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Simultaneous detection and identification of *Pepino mosaic virus* (PepMV) isolates by multiplex one-step RT-PCR

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Abstract A RT-PCR was developed for the simultaneous detection and identification of three groups of *Pepino mosaic virus* (PepMV): European/Peruvian, Chilean 1/US1 and Chilean 2/US2 groups, followed by a restriction analysis that allowed the separation of the European, Peruvian, Chilean 2 and US2 isolates (patent pending). The multiplex RT-PCR reaction was performed by a mix of six primers that amplified a part of the RNA-dependent RNA polymerase gene of PepMV plus an internal control. Amplifications resulted in a 980 bp, 703 bp or 549 bp PCR product for European/Peruvian, Chilean 1/US1 or Chilean 2/US2 groups, respectively. For the identification of the isolates present within the European/Peruvian and Chilean 2/US2 groups, the amplified PCR fragments were directly digested with *Sac*I enzyme. The multiplex RT-PCR method presented higher sensitivity to detect CH1/US1 isolates in field samples than the RFLP-PCR method described by Hanssen et al. (European Journal of Plant Pathology 121:131–146, 2008). The detection limit observed with the multiplex RT-PCR was equal to or 3,125 times higher when compared to single RT-PCR or ELISA-DAS and molecular hybridisation methods, respectively. The use of the multiplex RT-PCR method in routine analysis of field tomato samples allowed the detection of 36.2 and 33.4% more positives when compared to the serological and molecular hybridisation methods, respectively, and the identification of plants infected with one, two or three isolates of PepMV.

Keywords ELISA · Dot-blot hybridisation · Multiplex RT-PCR · PepMV genotypes · Simultaneous identification

Introduction

Pepino mosaic virus (PepMV) is a *Potexvirus* which was first described on Pepino (*Solanum muricatum*) in Peru (Jones et al. 1980). In 1999, PepMV was reported to be infecting tomato crops (*Solanum lycopersicon*) in the Netherlands (Van der Vlugt et al. 2000). Since then, PepMV has spread rapidly through the main tomato production areas worldwide, where it produces significant economic losses.

PepMV is a positive, single-stranded RNA virus. The total genome of PepMV is approximately 6,500 bp which includes five open reading frames (ORFs). Different strains of PepMV have been identified. The

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biological and molecular differences first observed between the tomato and pepino isolates of PepMV suggested that the tomato isolate of PepMV was a different strain, and was named ‘tomato strain’ (Van der Vlugt and Berendsen 2002). Further studies demonstrated that PepMV tomato strain isolates from different locations (Europe, North America and Canada) showed clear differences in symptom aggressiveness and nucleotide sequences when compared to the original pepino isolate (hereafter referred to as Peruvian isolate, PE) but only minor differences among them (Verhoeven et al. 2003). Furthermore, the North American strains US1 and US2, which showed only a 79–83% overall sequence homology to both European (EU) and Peruvian strains (PE), have been described (Table 1; Maroon-Lango et al. 2005). Recently, new isolates have been identified as Chilean isolates, CH1 (GenBank accession number DQ000984) and CH2 (GenBank accession number DQ000985), from a commercial tomato seed lot produced in Chile (Ling 2007), the highest sequence homology of which was to isolates US1 (98%) and US2 (91%), respectively (Table 1; Ling 2007). Moreover, a new Polish isolate highly distinct from the currently identified isolates has been described and referred to as the PK isolate (GenBank accession number EF408821). The Polish isolate PK shows 81% or less nucleotide sequence identity with EU, PE or US strains (Pospieszny and Borodynko 2006). Further studies revealed that PepMV-PK shows a high nucleotide sequence identity (98%) with the CH2 isolate, which suggests their common origin (Hasiów et al. 2007). In 2007, the presence of an isolate of the US1 strain of PepMV was reported in tomato in the Canary Islands (Spain), located in a different continent to that where it was originally reported in North America (Alfaro-Fernández et al. 2008).

In 2002, a RT-PCR-RFLP assay was proposed as a rapid method to detect and identify new isolates of PepMV. Three different RFLP patterns were identified (P1, P2 and P3 types). Most of the samples analysed were included in the P1 type that corresponded to the tomato strain widely spread throughout Europe. Some samples showed the P2 type which was identified as the Peruvian isolate, and the third pattern, type P3, that showed one different isolate of PepMV and appeared rarely in the tested samples and almost always in mixed infections with the P1 type (Martínez-Culebras et al. 2002). Types P1, P2 and P3 have been suggested to correspond to the EU, PE and US2 strains, respectively (Pagán et al. 2006).

The genetic variability and population structure of PepMV infecting tomato crops in Spain was analysed by sequencing and studying three genomic regions: a part of the RNA-dependent RNA polymerase (RdRp) gene, the triple-gene block (TGB) and the capsid protein (CP) gene. The results showed that the most prevalent genotype in Spain is the EU strain (>80% of the population). PE and US2 strains were also detected at a less relative frequency and were always found in mixed infections with the EU strain. Some recombinant isolates were also reported (Pagán et al. 2006). However, in

Table 1 Homology among different complete nucleotide sequences of *Pepino mosaic virus* strains obtained from the GenBank database

	EU[a] Acc.No. AJ606360	**PE** Acc.No. AM109896	**CH1** Acc.No. DQ000984	**US1** Acc.No. AY509926	**CH2** Acc.No. DQ000985	**US2** Acc.No. AY509927
EU Acc.No. AJ606360	100	95	82	82	84	81
PE Acc.No. AM109896		100	82	82	83	80
CH1 Acc.No. DQ000984			100	98	84	90
US1 Acc.No. AY509926				100	85	90
CH2 Acc.No. DQ000985					100	91
US2 Acc.No. AY509927						100

[a] EU, PE and CH correspond to European, Peruvian and Chilean isolates, respectively

North America all the major genotypes of PepMV (EU, US1, US2 and CH2) were identified, although the EU was also the predominant strain (Ling et al. 2008).

The low sequence homology observed between different PepMV genotypes has not been correlated to different symptomatology in infected plants. However, co-infection with several genotypes resulted in more severe PepMV symptoms and revealed the presence of the PepMV recombinant (Hanssen et al. 2008). In this sense, the incorporation of routine detection techniques that permits not only the detection of the virus but also identifies the corresponding isolates of PepMV is desirable. Different approaches have been used to detect and identify the virus: immunosorbent electronic microscopy (ISEM) (Van der Vlugt et al. 2000), enzyme-linked immunosorbent assay (ELISA) (Jordá et al. 2001) and different molecular methods including RT-PCR (Mumford and Metcalfe 2001; Van der Vlugt et al. 2000) or one-step immunocapture real-time TaqMan RT-PCR assay designed to use two primers targeting a conserved region of the TGB2 gene plus a single TaqMan™ probe that covered all strains of PepMV (Ling et al. 2007). In order to identify the exact PepMV strain present in one sample, these molecular methods had to be followed by DNA sequencing. However, so far there is only methodology based on a single RT-PCR-RFLP assay adjusted for simultaneous detection and identification of three (Martínez-Culebras et al. 2002) or five (Hanssen et al. 2008; hereafter RT-PCR-Hanssen) PepMV strains. The development of multiplex RT-PCR has been used successfully for the routine diagnosis of plant viruses (see James et al. 2006 for review; Ferrer et al. 2007; Nie and Singh 2000; Sánchez-Navarro et al. 2006; Uga and Tsuda 2005). This diagnostic method allows the simultaneous detection and identification of different viruses with less time and cost waste. Furthermore, a multiplex RT-PCR assay has been developed to identify different strain types of a single virus (Lorenzen et al. 2006; Ratti et al. 2005; Rigotti and Gugerli 2007) and even different species of a virus (Martínez-Culebras et al. 2001). In the present work we have developed a one-step multiplex RT-PCR reaction plus a restriction analysis that permits the simultaneous detection and identification of five different PepMV isolates (hereafter RT-PCR-*Sac*I). The use of the multiplex reaction in routine diagnosis has revealed that mixed infection of PepMV strains is a common situation in the field.

