

## SHORT COMMUNICATION

## DIFFERENTIATION OF CUCUMBER MOSAIC VIRUS SUBGROUPS BY RT-PCR RFLP

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## SUMMARY

A new cucumber mosaic virus (CMV) subgroup based on recent sequence data has been proposed by Palukaitis and Zaitlin (1997) to distinguish between some strains in the former subgroup I. The three subgroups are tentatively named IA, IB and II. Here we describe a simple and rapid procedure based on the reverse transcriptase-polymerase chain reaction (RT-PCR) followed by enzymatic digestion (RFLP) to identify and classify CMV isolates accurately into the three subgroups. Two specific primers that target CMV RNA-2, amplify a DNA fragment of approximately 650 bp which yields three different restriction patterns after digestion with *Mlu*I. Each pattern proved specific for CMV strains of each subgroup.

This method proved innovative in differentiating CMV strains in subgroups IA, IB and II rather than only in subgroups I and II and may provide a simple way to investigate the dynamics of CMV populations in nature.

## RIASSUNTO

**IDENTIFICAZIONE DEL SOTTOGRUPPO DI APPARTENENZA DEGLI ISOLATI DEL VIRUS DEL MOSAICO DEL CETRIOLO MEDIANTE RT-PCR RFLP.** Gli isolati del virus del mosaico del cetriolo (CMV) vengono distinti in due sottogruppi denominati I e II in base alle caratteristiche sierologiche e biomolecolari. Tuttavia, il sempre maggiore numero di ceppi virali sequenziati ha messo in evidenza che all'interno del sottogruppo I alcuni ceppi differiscono per più del 7% della loro sequenza nucleotidica da altri dello stesso sottogruppo. Su questa base, Palukaitis e Zaitlin (1997) hanno proposto di suddividere i ceppi del sottogruppo I in IA e IB. Questa ipotesi ha trovato conferma nei risultati di una recente analisi filogenetica (L. Barbarossa, C. La Nave e D. Gallitelli, manoscritto in preparazione).

Qui viene descritto un metodo rapido per assegnare correttamente gli isolati di CMV ad uno dei tre sottogruppi proposti. Impiegando due primers specifici in una reazione di RT-PCR, il metodo prevede l'amplificazione di un frammento di DNA di circa 650 bp dall'RNA-2 del virus che, a seguito di digestione con *Mlu*I, produce profili di restrizione caratteristici per i ceppi allocati in ciascuno dei tre sottogruppi.

Nei confronti di metodi descritti in precedenza, quello proposto in questa sede risulta innovativo in quanto permette la differenziazione fra ceppi dei sottogruppi IA, IB e II e non soltanto fra quelli dei sottogruppi I e II; per questo motivo, esso può anche risultare utile per monitorare la dinamica delle popolazioni di CMV in natura.

*Key words:* Cucumovirus, RT-PCR RFLP, subgrouping.

Cucumber mosaic virus (CMV), type species of the genus *Cucumovirus*, family *Bromoviridae* (Murphy *et al.*, 1995) is recognised world-wide as a threat to the health of many crops.

CMV particles encapsidate three linear plus-sense single-stranded genomic RNAs, designated RNA1, RNA2, RNA3 in order of decreasing size (reviewed by Palukaitis *et al.*, 1992).

Strains of CMV can be divided into two subgroups, I (or WT) and II (or S), on the basis of sequence similarity and serological relationships. There appear to be no clear differences in the host range of subgroup I and II isolates but they can be differentiated by several means (reviewed by Gallitelli, 1998) including serology (Dev-ergne and Cardin, 1975; Edwards and Cooper, 1985; Kearney *et al.*, 1990; Daniels and Campbell, 1992; Hu *et al.*, 1995; Ilardi *et al.*, 1995; Anonymous, 1998), molecular hybridization analysis (MHA) (Piazzolla *et al.*, 1979; Owen and Palukaitis, 1988; Crescenzi *et al.*, 1993a; Hu *et al.*, 1995; Rodriguez Alvarado *et al.*, 1995), RNase protection assay (RPA) (Fraile *et al.*, 1997), RT-PCR (Hu *et al.*, 1995; Singh *et al.*, 1995) and RT-PCR followed by enzymatic digestion of the amplified product (RT-PCR RFLP) (Rizos *et al.*, 1992; Ilardi

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*et al.*, 1995, Anonymous, 1998).

Recent sequence data show that a number of CMV strains within subgroup I originating from Asia (here referred to as 'Asian strains') differ by 7-12% in sequence from other subgroup I strains. On this basis, Palukaitis and Zaitlin (1997) proposed to split subgroup I by placing the Asian strains in subgroup IB and the others in subgroup IA. Strains in the same subgroup differ only by 2-3% in sequence homology.

In the Mediterranean basin, the economic importance of CMV correlates with recurrent outbreaks in tomato crops (Gallitelli, 1998). In addition to the well known fern leaf/shoestring and tomato necrosis (synonym: tomato lethal necrosis) diseases (Kaper and Waterworth, 1981; Palukaitis *et al.*, 1992), tomato fruit necrosis has been observed during CMV epidemics (Jordà *et al.*, 1992; Crescenzi *et al.*, 1993).

