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Specific detection of Lettuce mosaic virus isolates belonging to the “Most” type

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Lettuce mosaic virus (LMV)-Most isolates can infect and are seed-borne in cultivars containing the *mo*1 gene. A reverse transcription and polymerase chain reaction (RT-PCR)-based test was developed for the specific detection of LMV-Most isolates. Based on the complete genome sequences of three LMV isolates belonging respectively to the Most type, the Common type and neither of these two types, three different assays were compared: (i) presence of a diagnostic restriction site in the region of the genome encoding the variable N-terminus of the capsid protein, in the 3′ end of the genome, (ii) RT-PCR using primers designed to amplify a cDNA corresponding to a portion of the P1 coding region, in the 5′ end of the genome and (iii) RT-PCR using primers designed to amplify a central region of the genome. The assays were performed against a collection of 21 isolates from different geographical origins and representing the molecular variability of LMV. RT-PCR of the central region of the genome was preferred because its results are expected to be less affected by natural recombination between LMV isolates, and it allows sensitive detection of LMV-Most in situations of single as well as mixed contamination.

Keywords: Lettuce; Potyvirus; LMV; RT-PCR; Detection; Resistance breaking

Lettuce mosaic virus (LMV) is one of the most important pathogens of lettuce (*Lactuca sativa*) worldwide (Dinant and Lot, 1992). LMV belongs to the genus *Potyvirus* within the family Potyviridae (Barnett et al., 1995). The genomic organization of LMV is typical of potyviruses, with a single positive-sense genomic RNA of 10.080 nucleotides (nt) encapsidated as flexuous rods (Revers et al., 1997b). The viral genomic RNA has a virally encoded protein linked covalently at its 5′ end, a poly-A tail at its 3′ end, and contains a single open reading frame (ORF) which encodes a large polyprotein with 3255 amino acids (Revers et al., 1997b). LMV is transmitted by aphids in a non-circulative manner, and is seed-borne in lettuce (Dinant and Lot, 1992; Ryder, 1973).

The control of lettuce mosaic relies on prophylactic measures such as the elimination of contaminated commercial seed lots (Tomlinson, 1962) and on genetic resistance (Dinant and Lot, 1992; Ryder, 1970). Two alleles of the recessive gene *mo*1 (*mo*1^1, formerly named *g*, and *mo*1^2, formerly named *mo*) were introgressed into different lettuce cultivars, conferring either tolerance (systemic virus accumulation but no symptoms) or resistance (no systemic virus accumulation), depending on the virus isolate considered (Dinant and Lot, 1992; Pink et al., 1992a; Pink et al., 1992b; Revers et al., 1997a). The *mo1* alleles from resistant and susceptible lettuce cultivars were isolated recently and shown to encode the cap-binding protein, eIF4E (Nicaise et al., 2003). The *mo*1 alleles also provide control of seed transmission because, even in the tolerance cases, LMV accumulates in the mother plants containing *mo*1^1 or *mo*1^2, but does not access the embryo (Dinant and Lot, 1992; Pink et al., 1992b).

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As a result of the introgression of the *mo*1 alleles into commercial lettuce cultivars, lettuce mosaic was generally considered to be under control (Walkey et al., 1985). In the last decade, however, LMV isolates capable of overcoming the resistance afforded by *mo*1 have been described in various parts of the world, including Europe (Dinant and Lot, 1992; Pink et al., 1992a; Pink et al., 1992b; Revers et al., 1997a; Varveri et al., 2002), South America (Stangarlin et al., 2000) and North Africa (Fakhfakh et al., 2001). While generally resistance-breaking isolates are not seed-borne, limiting their economic significance to local outbreaks (Dinant and Lot, 1992; Pink et al., 1992b), these newly observed LMV isolates combine resistance breaking and efficient seed transmission in resistant hosts (Dinant and Lot, 1992; Revers et al., 1997a). In a study of the genetic diversity within LMV isolates collected on a worldwide scale (Krause-Sakate et al., 2002), such isolates clustered separately, suggesting a monophyletic origin of this group of isolates for which the name LMV-Most (for *mo*1-breaking, seed transmitted) was proposed. Similarly, the name LMV-Common was proposed for another monophyletic group corresponding to the seed-borne isolates that are unable to infect *mo*1 plants. It should be stressed that although no seed-borne and *mo*1-breaking isolate was found outside of the LMV-Most clade in this study (Krause-Sakate et al., 2002), it is conceivable that such isolates could exist and, because the name "Most" is based on cladistics and not biology, despite their biological properties such isolates would be considered non-Most. The complete nucleotide sequences of LMV-0, a Common-type isolate from France, and of LMV-AF199, a Most-type isolate from Brazil, have been determined (Krause-Sakate et al., 2002; Revers et al., 1997b).