Material and methods

Virus sources and RNA preparations

Tomato PepMV isolates from different geographical origins were included as positive controls in the assay: DSMZ (German Collection Micro-organism and Cell Cultures, GMBH, Baunschweig, Germany) PV-0632 from Italy, DSMZ PV-0674 from UK, DSMZ PV-0716 from Italy and DSMZ PV-0730 from the Netherlands. A typical PepMV isolate from Peru (DSMZ PV-0554) obtained from *S. muricatum* was also studied. The new Polish PK-isolate, kindly provided by H. Prospieszny (Institute of Plant Protection, Poznań, Poland), was included in the assay. Eleven tomato samples infected with a well characterised PepMV isolate were also analysed in this study: ten isolates belonging to our virus isolate collection previously analysed: Mu 00.2; Mu 00.3; Mu 00.4; Mu 00.5; CI 01.1; CI 01.2; CI 01.3; Al 01.2; Ba 03.1 (Pagán et al. 2006); PepMV-Can1 isolate (Alfaro-Fernández et al. 2008) and Sp-13 isolate (GenBank accession number AF484251), kindly provided by Dr. M. Aranda, (CEBAS-CSIC, Murcia, Spain). Forty-two tomato samples with typical symptoms of PepMV were collected from the major tomato production areas in Spain. Infected leaves were previously tested by DAS-ELISA with specific antisera against PepMV (DSMZ GMBH, Baunschweig, Germany) according to the manufacturer´s instructions to verify the virus infection. ELISA readings were considered positive when the absorbance of sample wells was at least three times greater than the mean absorbance reading of three healthy controls. Samples were analysed from 0.05 g of infected tissue ml^{-1}, which corresponded to the original sample (undiluted) in the sensitivity assay explained below.

Total nucleic acid extraction was performed from 0.1 g of leaves using the silica capture extraction protocol (MacKenzie et al. 1997). The extracted nucleic acids were stored at −80°C until use.

Primer design

The characterisation of specific regions of the PepMV genome used to differentiate all genotypes was performed firstly, by the sequence alignment of PepMV isolate representatives of the different genotypes using the CLUSTAL X programme, and secondly, by the design of strain-specific primers using the OLIGO programme. The isolates of PepMV used for the sequence alignment were: Chilean isolates, CH1 (GenBank accession number DQ000984) and CH2 (GenBank accession number DQ000985); Peruvian isolates (PE), SM-74 (GenBank accession number AM109896) and LP-2001 (GenBank accession number AJ606361); European isolates (EU), Sp-13 (GenBank accession number AF484251), LE-2000 (GenBank accession number AJ606359) and LE-2002 (GenBank accession number AJ606360) and the North American (US) isolates, US1 (GenBank accession number AY509926) and US2 (GenBank accession number AY509927). A region of the RNA polymerase gene was selected to identify the different PepMV isolates. Three specific sense primers: PepMV-DEP, PepMV-D1 and PepMV-D2 and a common antisense primer (PepMV-R) were selected to differentiate the three EU/PE, CH1/US1 and CH2/US2 PepMV groups, respectively (Fig. 1). The selected primers amplified three amplicons of 980, 703 and 549 base pairs corresponding to the EU/PE, CH1/US1 and CH2/US2 PepMV groups, respectively. In addition, the specific PepMV isolate of the EU/PE and CH2/US2 groups could be discriminated by digesting the amplicons with the *Sac*I enzyme (Table 2 and Fig. 1). The expected amplicons of each PepMV group together with the nucleic acid fragments obtained after incubation with the *Sac*I enzyme and the sequence alignment of the different primers with the isolates of PepMV used, are indicated in Fig. 1.

For the amplification of the coat protein gene (CP), three different sense primers that specifically targeted the EU/PE group (sPepMVCP EU: 5′ TGT TCA CAA AAA TCA ACT TCA A 3′), CH1/US1 group (sPepMVCP CH1/US1: 5′ CTT TGA GCA CTT CAC AAT TAA G 3′) and the CH2/US2 group (sPepMVCP CH2/US2: 5′ CTA TGG AAA ACC AAC CTA CAG C 3′) in combination with the common reverse primer described by Pagán et al. (2006), were designed in order to analyse and check the results obtained with the multiplex RT-PCR-*Sac*I.

Fig. 1 Schematic representation of the PepMV genome with the localisation of the four primers used in the multiplex RT-PCR and the amplified PCR fragments. The sequence of the direct primers PepMV-D1, PepMV-D2 and PepMV-DEP together with the reverse primer PepMV-R is underlined in the alignment performed with Clustal X using PepMV isolates representative of all genotypes. Sequences included in the alignment belong to European (EU) strains (Sp-13, GenBank accession number AF484251; LE-2002, GenBank accession number AJ606360; LE-2000, GenBank accession no. AJ606359), Peruvian (PE) strains (SM-74, GenBank accession number AM109896; LP-2001, GenBank accession number AJ606361), Chilean 1 (CH1) strain (GenBank accession number DQ000984), US1 strain (GenBank accession number AY509926), Chilean 2 (CH2) strain (GenBank accession number DQ000985) and US2 strain (GenBank accession number AY509927). Dots indicate identical nucleotides to the Sp-13 isolate, and numbers on the right represent the position of the last nucleotide in the PepMV genome. The amplified PCR products obtained with the different direct and reverse primer combinations together with the resultant DNA fragments after the *Sac*I digestion is schematised. Numbers inside the schematic boxes represent the base pairs (bp) size. The coding sequences corresponding to the RNA-dependent RNA polymerase, triple gene block and the coat protein are indicated as RdRp, TGB and CP, respectively. Numbers on the top of the schematic PepMV genome correspond to the nucleotide sequence. Untranslated regions are indicated as UTR