In Italy, tomato fruit necrosis is consistently associated with a subgroup I strain (CMV-Tfn) which supports a 390 nucleotide satellite named Tfn-satRNA (Crescenzi *et al.*, 1993). The complete sequence of CMV-Tfn has been determined (L. Barbarossa, C. La Nave and D. Gallitelli, unpublished results) and its phylogenetic relationships with other CMV strains, for which complete sequences have been reported, also determined. The consensus trees clearly show that three primary branches depart from the bulk of all CMV sequences, and virus strains cluster in three distinct subgroups. Within each subgroup, sequence heterogeneity is 0-2% whereas intersubgroup differences are 7% or more. Comparable consensus trees were obtained with all CMV genomic RNAs within two extremes represented by CMV-RNA-2 (high differentiation) and CMV CP sequence (poor differentiation). Thus, phylogenetic analysis seems to support the existence of a third subgroup of strains and places CMV-Tfn among the Asian strains, in subgroup IB (L. Barbarossa, C. La Nave and D. Gallitelli, manuscript in preparation).

From this comparison it was noted that the *Mlu*I restriction map of RNA-2 of all CMV strains considered, correlates with the three subgroups. In fact *Mlu*I restriction sites are not present in RNA-2 of subgroup IA strains (CMV-MB8, Acc. no. D86613; CMV-O, Acc. no. D10209; CMV-Y, Acc. no. D12538; CMV-Kor, Acc. no. U66287; CMV-B, Acc. no. U59740; CMV-Legume, Acc. no. D16406; CMV-Fny, Acc. no. D00355) whereas one site (position 159) is present in RNA-2 of strains of subgroup IB (CMV-Tfn, Acc. no. Y16925; CMV-NT9, Acc. no. D2879; CMV-K, Acc. no. S72187; CMV-As, Acc. no. AF033667; CMV-SD, Acc. no. D86330) and two sites (positions 173 and 322) are present in RNA-2 of subgroup II strains (CMV-Q, Acc. no. X00985; CMV-S, Acc. no. Y10885; CMV-TrK7,

Acc. no. AJ007934). The exception is CMV-Ixora (Acc. no. U20218) which clusters with subgroup IB strains, according to our phylogenetic analysis, but does not carry any *Mlu*I site.

Here we show that differentiation of CMV subgroups can readily be achieved by targeting RNA-2 sequences flanking *Mlu*I restriction sites with RT-PCR RFLP.

Using primers synthesised on the basis of EMBL Data bank sequences, RNA preparations extracted from purified virions of CMV strain S (subgroup II), Fny (subgroup IA) (supplied by Dr. Peter Palukaitis, Scottish Crop Research Institute, Dundee, UK) and Tfn (subgroup IB) were retrotranscribed to cDNA and then amplified by 35 cycles of PCR. Three microlitres of RNA preparation suspended in RNase-free water were mixed with 50 pmol of the degenerate primer 5'-GGTTCGAA(AG)(AG)(AT)ATAACCGGG-3'(RW8), complementary to positions 618-637 of CMV-Fny RNA-2, heated at 95°C for 3 min, chilled on ice and reverse-transcribed with 10 units of AMV reverse transcriptase (Amersham Life Science, UK) according to the manufacturer. Ten µl of this mixture were subjected to PCR amplification with 25 pmol of primer RW8, 50 pmol of the primer 5'-GTTTATTTACAAGAGCGTACGG-3' (RV11), homologous to positions 1-22 of CMV-Fny RNA-2, and 2.5 units of *Taq* polymerase (Promega Corp., USA) as recommended by the manufacturer. DNA was amplified in a Perkin Elmer Cetus thermal cycler with 1 cycle at 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 64°C for 1 min, 72°C for 2 min. In the last cycle, time extension at 72°C was 10 min. Ten µl of the amplified product were digested with 5 units of *Mlu*I (Boehringer Mannheim, Germany) as recommended by the manufacturer and analysed by electrophoresis in 1.2% agarose gel in 90 mM Tris, 90 mM boric acid, 1 mM Na<sub>2</sub> EDTA, pH 8.3.

The RT-PCR RFLP assay amplified a DNA fragment of approximately 650 bp for the three CMV strains (Fig. 1, lanes a, c, e) which after digestion with *Mlu*I yielded three distinct restriction patterns *i.e.* two fragments of approximately 470 and 160 bp for CMV-Tfn (Fig. 1, lanes d, h), three fragments of approximately 320, 170 and 150 bp for CMV-S (Fig. 1, lanes f, i) and one undigested sequence of approximately 650 bp for CMV-Fny (Fig. 1, lanes b, g). Similar results were obtained when the RT-PCR RFLP protocol was applied to DNA preparations extracted (White and Kaper, 1989) from fresh or dried plant tissues infected by the same CMV strains (not shown).