Because of their ability to spread in seed lots even in the presence of the two available resistance genes, Most-type isolates are an increasing threat to lettuce production worldwide. In addition, at least one recombinant isolate between Common and Most types has been detected in an area heavily contaminated by both types of isolates (Krause-Sakate et al., 2004), suggesting that the co-existence of these two types of isolates in fields could generate new LMV forms with novel combinations of biological and/or epidemiological properties. Therefore, a strict control of LMV-Most is necessary.

The objective of this study was to develop an assay for the specific detection of Most-type isolates. LMV-Common and LMV-Most have virtually identical sequences in the highly variable immunogenic N-terminus of the capsid protein, suggesting that they would be difficult to distinguish immunologically. This was confirmed when monoclonal antibodies raised against LMV-0 were used (Candresse et al., in preparation). Therefore an assay relying on nucleotide differences, and based on the polymerase chain reaction (PCR) was developed, in order to benefit from the high sensitivity afforded by this technique, and from the existence of potentially translationally silent nucleotide differences.

The LMV isolates used in this study have been described (Krause-Sakate et al., 2002). Additional isolates obtained from further field collections were similarly typed, based on the sequence of a variable 216-nucleotide portion of their genome, at the junction between the polymerase and capsid protein coding regions (Fakhfakh et al., 2001; Varveri et al., 2002); data not shown). Twenty-one LMV isolates were selected to represent the range of geographical and phylogenetic variation of LMV. Fig. 1 shows their positions in a dendrogram derived from the variable nucleotide sequence at the junction between the NIb and CP coding regions.

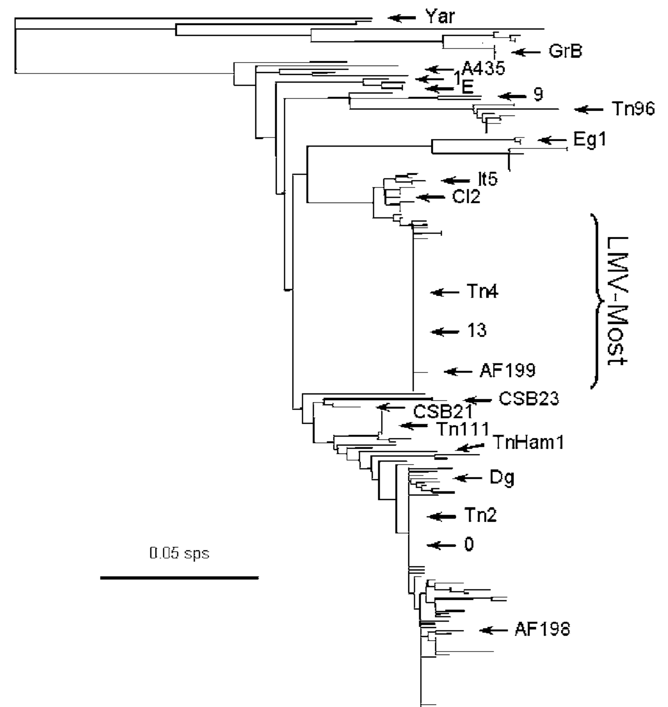

Fig. 1. Hierarchical clustering of 204 LMV isolates derived from the 216-nucleotide sequence at the junction between the NIb and CP coding regions (Krause-Sakate et al., 2002), showing the positions of the 21 isolates chosen as representatives of the geographical and phylogenetic variation within LMV. The scale bar shows 0.05 substitutions per site (sps). LMV-Most isolates are indicated.