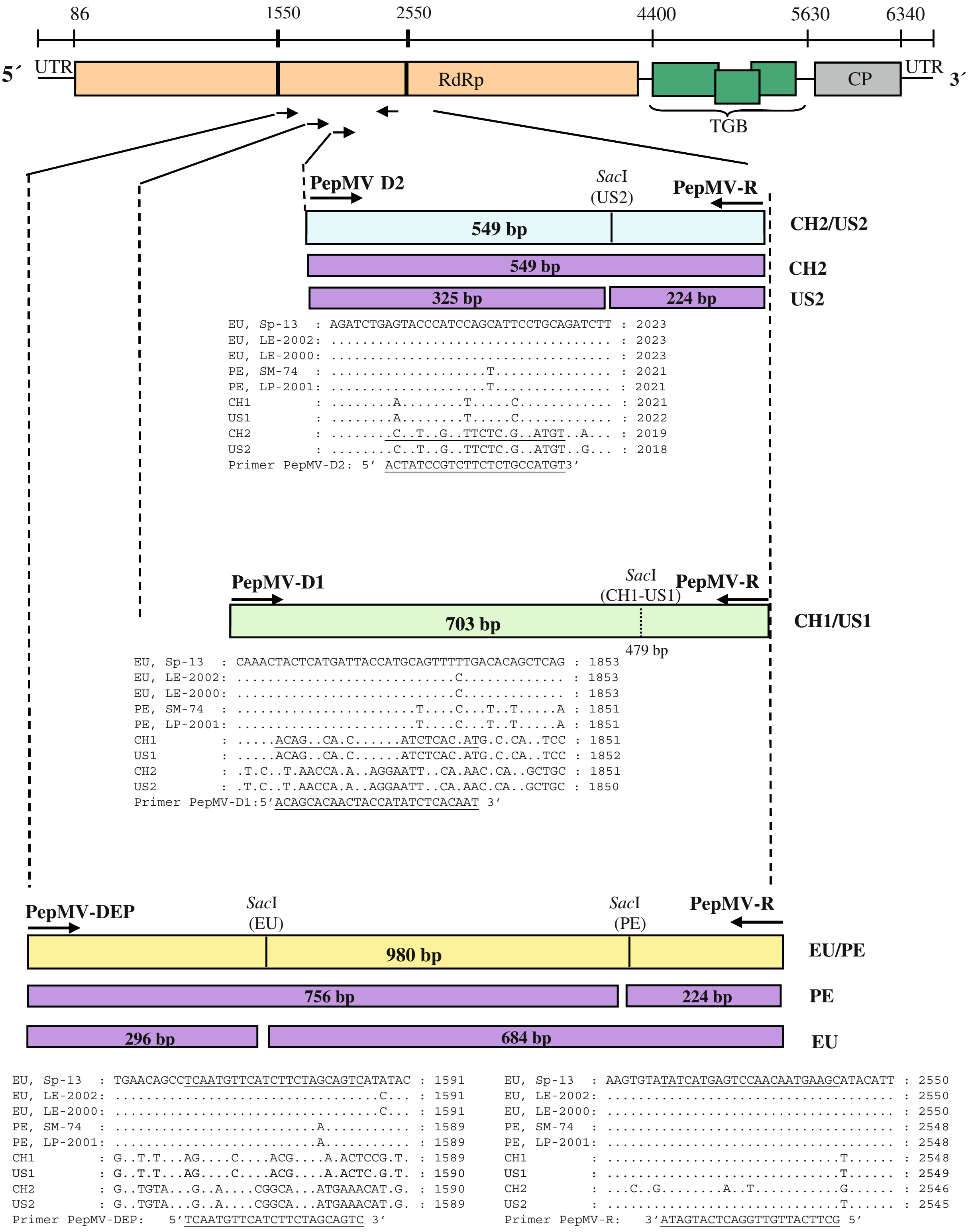
RT-PCR and restriction digestion

RT-PCR reaction was performed using the SuperScript III one step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen Life Technologies. Barcelona, Spain). The reaction was carried out with a mixture of all the primers listed in Fig. 1 at a final

Table 2 PepMV genotypes by *Sac*I restriction endonuclease digestion of multiplex RT-PCR products

	PepMV amplified fragments					
	549[a]		**703**		980	
	CH2	US2	CH1	US1	EU	PE
cDNA *fragments* *after Sac*I digestion		**325**	**479**	**479**	296	**756**
	549[b]	224	224	224	**684**	224

[a] Base pairs of cDNA fragments are based on theoretical digests of reference sequences retrieved from GenBank. [b] Underlined numbers represent cDNA fragments representative of the corresponding PepMV genotype



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EU, Sp-13  : AGATCTGAGTACCCATCCAGCATTCCTGCAGATCTT : 2023
EU, LE-2002: .................................... : 2023
EU, LE-2000: .................................... : 2023
PE, SM-74  : ....................T............... : 2021
PE, LP-2001: ....................T............... : 2021
CH1        : ........A........T.....C............ : 2021
US1        : ........A........T.....C............ : 2022
CH2        : ........C..T..G..TTCTC.G..ATGT..A... : 2019
US2        : ........C..T..G..TTCTC.G..ATGT..G... : 2018
Primer PepMV-D2: 5’ ACTATCCGTCTTCTCTGCCATGT3’
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EU, Sp-13  : CAAACTACTCATGATTACCATGCAGTTTTTGACACAGCTCAG : 1853
EU, LE-2002: ............................C............. : 1853
EU, LE-2000: ............................C............. : 1853
PE, SM-74  : .......................T....C...T..T.....A : 1851
PE, LP-2001: .......................T....C...T..T.....A : 1851
CH1        : .....ACAG..CA.C......ATCTCAC.ATG.C.CA..TCC : 1851
US1        : .....ACAG..CA.C......ATCTCAC.ATG.C.CA..TCC : 1852
CH2        : .T.C..T.AACCA.A..AGGAATT..CA.AAC.CA..GCTGC : 1851
US2        : .T.C..T.AACCA.A..AGGAATT..CA.AAC.CA..GCTGC : 1850
Primer PepMV-D1:5’ACAGCACAACTACCATATCTCACAAT 3’
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EU, Sp-13  : TGAACAGCCTCAATGTTCATCTTCTAGCAGTCATATAC : 1591
EU, LE-2002: ..................................C... : 1591
EU, LE-2000: ..................................C... : 1591
PE, SM-74  : ..........................A........... : 1589
PE, LP-2001: ..........................A........... : 1589
CH1        : G..T.T...AG....C....ACG....A.ACTCCG.T. : 1589
US1        : G..T.T...AG....C....ACG....A.ACTC.G.T. : 1590
CH2        : G..TGTA...G..A....CGGCA...ATGAAACAT.G. : 1590
US2        : G..TGTA...G..A....CGGCA...ATGAAACAT.G. : 1589
Primer PepMV-DEP:   5’TCAATGTTCATCTTCTAGCAGTC 3’
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EU, Sp-13  : AAGTGTATATCATGAGTCCAACAATGAAGCATACATT : 2550
EU, LE-2002: ..................................... : 2550
EU, LE-2000: ..................................... : 2550
PE, SM-74  : ..................................... : 2548
PE, LP-2001: ..................................... : 2548
CH1        : ..............................T...... : 2548
US1        : ..............................T...... : 2549
CH2        : ...C..G........A..T...........G...... : 2546
US2        : ..............................T...... : 2545
Primer PepMV-R:   3’ATAGTACTCAGGTTGTTACTTCG 5’
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concentration of 0.25 pmol μl^{-1} and the primers of the internal control Rbcl gene (0.05 pmol μl^{-1}) corresponding to partial sequence of the ribulose 1.5-biphosphate carboxylase chloroplast gene (Sánchez-Navarro et al. 2005). The PCR programme consisted of an initial incubation at 50°C for 30 min followed by 2 min at 94°C and 40 cycles of 94ºC for 15 s, 50°C for 30 s and 68°C for 1 min. A final incubation at 68°C for 10 min was introduced to finish the incomplete PCR fragments. The amplified PCR products were analysed on 1.2% agarose/TAE gels stained with ethidium bromide. To confirm the viral-strain origin, amplified products of each group were purified with High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and directly sequenced. Ten microliters of the PCR reaction were directly digested with the *Sac*I enzyme (MBI Fermentas, Vilnius, Lithuania) in a total volume of 20 µl, following the manufacturer's instructions. All the digestion reaction was analysed in a 5% TAE polyacrilamide gel and stained with ethidium bromide.

Dot-blot hybridisation

Dot-blot hybridisation was used to compare the sensitivity and the end-point detection limit with the multiplex RT-PCR detection method described by Sánchez-Navarro et al. (1998) Total RNA extractions of three field samples were five-fold diluted using total RNA extracted from healthy tissue. The undiluted sample corresponded to 0.05 g of infected tissue ml^{-1}. One microliter of the non-diluted RNA extraction and of each dilution was first denatured with formaldehyde and then directly applied to a nylon membrane (Más et al. 1993). Analysis of total nucleic acids by non-isotopic dot-blot hybridisation was performed as described previously by Sánchez-Navarro et al. (1998) using a dig-RNA probe complementary to a fragment of RdRp of PepMV.

