coding region with ENMV. The identity threshold for species demarcation in the genus *Potyvirus* is 76% at the nucleotide level and 80% at the amino acid level in the CP or the whole genome (Adams *et al.*, 2005); therefore, isolate 7098 could be considered as an isolate of ENMV. However, CP identity between isolate 7098 and OOSMV (75% nt, 80% aa), 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 to distinguish closely related species (Romay *et al.*, 2014). Because 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 lesser extent, VDMV clearly belong to a cluster of related viruses. Considering that isolate 7098 belongs to a divergent strain of ENMV, it is suggested that it be named ENMV-Fr. Its status may change when the complete sequence of the original ENMV isolate is available.

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

Endive necrotic mosaic virus 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; GenBank accession number AJ223827). It was then observed in iceberg lettuce cultivars in several regions of Germany and in *L. sativa* 'Commander' in South Africa in 2011 (GenBank accession numbers JQ437576 and JQ437577). Isolates from Germany and South Africa were closer to each other (92% identity on a 610 nucleotide long alignment) than to ENMV-Fr (79% and 84% identity on a 613 and a 1352 nucleotide long alignments, respectively); this indicates that the presence of ENMV in France is not the result of a recent exchange between France and one of these countries, unlike the situation with French and Italian isolates of LINV. In agreement with this hypothesis, the sequences of the 413-nt part of the genome at the junction of the NIb and CP coding regions showed that the ENMV isolate collected in France in 1999 (used to produce the antiserum) was closer to ENMV-Fr (92% nt identity) than to the German isolate (73% nt identity, data not shown).

How long ENMV-Fr has been present in southeastern 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 in the present investigation 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 cultivated crops; this is supported by the fact that most lettuce cultivars grown in France have the *Tu* gene and so are probably resistant to ENMV-Fr.

In contrast to ENMV-Fr, LINV has only been found in cultivated lettuce. No natural wild plant or weed was found to host this virus in the present survey, which is consistent with a recent introduction. However, its efficient transmission by aphids and its ability to infect most lettuce cultivars provide an opportunity for this virus to move from one lettuce crop to another, because lettuce is grown all year round. Results from the host range experiments also suggest putative alternative hosts for LINV, especially *L. serriola*, sunflower and other ornamental or vegetable species in the family Asteraceae. The presence of closely related isolates in France and Italy is also compatible with putative seed transmission.

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

The present investigation has identified possible sources of resistance to ENMV and/or LINV in the *Cichorium* and *Lactuca* genera that may be used for breeding purposes. 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, where all *C. endivia* tested (23/23) were susceptible to TuMV, while most *C. intybus* (35/36) were resistant to both TuMV and BiMoV (Zitter & Guzman, 1977; Provvidenti *et al.*, 1979).

Interestingly, in *Lactuca* spp., most resistant genotypes showed resistance to several viral species. However, in most cases, if wild *Lactuca* species showed resistance to a given potyvirus, 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. Similar results were 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. *Lactuca virosa* 'PIVT1398', which carries the *Mo3* gene conferring resistance to all tested LMV isolates, was also resistant to ENMV-Fr and LINV-Fr. Finally, *L. perennis* 'LS297' was also resistant to both ENMV-Fr and LINV-Fr. Thus, important future research leading from this study would 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, two previously unidentified potyviruses of lettuce have been described. 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 such as *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-Fr has been shown in *L. sativa* cultivars, and its low prevalence could be due to a recent emergence in France, suggesting a still limited distribution.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Symptoms induced by isolates 7091 or 7098 on mechanically inoculated *Lactuca sativa* plants.

Table S1 Weeds or wild plants sampled in the region of Avignon (2009–2013) and tested for the presence of ENMV and LINV by antigen-coated plate-ELISA and RT-PCR.