All isolates were stored at 4 °C after desiccation (Bos, 1977) and propagated routinely under greenhouse conditions (16 h day length, 20–30 °C) in the susceptible lettuce cultivar "Trocadéro". Coupled reverse transcription and polymerase chain reaction (RT-PCR) was performed as described earlier (Krause-Sakate et al., 2002). The primer pairs used for the PCR are described in Table 1. The cDNA fragments amplified were analyzed by agarose or polyacrylamide gel electrophoresis.

We have described previously (Revers et al., 1999) that *Eco*R I digestion of a cDNA fragment obtained after RT-PCR, covering the 3′ part of the NIb coding region and the 5′ part of the CP coding region, provided a pattern characteristic of two isolates (LMV-13 and LMV-Aud) that have now been typed as Most (Krause-Sakate et al., 2002). The possibility that this could be a general diagnostic feature of the Most-type isolates was therefore investigated. This region was RT-PCR-amplified using the broad-spectrum primer pair 08894p-09171m and the product obtained was sequenced as

Table 1
Oligonucleotide primers used for the amplification of LMV cDNA fragments by RT-PCR

Name	Sequence	Position	Polarity	T_m (°C)	Size (nts)
08894p[a]	CCGTACATAGCIGARTGTGCT	8894	Sense	56	278
09171m[a]	GCGTTGATGTCGTCATCYTT	9171	Antisense		
Br535p[b]	GTTTCGGTACCAGAACCC	535	Sense	57	482
Br1016m[b]	CTTGATCTATAAGAGCTGC	1016	Antisense		
Most5930p	GATGGGGGTATTTTCGAT	5930	Sense	54	615
Most6544m	GACAAGATAAGCTCTAATTCCAC	6544	Antisense		

The name, sequence, position of the 5′ nucleotide along the complete LMV genome, polarity according to the LMV genome, annealing temperature used (T_m) and expected size in nucleotides of the amplified fragment are given. I stands for Inosine, R for a mixture of G and A, Y for a mixture of C and T.

[a] Described in (Krause-Sakate et al., 2002).

[b] Described in (Krause-Sakate et al., 2004).

in (Krause-Sakate et al., 2002) for a total of 204 isolates from worldwide origins (data not shown). Examination of the sequences obtained showed that among this set of isolates there were 11 different hexanucleotide sequences starting at position 9088 (numbered according to the complete genome) and confirmed the presence of an *Eco*R I site at this position in all 53 Most-type isolates tested (Table 2). Only one of the 151 non-Most isolates, LMV-CSB21, had the *Eco*R I site. Furthermore, a set of isolates from a sister clade to LMV-Most in the phylogenetic analysis (represented by isolates Cl2 and It5 in Fig. 1), which had an average of only four nucleotide differences with Most-type isolates in the 216-nucleotide region sequenced, lacked *Eco*R I_{9088} and had a GAACTC sequence instead. This confirmed further that the presence of an *Eco*R I site was highly correlated to the Most type. Indeed, it was confirmed experimentally in all Most-type isolates tested in routine by RT-PCR followed by *Eco*R I digestion according to Revers et al. (Revers et al., 1999) (data not shown).

Therefore, *Eco*R I digestion of the 08894p-09171m product provides a reliable diagnostic of Most-type isolates, with no false negatives identified to date, and only one false positive out of 151 isolates sequenced. However, scoring the presence of a restriction site implies a two-step procedure between sample preparation and gel electrophoresis, and the use of polyacrylamide instead of agarose gels to allow for a more consistent electrophoretic separation in the size range considered (the RT-PCR product, 278 nucleotides, yields two fragments of 194 and 84 nucleotides, respectively). It is, therefore, less amenable for routine detection than an assay that would be based on selective amplification since it requires more time and labor. In addition, a two-step procedure is by essence more error-prone than a single-step one. The development of primers that would allow specific amplification from LMV-Most but not from other types of LMV isolates was therefore attempted.