Results

Detection of groups of PepMV isolates with the multiplex RT-PCR

A multiplex RT-PCR reaction with a cocktail of primers PepMV-DEP, PepMV-D1, PepMV-D2 and PepMV-R (Fig. 1), including the primers to amplify the internal control, was first used to analyse different tomato tissues infected with the PepMV isolates DSMZ PV-0632, PepMV-Can1 and DSMZ PV-0730 representative of the CH2/US2, CH1/US1 and EU/PE groups, respectively. Electrophoretical analysis revealed three PCR products of 549 bp, 703 bp and 980 bp that corresponded to the three PepMV groups, plus the internal control (Fig. 2a, lanes 2, 3 and 4). In a second step, we analysed the capacity of the multiplex reaction to amplify the corresponding PCR fragments in a mixed infection. To do this, we obtained simulated multiple infections by combining the total RNA of samples analysed in lanes 2, 3 and 4 of Fig. 2a. All simulated infections carrying two or three isolates of the three PepMV groups were clearly detected (Fig. 2a, lanes 5–8). In addition, we analysed a sample carrying the two PepMV isolates of the EU/PE group. We observed a single PCR amplicon of the expected size (980 bp, Fig. 2a, lane 9) that rendered the corresponding fragments for the two isolates when it was digested with the *Sac*I enzyme (see below). All analysed samples showed the internal control amplification fragment with the expected size of 186 pb. No extra bands interfering with the specific virus DNA fragment were detected. In addition to the previously analysed PepMV isolates originating from Spain (PepMV-Can1), Italy (DSMZ PV-0632) and the Netherlands (DSMZ PV-0730) (Fig. 2a), we decided to use the multiplex RT-PCR to analyse several isolates of PepMV from Peru (DSMZ PV-0554), UK (DSMZ PV-0674), Italy (DSMZ PV-0716) and Poland (PK), together with the Spanish EU isolates Mu 00.2, CI 01.1, Ba 03.1 and Sp-13 (Pagán et al. 2006). All the isolates rendered a unique DNA fragment corresponding to either the EU/PE or the CH2/US2 groups (Fig. 2b, lanes 1–8). With the exception of the Spanish PepMV-Can1 isolate, the remaining isolates originating from Spain, Peru, The Netherlands, and UK were classified in the EU/PE group, whereas isolates from Italy and Poland were in the CH2/US2 group. All the EU or CH2 isolates previously classified by Pagán et al. (2006) or Hasiów et al. (2007) rendered the expected amplicons of 980 bp and 549 bp, respectively.

Identification of the specific PepMV isolate by restriction analysis

The EU, PE, CH2, CH1/US1 or US2 isolates of PepMV could be identified by direct treatment of the

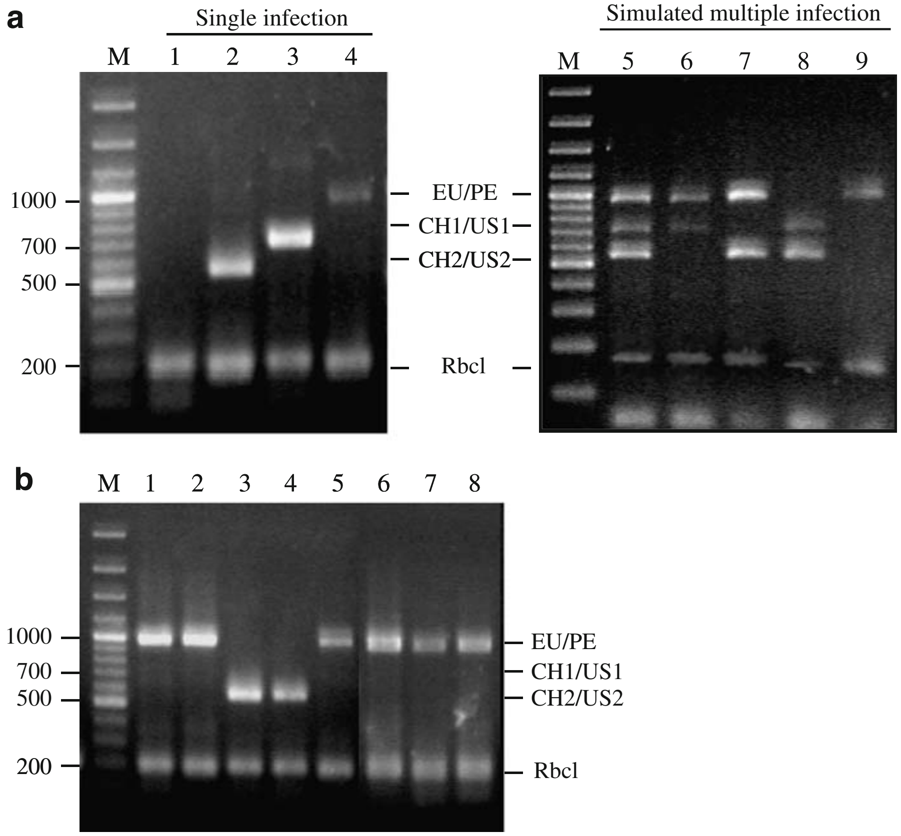

Fig. 2 Analysis by one-step multiplex RT-PCR of tomato samples infected with PepMV genotypes representative of the EU/PE, CH1/US1 or CH2/US2 groups. The multiplex RT-PCR analysis was performed by using a cocktail of primers listed in Fig. 1 plus two primer targets to the ribulose 1.5-biphosphate carboxylase chloroplast gene (0.05 pmol μl^{-1}) as an internal control (Sánchez-Navarro et al. 2005). **a** Analysis of tomato samples infected with PepMV isolates representative of CH2/US2 (lane 2, DSMZ PV-0632) , CH1/US1 (lane 3, PepMV-Can1) or EU/PE (lane 4, DSMZ PV-0730) groups; lane 1 corresponds to healthy tomato. **b** Analysis of simulated multiple infections created by mixing single-infection extracts of samples analysed in lanes 1–3. Lane 5: triple infection; lanes 6–8: double infection carrying the three possible combinations; lane 9: double infection of EU (DSMZ PV-0730) and PE (DSMZ PV-0554) isolates. **c** Analysis of tomato samples infected with PepMV isolates originating from Peru (lane 1, isolate DSMZ PV-0554), UK (lane 2, isolate DSMZ PV-0674), Italy (lane 3, isolate DSMZ PV-0716), Poland (lane 4, isolate PK) and Spain (lanes 5–8, isolates Mu 00.2, CI 01.2, Ba 03.1 and Sp-13). The amplicons corresponding to the EU/PE, CH1/US1 and CH2/US2 PepMV groups plus the internal control (Rbcl) are indicated. Lanes M: 100 bp molecular weight marker

multiplex RT-PCR reaction with the *Sac*I enzyme (Fig. 1 and Table 2). To observe the different DNA fragments obtained in a multiple infection we performed the multiplex RT-PCR analysis plus the *Sac*I digestion of two simulated samples containing total RNA of samples infected with PE, EU and CH2 or EU, CH2 and CH1/US1 isolates (Fig. 3a and b, respectively). Figure 3 shows the analysis of the *Sac*I digestion in which the representative DNA fragments of the PE, EU, CH2 and CH1/US1 are indicated. Furthermore, we decided to identify the isolate of PepMV present in all the samples analysed in Fig. 2. Figure 3c shows the analysis of RT-PCR-*Sac*I. All the samples contained a unique isolate corresponding to the EU (samples: DSMZ PV-0674, DSMZ PV-0730, Mu 00.2, CI 01.2, Ba 03.1, Sp-13), CH2 (samples: DSMZ PV-0632, DSMZ PV-0716 and PK), PE (sample DSMZ PV-0554) or CH1/US1 (sample PepMV-Can1). All the PepMV isolates previously classified as EU (Pagán et al. 2006) rendered the expected fragment of 684 bp (Fig. 3c, lanes 8–11). All the analysed samples rendered one of the predicted