Since CMV-Ixora seemed exceptional in lacking the *Mlu*I site present in all other subgroup IB strains, its RNA-2 was amplified from an RNA preparation sup-

plied by Dr. F. Cellini (Metapontum Agrobios, Italy) and digested. The restriction pattern showed two fragments of *ca* 180 and 470 bp (not shown), proving that this RNA contains an *Mlu*I site like other CMV strains of subgroup IB. From analysis of the published sequence (McGarvey *et al.*, 1995 and Acc. no. U20218) it can be noted that a T/C substitution at position 180 should abolish the *Mlu*I site. This is in contrast with our results and clearly indicates a cloning artefact, also because the C/T substitution necessary to restore the *Mlu*I site does not disrupt the 2a ORF.

Three characterized CMV strains denoted CMV-TTS (Grieco *et al.*, 1992; Cillo *et al.*, 1994), CMV-FL (Crescenzi *et al.*, 1993) and CMV-77 (Grieco *et al.*, 1997) and assigned to CMV subgroup I on the basis of MHA were also analysed by the RT-PCR RFLP. Fig. 1 shows that amplified sequence of CMV-77 (lane j) was not digested by *Mlu*I (lane k) whereas those of CMV-FL and CMV-TTS (lanes l, n) yielded two fragments of approximately 470 and 160 bp (lanes m, o). Thus CMV-77 can be assigned to subgroup IA and CMV-FL and CMV-TTS to subgroup IB.

The use of PCR with a single pair of CMV-specific primers coupled with RFLP analysis of amplified sequences offers a simple and accurate means of identifying and grouping CMV isolates. As already mentioned,

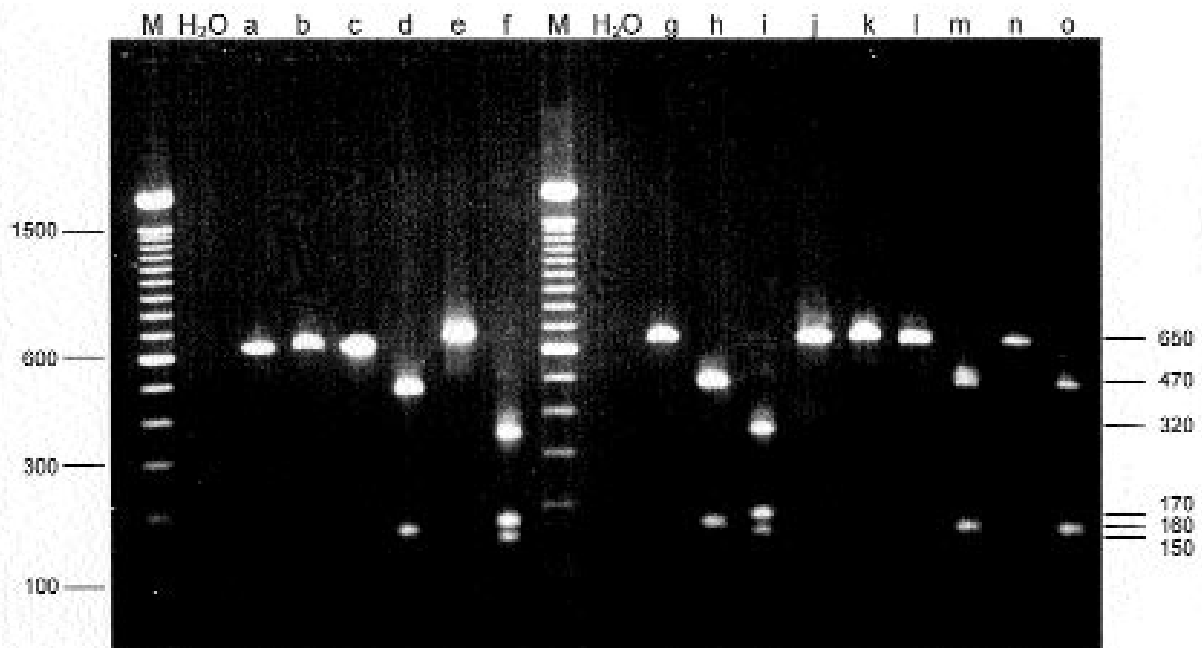
this strategy has been successfully applied in several instances but the approach described here is innovative in differentiating CMV strains in subgroups IA, IB and II rather than only in subgroups I and II.

The method we have developed could be applied to study the dynamics of CMV in natural populations. This is an emerging necessity, since better knowledge of CMV biodiversity may be fundamental in establishing a correct baseline for risk assessment, in anticipation of large scale deployment of transgenic virus-resistant plants (Tepfer and Balazs, 1997).

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**Fig. 1.** Electrophoretic analysis in 1.2% agarose gel of amplified products (lanes a, c, e, j, l, n) and respective restriction patterns (lanes b, d, f, g, h, i, k, m, o) obtained for the following strains of CMV: Fny (lanes a, b, g), Tfn (lanes c, d, h), S (lanes e, f, i), 77 (lanes j, k), FL (lanes l, m), TTS (lanes n, o). M: 100 bp ladder (BRL); H<sub>2</sub>O: negative control, no RNA.

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