A representative collection of 21 LMV isolates from all over the world and covering the range of LMV molecular diversity described by Krause-Sakate et al. (Krause-Sakate et al., 2002) was first established (Fig. 1). Three Most-type (from North Africa, Western Europe and South America) and three Common-type (from Western Europe and South America) LMV isolates were included in this collection, in addition to a number of isolates belonging to other sequence types. In particular two isolates from a sister clade to LMV-Most, one from South America (Cl2) and one from Southern Europe (It5) were included in the collection. In addition, two atypical isolates were also included: CSB21, the only non-Most isolate that had an *Eco*R I site at position 9088 (see above), and Tn2, a natural recombinant between the Common and Most types (Krause-Sakate et al., 2004).

In a first attempt to design Most-specific oligonucleotide primers, the entire LMV genome sequence was examined for the presence of short regions with higher-than-average nucleotide divergence. As a result of this examination, the primer pair Br535p–Br1016m was designed (Krause-Sakate et al., 2004) (Table 1). These primers are specific for LMV-AF199 (of the LMV-Most type), with 5 and 6 nucleotide differences compared to LMV-0 (4 and 3 compared to the non-Most, non-Common isolate LMV-E), respectively. They allow the amplification of a 482-nucleotide product from the P1 coding region.

This pair of primers was assayed against the representative LMV collection and 28 additional isolates. Within this set of isolates, these primers yielded an amplification product only for those belonging to the Most type (Fig. 2 and Table 3), with the exception of two non-Most isolates that yielded a positive amplification, LMV-Cl2 and LMV-Tn2 (Fig. 2). LMV-Cl2,

Table 2
Hexanucleotides found at position 9088 in 204 LMV isolates of world-wide origin, typified according to the sequence between positions 8936 and 9151 as in (Krause-Sakate et al., 2002)

Hexanucleotide at position 9088	Isolate type		
	Most	non-Most	Total
AAATGC	0	8	8
AAATTC	0	3	3
AGGCCC	0	1	1
AGGCTC	0	9	9
GAACAC	0	1	1
GAACCC	0	9	9
GAACTC	0	110	110
GAATTC	**53**	**1**	**54**
GAGCTC	0	6	6
GAGTTC	0	1	1
GGGCTC	0	2	2
Total	53	151	204

The *Eco*R I site is outlined in bold.

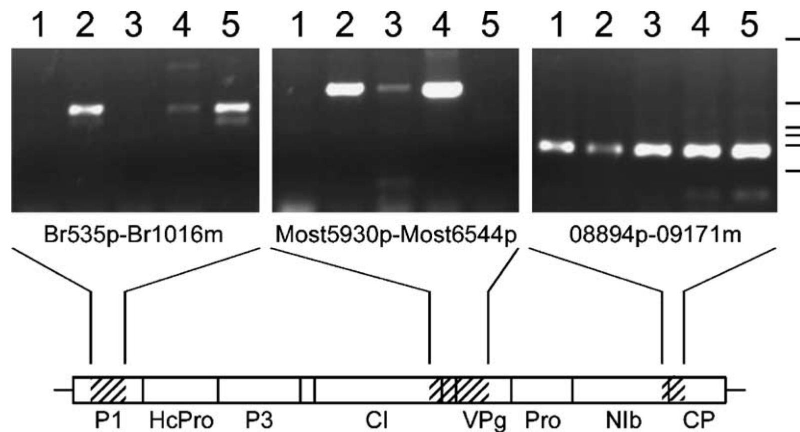

Fig. 2. Specificity of the RT-PCR amplification using the three pairs of primers 08894p-09171m, Br535p–Br1016m and Most5930p–Most6544m, respectively. The positions of the amplified regions along the LMV genome are shown in the bottom panel. The lanes contain the RT-PCR products from: (1) LMV-0 (Common); (2) LMV-AF199 (Most); (3) LMV-E (non-Common: non-Most); (4) LMV-Cl2 (non-Common, phylogenetically close to Most) and; (5) LMV-Tn2 (natural recombinant between Most and Common). The positions of size markers are shown on the right: from top to bottom, 1018 bp, 517/506 bp, 396 bp, 344 bp, 298 bp and 220 bp.

one of the isolates from the sister clade to LMV-Most discussed above, yielded a faint but consistent signal which was not observed for the other isolates of this clade (Table 3 and data not shown) and was probably due to a sequence fortuitously similar enough to LMV-AF199 to allow annealing of the primers. The situation regarding LMV-Tn2 is different since this isolate (with biological properties and a NIb-CP sequence of the Common type) is a natural recombinant with the 3′ half of the genome deriving from the Common type and the 5′ part from the Most type, a situation that was discovered using the primer pair Br535p–Br1016m (Krause-Sakate et al., 2004).