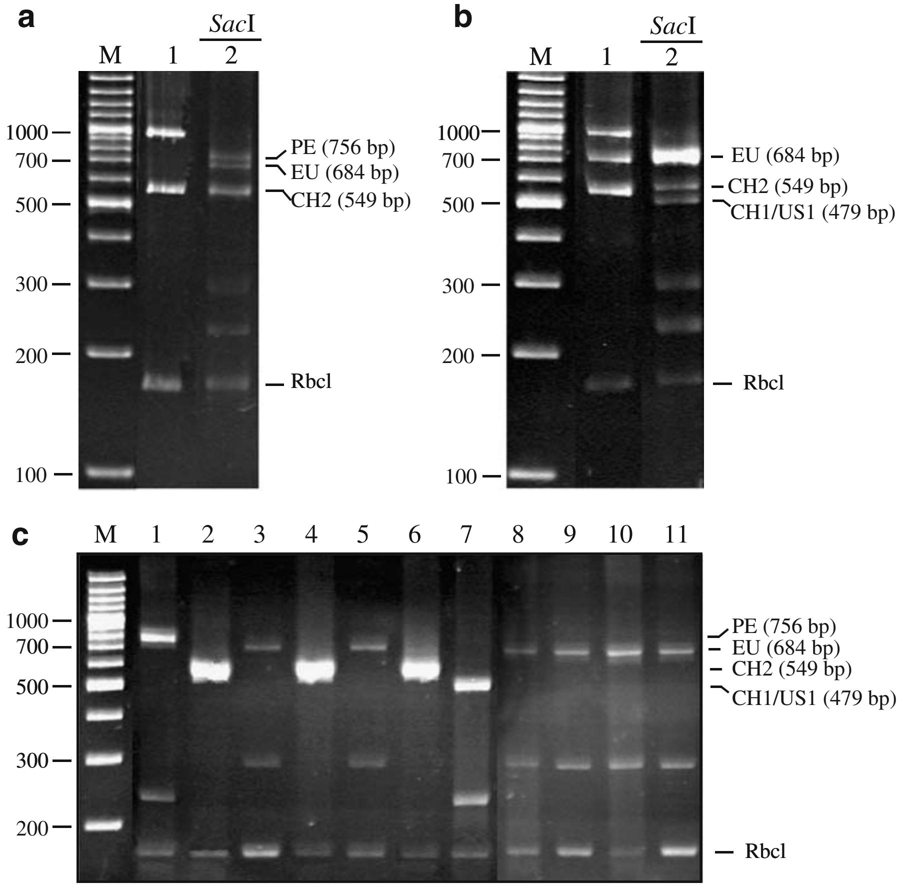

Fig. 3 Restriction analysis of the multiplex RT-PCR products. Simulated triple infection extracts created by mixing total RNA extracted from tomato samples infected with PepMV isolates EU, PE and CH2 (**a**) or EU, CH2 and CH1/US1 (**b**) were subjected to multiplex RT-PCR and direct *Sac*I restriction digestion. Lane 1, undigested sample; lane 2, *Sac*I digestion products. **c** *Sac*I restriction patterns exhibited by amplified PCR products of tomato samples analysed in Fig. 2. Lane 1: DSMZ PV-0554; lane 2: DSMZ PV-0632; lane 3: DSMZ PV-0674; lane 4: DSMZ PV-0716; lane 5: DSMZ PV-0730; lane 6: PK; lane 7: PepMV-Can1; lane 8: Mu 00.2; lane 9: CI 01.2; lane 10: Ba 03.1; lane 11: Sp-13. The DNA fragments representative of each PepMV isolate are indicated (see Table 2). Lanes M: 100 bp molecular weight marker. Numbers represent the base pairs (bp) of the DNA fragment. The analyses were performed in 5% TAE poliacrylamide gels stained with ethidium bromide

DNA fragments for the corresponding PepMV isolate.

Sensitivity of one-step multiplex RT-PCR-*Sac*I and comparison with one-step single RT-PCR, dot-blot hybridisation and ELISA

The simultaneous amplification of several amplicons together with the use of a cocktail of primers could affect the detection limit of the multiplex RT-PCR reaction. To analyse how both aspects influence the sensitivity of the multiplex reaction we have analysed, by either multiplex or single RT-PCR, a serially diluted tomato sample infected with two isolates of PepMV assigned to the EU/PE or CH2/US2 groups. The dilutions were performed by using healthy tomato extract in order to reduce the virus titer. To have a direct comparison between the main virus detection techniques, the same or comparable extract dilutions, in terms of grams of infected tissue ml^{-1}, were analysed by dot-blot hybridisation and ELISA, respectively. Figure 4 shows the detection limit obtained with all the techniques. The end-point dilution limit observed in the multiplex RT-PCR was 5^{-7} (0.64 μg ml^{-1}) and 5^{-6} (3.2 μg ml^{-1}) for the EU/PE or CH2/US2 isolates, respectively (Fig. 4a). The same detection limits were

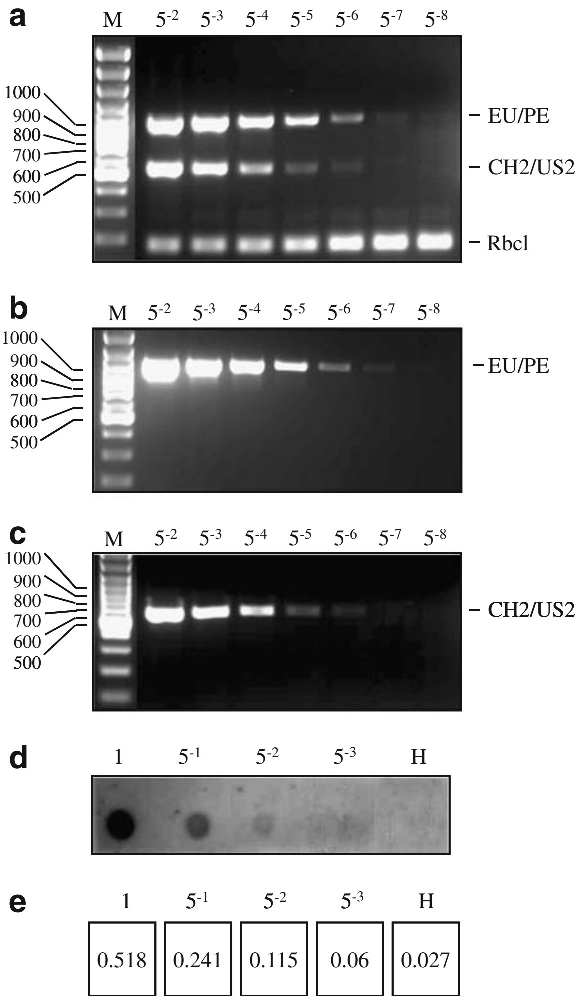

Fig. 4 Comparison of the sensitivity detection limit for PepMV detection by multiplex or single RT-PCR, non-isotopic molecular hybridisation and DAS-ELISA. Total RNA extracted from a tomato sample infected with two PepMV isolates representative of the EU/PE and CH2/US2 groups was five-fold serially diluted using total RNA extracted form healthy tomato. Five-fold serial dilutions were analysed by multiplex RT-PCR (**a**), single RT-PCR using the specific primers for the EU/PE (**b**) or CH2/US2 (**c**) PepMV genotypes, and non-radioactive molecular hybridisation (**d**). In **e**, comparable five-fold dilution series in phosphate buffer of the same tomato infected sample were analysed by ELISA. Each point is the mean of three replications. The last positive signal for the EU/PE or CH2/US2 isolates was 5^{-7} (1:78125) in (**a**) and (**b**) or 5^{-6} (1:15625) in (**a**) and (**c**), respectively. In (**d**) and (**c**), the last dilution with a positive signal corresponded to 5^{-2} (1:25). The original dilution (1) corresponded to a tissue concentration of 0.05 g ml^{-1}. The PCR products corresponding to the EU/PE, CH2/US2 PepMV isolates or the internal control (Rbcl) are indicated. H, healthy tomato plant. Numbers at the top indicate the dilution performed on the original undiluted sample. Lanes M: 100 bp molecular weight marker

obtained using a single RT-PCR in which we included only the pair of primers required to amplify either the EU/PE or CH2/US2 isolates (Fig. 4b and c). Apparently, neither the multiple infections nor the presence of a cocktail of six different primers affect the limit detection of the multiplex reaction, regardless of the amount of EU/PE or CH2/US2 isolate present in the infected tissue. When the serial dilutions were analysed by dot-blot hybridisation or ELISA, PepMV was detected up to the 5^{-2} dilution (2 mg ml^{-1}; Fig. 4d and e), representing a detection limit 3,125 (5^5 times) less sensitive than the multiplex RT-PCR.

Analysis of field samples and previously characterised PepMV isolates by both multiplex RT-PCR-*Sac*I and RT-PCR-Hanssen, dot-blot hybridisation and ELISA tests

The robustness of the multiplex RT-PCR-*Sac*I to be used in routine diagnosis was analysed by performing the analysis of several PepMV isolates from six different countries previously described (samples 1–17) plus 42 field tomato samples harvested in three different Spanish regions (samples 18–59). Total RNA, extracted as described above, was used to perform multiplex RT-PCR and dot-blot hybridisation analysis. Comparable extracts in terms of grams of tissue ml^{-1} were analysed by ELISA procedure. In addition, the multiplex RT-PCR-*Sac*I was compared with the RT-PCR-Hanssen. Briefly, this methodology consisted of the amplification by RT-PCR with two pairs of primers common to all the genotypes of two different fragments of PepMV genome: a part of the RpRd and the complete CP gene. Later, an RFLP analysis with two and four restriction endonucleases for RdRp and CP-amplified PCR products, respectively, was performed and theoretically five different genotypes could be distinguished. The comparison between both methods is detailed in Table 3.

Regarding the PepMV isolates previously characterised (samples 1–17, Table 3), all diagnostic techniques were able to detect PepMV. Both RT-PCR-*Sac*I and RT-PCR-Hanssen identified the same PepMV isolates except for the samples DSMZ PV-0632 and DSMZ PV-0716. The PepMV isolates present in both samples were identified as CH2 by RT-PCR-*Sac*I; meanwhile the RT-PCR-Hanssen rendered a restriction pattern of the CP-amplified product not associated with any of the described PepMV isolates. The analysis

Table 3 Comparative analysis for the presence of PepMV performed by DAS-ELISA, Molecular Hybridisation (MH), multiplex RT-PCR with *SacI* restriction (RT-PCR-*Sac*I) and RFLP-PCR method described by Hanssen et al. (2008) (RT-PCR-Hanssen) to previously described PepMV isolates and field tomato samples

	Sample code	Area	Variety	ELISA	MH	RT-PCR-*Sac*I			RT-PCR-Hanssen		
1	PV-0632	Italy	Unknown	+	+	CH2	-	-	nd[a]	-	-
2	PV-0674	UK	Unknown	+	+	-	-	EU	-	-	EU
3	PV-0716	Sardinia, Italy	Unknown	+	+	CH2	-	-	nd[a]	-	-
4	PV-0730	The Netherlands	Unknown	+	+	-	-	EU	-	-	EU
5	PV-0554	Peru	*S. muticatum*	+	+	-	-	PE	-	-	PE
6	PK	Poland	Unknown	+	+	CH2	-	-	CH2[b]	-	-
7	Mu 00.2	Murcia, Spain	Unknown	+	+	-	-	EU	-	-	EU
8	Mu 00.3	Murcia, Spain	Unknown	+	+	-	-	EU	-	-	EU
9	Mu 00.4	Murcia, Spain	Unknown	+	+	-	-	EU	-	-	EU
10	Mu 00.5	Murcia, Spain	Unknown	+	+	-	-	EU	-	-	EU
11	CI 01.1	Canary Islands, Spain	Unknown	+	+	-	-	EU	-	-	EU
12	CI 01.2	Canary Islands, Spain	Unknown	+	+	-	-	EU	-	-	EU
13	CI 01.3	Canary Islands, Spain	Unknown	+	+	-	-	EU	-	-	EU
14	Al 01.2	Alicante, Spain	Unknown	+	+	-	-	EU	-	-	EU
15	Ba 03.1	Barcercelona, Spain	Unknown	+	+	-	-	EU	-	-	EU
16	Can-1	Tenerife, Spain	Unknown	+	+	-	CH1/US1[e]	-	-	CH1/US1	-
17	Sp-13	Spain	Unknown	+	+	-	-	EU	-	-	EU
18	9394	Arico (Tenerife, Spain)	Unknown	-	-	CH2	-	EU	CH2	-	EU
19	9405	Arico (Tenerife, Spain)	Mariana	+	+	-	CH1/US1[c]	EU	nd	EU	
20	9415	Arico (Tenerife, Spain)	Unknown	-	-	-	CH1/US1[d]	EU	nd	EU	
21	9416	Arico (Tenerife, Spain)	Unknown	-	-	-	-	EU	-	-	EU
22	9423	Arico (Tenerife, Spain)	Mariana	+	+	-	CH1/US1[d]	EU	nd	EU	
23	9432	Arico (Tenerife, Spain)	Boludo	+	+	-	-	EU[e]	-	-	EU
24	9433	Arico (Tenerife, Spain)	Boludo	-	-	-	-	-	-	-	-
25	9435	Arico (Tenerife, Spain)	Boludo	+	+	-	-	EU[f]	-	-	EU
26	9453	Agüimes (Gran Canaria, Spain)	Boludo	-	-	CH2	-	EU	CH2	-	EU
27	9454	Agüimes (Gran Canaria, Spain)	Boludo	-	-	CH2	-	EU	CH2	-	EU
28	9494	S. Lucía (Gran Canaria, Spain)	Unknown	+	+	-	CH1/US1[c]	EU*	nd	EU	
29	9495	Los abrigos (Tenerife, Spain)	Unknown	+	+	-	CH1/US1[c]	EU	nd	EU	
30	9503	Los abrigos (Tenerife, Spain)	Unknown	+	+	-	CH1/US1[c]	EU*	nd	EU	
31	9506	Los abrigos (Tenerife, Spain)	Unknown	+	+	-	CH1/US1[c,e]	EU	nd	EU	
32	9507	Abades (Tenerife, Spain)	Dorothy	-	-	-	-	-	-	-	-
33	9509	Abades (Tenerife, Spain)	Dorothy	-	-	-	-	-	-	-	-
34	9511	Abades (Tenerife, Spain)	Dorothy	-	-	CH2	-	EU	CH2	-	EU
35	9516	Tenerife, Spain	Dorothy	+	+	-	-	EU	-	-	EU
36	9527	Tenerife, Spain	Unknown	-	-	-	CH1/US1[e]	EU	nd	EU	
37	9533	Tamaimo (Tenerife, Spain)	Boludo	-	-	CH2	CH1/US1	EU	CH2	CH1/US1	EU
38	9537	Tamaimo (Tenerife, Spain)	Mariana	-	-	-	-	-	-	-	-
39	9538	Tamaimo (Tenerife, Spain)	Mariana	-	-	CH2	-	EU	CH2	-	EU
40	9539	Tamaimo (Tenerife, Spain)	Mariana	-	-	-	-	-	-	-	-
41	9551	Tamaimo (Tenerife, Spain)	Maya	-	-	-	-	-	-	-	-
42	9636	Águilas (Murcia, Spain)	Boludo	-	-	CH2	-	EU	CH2	-	EU
43	9641	Mazarrón (Murcia, Spain)	Unknown	+	+	CH2[e]	-	-	CH2	-	-