Therefore, the primer pair Br535p–Br1016m allowed the specific detection of isolates that had the cognate sequence, but was shown in at least one case to produce a false positive due to recombination between viral isolates. Conceptually, the opposite situation could also have happened depending on the position of the recombination point and of the structure of the recombinant (i.e. an isolate with biological properties of the Most type but with a P1 sequence of the Common type would have resulted in a false negative detection). To avoid the risk of false positive or false negative signals associated with such situations that are uncontrollable in practice, oligonucleotide primers were designed that were as specific as possible to the sequences directly related to the biological characteristics of LMV-Most, namely the ability to infect plants with the *mo*1 gene. Seed transmission was considered a second priority because if the assay is to be used in seed control, by definition only seed-borne isolates will be concerned.

In the case of several potyviruses, it has been shown that the ability to infect plants with a recessive resistance gene was related to amino acid changes in the central domain of the genome-linked viral protein, VPg (Borgstrom and Johansen, 2001; Nicolas et al., 1997). For LMV, a central domain of the genome comprising the VPg coding region is also involved in the ability of an isolate to infect *mo*1 plants (Redondo et al., 2001). The central region of the genome contains relatively less nucleotide differences between LMV-Most and other types of isolates compared to the P1 coding region (Krause-Sakate et al., 2002). Nevertheless the primer pair Most5930p–Most6544m (Table 1), specific for LMV-AF199, contains 5 and 2 differences relative to the LMV-0 sequence, and 3 and 2 differences relative to the LMV-E sequence, respectively. Most6544m corresponds to nucleotides

Table 3
Results of the RT-PCR typing for the LMV isolates in the representative collection, using the three methods described in this paper

Isolate	*Eco*R I_{9088}	Br535p–Br1016m	Most5930p–Most6544m
Yar	−	−	−
GrB	−	−	−
A435	−	−	−
1	−	−	−
E	−	−	+/−
9	−	−	nt
Tn96	−	−	−
Eg1	−	−	+/−
It5	−	+	+
Cl2	−	+/−	+
Tn4	+	+	+
13	+	+	+
AF199	+	+	+
CSB23	−	−	−
CSB21	+	−	−
Tn111	−	−	−
TnHam1	−	−	−
Dg	−	−	−
Tn2	−	+	−
0	−	−	−
AF198	−	−	−

A minus sign indicates a negative result (no *Eco*R I site or no RT-PCR amplification, respectively), a plus sign indicates a positive result, +/− indicates a poor amplification; nt: not rested. LMV-Most isolates are in bold.

characteristic of the central domain of the Most-type VPg. These primers allow the amplification of a 615-nucleotide product comprising the 3′ end of the CI coding region, the entire 6K2 coding region and the 5′ half of the VPg coding region.

This pair of primers was first assayed against the collection of 21 isolates described above (Fig. 2 and Table 3). The Most-type isolates reacted positively and the Common-type isolates reacted negatively, but some non-Most, non-Common resistance-breaking isolates also reacted positively (Fig. 2 and Table 3). This was the case of LMV-E, which yielded an inefficient but reproducible amplification, and of the isolates from the sister clade to LMV-Most, LMV-Cl2 and LMV-It5. In addition, these primers have been used successfully to screen field isolates from Brazil (Krause-Sakate et al., in preparation).

To evaluate the sensitivity of the assay, extracts of plants infected with LMV-AF199 were diluted in an extract from healthy plants ("absolute" sensitivity, important to detect low-level contaminations) or in extracts from plants infected with LMV-0 ("relative" sensitivity, important to detect LMV-Most in mixed contaminations). The infected extract could be diluted 10^4 times in healthy plant extract and still give a positive amplification signal using the primer pair 08894p-09171m, and only 10^3 times using the primer pair Most5930p–Most6544m, whereas an additional 10-fold dilution resulted in negative amplification (data not shown). This suggested that the absolute sensitivity of the Most-specific primers was lower by a factor of about 10 compared to that of the broad-spectrum primers developed earlier.