Table 3 (continued)

	Sample code	Area	Variety	ELISA	MH	RT-PCR-*Sac*I			RT-PCR-Hanssen		
44	9645	Mazarrón (Murcia, Spain)	Unknown	+	+	CH2	-	EU	CH2	-	EU
45	9646	Mazarrón (Murcia, Spain)	Unknown	+	+	CH2	-	EU	CH2	-	EU
46	9647	Mazarrón (Murcia, Spain)	Unknown	+	+	-	-	EU	-	-	EU
47	9648	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
48	9649	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2[e]	-	EU[e]	CH2	-	EU
49	9653	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
50	9654	Mazarrón (Murcia, Spain)	Pitenza	+	+	CH2	-	EU	CH2	-	EU
51	9658	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
52	9659	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
53	9661	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
54	9662	Mazarrón (Murcia, Spain)	Unknown	+	+	-	-	EU	-	-	EU
55	9665	Mazarrón (Murcia, Spain)	Unknown	-	+	CH2	-	EU	CH2	-	EU
56	9674	Lorca (Murcia, Spain)	Flortyl	+	+	CH2	-	EU	CH2	-	EU
57	9679	Lorca (Murcia, Spain)	Velasco	-	-	CH2	-	-	CH2	-	-
58	9680	Lorca (Murcia, Spain)	Unknown	-	-	CH2	-	EU	CH2	-	EU
59	9682	Lorca (Murcia, Spain)	Flortyl	+	+	CH2	-	EU	CH2	-	EU
Partial positive results of the previously characterized PepMV isolates (samples 1-17)						3	1	12/1[g]	1	1	12/1[g]
				17	17		17			17	
Partial positive results of the field samples (18-59)						22	9	34	22	1	34
				23	24		36			36	
Total positive results						25	10	46/1[g]	23	2	46/1[g]
				40	41		53			53	

nd pattern not described by Hanssen et al. (2008), *EU** Amplified fragment of 980 bp not digested with *Sac*I enzyme. Sequenced and compared with isolates of GenBank Database being grouped with the EU isolates

[a] Amplified fragment from the CP gene, directly sequenced with the primers described by Pagán et al. (2006)

[b] Result confirmed by RT-PCR for the CP gene with specific primer for CH2/US2 isolates sPepMVCP CH2/US2 and common reverse primer (Pagán et al. 2006)

[c] Samples tested positive to CH1/US1 isolates analysed by multiplex RT-PCR, however that isolate was not determined when analysed with Hanssen et al. (2008). Result was confirmed by RT-PCR for the coat protein gene with specific primer for CH1/US1 isolates sPepMVCP CH1/US1, and common reverse primer (Pagán et al. 2006), cloned and sequenced

[d] Samples tested positive to CH1/US1 isolates analysed by multiplex RT-PCR, however that isolate was not determined when analysed with Hanssen et al. (2008). Result was not determined with the analysis by RT-PCR for the coat protein gene with specific primer for CH1/US1 isolates sPepMVCP CH1/US1 and common reverse primer (Pagán et al. 2006)

[e] PCR products amplified with the mixture of all the primers of the multiplex RT-PCR, purified and directly sequenced

[f] Result confirmed by RT-PCR for the CP gene with specific primer for EU isolates sPepMVCP EU and common reverse primer (Pagán et al. 2006)

[g] Number of EU/number of PE isolates

of the nucleotide sequence of the CP-amplified products showed a change at nucleotide 5711 (in a CH2 genome) responsible for the unclassified restriction pattern (GenBank Accession number DQ000985). However, the CP sequences of PepMV isolates DSMZ PV-0632 and DSMZ PV-0716 showed 98% nt identity with CH2 and US2 isolates (GenBank accession numbers DQ000985 and AY509927).

Regarding the field tomato samples, we obtained 23, 24 or 36 samples out of 42 positive by ELISA, dot-blot hybridisation and multiplex RT-PCR-*Sac*I, respectively (Table 3; samples 18–59). All positive samples detected by ELISA and dot-blot were also detected by multiplex RT-PCR-*Sac*I. Considering the total number of positive samples detected by multiplex RT-PCR-*Sac*I, the percentage of positives detected by ELISA and dot-blot corresponded to 63.8% and 66.6%, respectively. The capacity of the multiplex reaction to discriminate among the five PepMV genotypes allows us to identify the corresponding isolates present in the infected samples. Thus, eight samples out of 36 were single-infected, and 27 and one sample(s) were double- and triple-infected, respectively. The majority of the single-infected samples showed the EU isolate (six out of eight) the remainder being infected with the CH2 (two out of eight, respectively). For the double-infection we found two different combinations: CH2 with EU (19 out of 27) or CH1/US1 with EU (8 out of 27). Interestingly, the two double-infection combinations corresponded to two different geographic regions in which the presence of CH2 and EU isolates was representative of the Murcia area, whereas the combination CH1/US1 with EU was specific for the Canary Islands (Gran Canaria and Tenerife). One sample from Tenerife (Canary Islands) showed a PepMV infection with three isolates that corresponded to CH2, CH1/US1 and EU genotypes.

All positive samples rendered the expected DNA fragment after the *Sac*I digestion during the identification of the corresponding PepMV genotype except two samples from Gran Canaria and Tenerife islands (sample numbers 9494 and 9503). In both cases, the amplified fragment of 980 bp characteristic of the EU/PE group was not digested with the *Sac*I enzyme. The PCR products were sequenced and compared to PepMV isolates representative of both EU and PE genotypes, showing 98–97% nt identity with isolates of the European tomato strain published in the GenBank database (Accession numbers AF484251, AJ606360). Figure 5a shows the phylogenetic analysis in which both isolates grouped with the EU isolates.

To confirm the correct classification of PepMV isolates, PCR products of isolates classified into the three different genotypes found in the field samples, were sequenced and compared with the PepMV isolates published in GenBank (Table 3). Two EU isolates (sample numbers 9432 and 9649) showed 98% nt identity with isolates of the European tomato strain (GenBank accession numbers AF484251, AJ606360 and AJ438767). Two isolates (sample number 9506 and PepMV-Can1 isolate), classified as CH1/US1, showed 99% nt identity with the US1 (GenBank accession number AY509926) and CH1 (GenBank accession number DQ000984) sequences, respectively. Two isolates (sample numbers 9641 and 9649) classified as CH2 showed 99–98% nt identity with a recently published Belgian isolate (GenBank accession number EF599605), CH2 and PK isolates (GenBank accession numbers DQ000985 and EF408821).

Finally, all field tomato samples were analysed by the RT-PCR-Hanssen method (Table 3). Results were similar in 34 out of the 42 samples analysed; however the CH1/US1 isolate identified by RT-PCR-*Sac*I restriction analysis in eight out of the 42 samples was not determined by the RT-PCR-Hanssen method. In order to check the presence or absence of this isolate in these samples, an RT-PCR with sense-specific primers designed for the CP gene of PepMV groups CH1/US1 (sPepMV CP CH1/US1), EU/PE (sPepMV EU) and CH2/US2 (sPepMV CH2/US2) and the common reverse primer described by Pagán et al. (2006) was performed. Previous results obtained with single-infected samples representative of the three PepMV groups (samples 9435 for the EU group, Can-1 for the CH1/US1 and PK for the CH2/US2; Table 3) showed the specificity of the sense primers. Only the specific CP amplicon of 770 bp corresponding to the CH1/US1 isolate was obtained in five out of these eight isolates. PCR products were cloned and sequenced. The phylogenetic analysis grouped all amplified CP fragments with the CH1/US1 strain as shown in Fig. 5b. In the remaining samples (three out of eight) which were positive to CH1/US1 by multiplex RT-PCR-*Sac*I, but were not determined by RT-PCR-Hanssen, there was no amplification with the specific primers for the CH1/US1 CP gene (Table 3).