When the extract of plants infected with LMV-AF199 was diluted in an extract of plants infected with LMV-0, RT-PCR amplification performed using the broad-spectrum primer pair and the resulting product digested with *Eco*R I, a faint band representing the digested product was observed in the first 10-fold dilution, but not in further dilutions (data not shown). This suggested that this technique could detect LMV-Most in mixed contaminations with LMV-Common, for instance in seed batches coming from plants infected with one or another type of isolates, as long as it represents about 10% of the virus population in the sample. On the other hand, the Most-specific primer pair allowed detection of LMV-AF199 at a 10^3-fold dilution (data not shown). Dilution in an infected plant extract did therefore not affect the sensitivity of the Most-specific detection. This result suggested that LMV-Most can be detected when it represents as little as 0.1% of the total LMV in a complex sample.

Since both primer pairs, the broad-spectrum 08894p-09171m and the most-specific Most5930p–Most6544m, allow a sensitive detection of LMV-Most, it was tempting to propose to use them together in order to allow simultaneous detection and typing of LMV in plant extracts: LMV-Common would give only one product of 278 nucleotides, while LMV-Most would yield this product and an additional one of 615 nucleotides. In addition, the broad-spectrum primers would provide an internal amplification control for the Most-specific amplification. For this purpose, the two primer pairs were mixed in a single reaction tube. In the first attempts, when standard concentrations of both primer pairs were used, it was evident that the amplification driven by the pair 08894p-09171m took over the one driven by the Most5930p–Most6544m pair since LMV-AF199 yielded only the 278-nt band and no detectable 615-nt product (data not shown). Therefore both primer pairs were mixed in various proportions in a second set of experiments. The best results were obtained when the Most5930p–Most6544m pair was used at a concentration of 2.0 mM each, and the 08894p-09171m pair at a concentration of 0.5 mM each (data not shown).

Finally, we propose three approaches to the specific detection of LMV-Most, and compare their specificities, their sensitivities as well as their ability to detect LMV-Most in mixed samples. This is an important feature especially for seed batches, because this is a situation where the product of several plants are mixed, and therefore possibly results in mixed sample even if each individual plant is infected with only one type of LMV.

*Eco*R I digestion of a RT-PCR product corresponding to the junction of the polymerase and capsid protein coding regions provides a reliable assay, with only one false positive out of 151 non-Most isolates collected. It is also a very sensitive one to detect simple contaminations, but can only detect LMV-Most when it represents more than 10% of the LMV composition of a sample. However, it involves more sample manipulations and is therefore not easily amenable to routine detection.

Although RT-PCR targeting the P1 coding region was sequence-specific, it was abandoned because it provided evidence for natural recombination in LMV, which opened the possibility that other recombination events could also yield false negative results. In fact, this also holds for detection based on the polymerase-capsid junction (i.e. the *Eco*R I assay). Together, this prompted us to base an assay on a region as close as possible to the one governing the key biological feature of LMV-Most compared to other seed-borne LMV, namely, its ability to overcome the *mo*1 resistance.

An RT-PCR targeting the central region of the LMV genome, and using at least one primer specific for the VPg central region was therefore developed. It was sensitive even to detect LMV-Most in mixed contaminations, and was amenable to simultaneous detection and typing of LMV when used in a mixture with broad-spectrum primers. However, because this pair of primers addresses more the key biological feature than the phylogenetic relationship on which the Most type is based, it was expected that it would detect isolates of LMV sharing this property even without belonging to LMV-Most as defined phylogenetically. Indeed, this has been observed. In practice, however, the vast majority of field LMV isolates are seed-borne and belong either to LMV-Common or to LMV-Most, while other types of resistance-breaking isolates remain rare and local (and over-represented in laboratory collections). Detection of additional resistance-breaking

isolates places this assay on the safe side and could allow the detection of early epidemics of any other type of seed-borne resistance-breaking LMV isolates.

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