Discussion

The increasing number of PepMV genotypes so far characterised together with the observation that mixed infection is a common phenomenon in field samples (here and Hanssen et al. 2008) that could result in more severe symptoms and/or recombination events, entirely justify the incorporation of a routine detection method that permits the identification of the corresponding PepMV genotype. In the present work we have used the variability observed in the polymerase gene to design specific primers for the CH2/US2, CH1/US1 and EU/PE groups. In addition, the presence or absence of different *Sac*I restriction sites in the amplified DNA fragments permits the identi-

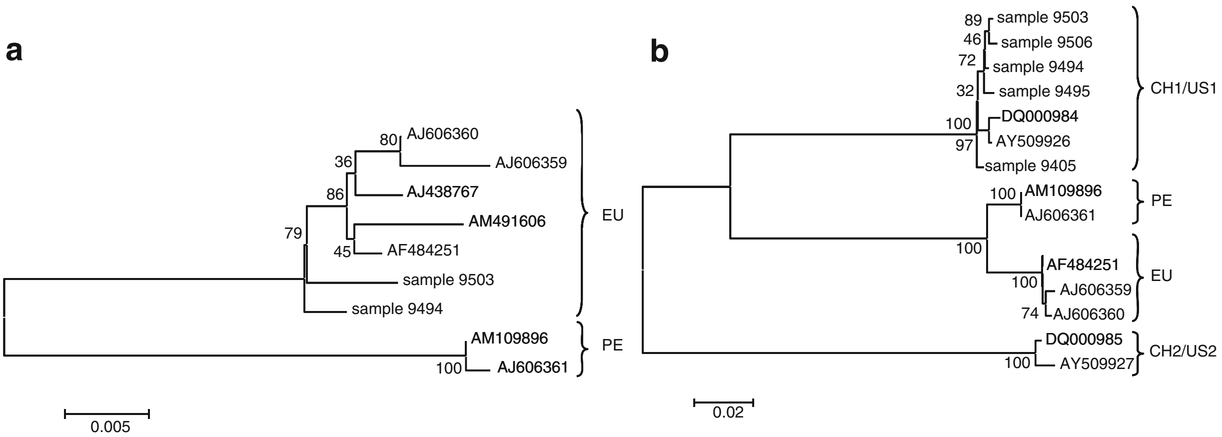

Fig. 5 **a** Phylogenetic analysis of the EU/PE isolates lacking the *Sac*I restriction site. The analysis was performed using the nucleotide sequence of 980 nt (nt 1,563–2,543 in the PepMV genome) corresponding to the specific PCR product of the EU/PE genotype of the RdRp gene. Nucleotide sequences corresponded to EU (GenBank accession numbers. AJ606360, AJ606359, AJ438767, AM491606 and AF484251) and PE (GenBank accession numbers AM109896 and AJ606361) genotypes, plus the two tomato samples 9494 and 9503. **b** Phylogenetic analysis of the CH1/US1 isolates not determined by the RFLP-PCR method described by Hanssen et al. (2008). The analysis was performed using the CP nucleotide sequence of 770 nt (nt 5593–6369 in the PepMV genome). Nucleotide sequences corresponded to EU (GenBank accession numbers. AJ606360, AJ606359 and AF484251), PE (GenBank accession numbers AM109896 and AJ606361), CH1 (GenBank accession number DQ000984), US1 (Accession number AY509926), CH2 (GenBank accession number DQ000985) and US2 (GenBank accession number AY509927) genotypes, plus the five tomato samples 9405, 9494, 9495, 9503 and 9506. Nucleotide sequences were aligned with CLUSTAL-X version 1.83 with its default parameters (Thompson et al. 1997). The neighbour-joining phylogenetic trees were obtained using the MEGA version 3.1. (Kumar et al. 2004). The statistical reliability of the constructed trees was assessed by the bootstrap method based on 10,000 pseudoreplicates. The number above the nodes indicates the percentage of bootstrap replicates which supported the branching

fication of the CH2, US2, CH1/US1, EU and PE isolates.

So far, there is only methodology available based on a single RT-PCR-RFLP assay adjusted for the simultaneous detection of the five different genotypes (Hanssen et al. 2008). However, this approach implies the amplification of two different coding sequences (polymerase and coat protein genes) that should be digested by six different restriction enzymes. The new approach presented here significantly reduces the analytical process since it is a multiplex reaction in which the RT-PCR products permit discrimination between EU/PE, CH1/US1 and CH2/US2 PepMV groups, and only a *Sac*I endonuclease is required to discriminate between EU-PE or CH2-US2 genotypes. The comparison between both RFLP-PCR methods revealed similar results; however multiplex RT-PCR-*Sac*I was more sensitive to identify the CH1/US1 isolate in eight out 42 field samples. This observation was confirmed in five samples by sequencing the CP gene. This difference could be caused by an unequal concentration of CH1/US1 and EU isolates present in mixed infected samples. Therefore, an RT-PCR with common primers, used in the Hanssen et al. (2008) method, will amplify more efficiently in mixed infections, the isolate present at a higher concentration, a disadvantage that could be overcome by using specific primers for each PepMV strain. However, we found three samples that were classified as CH1/US1 plus EU by RT-PCR-*Sac*I and were negative by using the specific CH1/US1 CP primers. This pattern could be explained by variation in the CP gene or by the presence of recombinant isolates between CH1/US1 and EU strains. The analysis of such samples by RT-PCR-Hanssen using both the RdRp and CP genes, detected only the EU strain, indicating the absence of recombinant isolates. This result reinforces the idea that the inability to amplify the CH1/US1 CP gene is due to nucleotide changes. In this sense, nucleotide variations in the CP gene have been found in the DSMZ-PV-0632 and DSMZ-PV-0716 samples, and were responsible for the unclassified CP restriction pattern obtained by RT-PCR-Hanssen.

The advantages mentioned above for the RT-PCR-*Sac*I approach are completed by the incorporation of two primer targets to a host mRNA as an internal control, a procedure that avoids the presence of false negatives. However, unlike the remaining PepMV genotypes, CH1 and US1 could not be discriminated by restriction analysis due to the high nucleotide sequence identity among them (98.7%) (Ling 2007). Taken together, the direct comparison between both RT-PCR-RFLP and RT-PCR-*Sac*I techniques, presents the new multiplex reaction as a simpler, more specific and sensitive methodology, and a clear alternative for the previous RT-PCR-RFLP assay. Other methods have been developed for diagnosis of PepMV infection such as the one-step immunocapture real-time TaqMan RT-PCR assay (Ling et al. 2007), which is a highly sensitive technique capable of detecting all the genotypes of PepMV in one single reaction. The new multiplex RT-PCR-*Sac*I method could be perfectly compatible with the previous Real time RT-PCR since the latter should be the option for routine diagnosis whereas the multiplex RT-PCR is more appropriate for an epidemiological survey. In addition, the observation of a high percentage of PepMV mixed infections in the field make the new RT-PCR-*Sac*I assay even more necessary to reduce the risk of severe PepMV symptoms and/or recombination events.

To validate the new approach we have analysed different aspects that should be taken into consideration before incorporating it into routine diagnosis. First, we have analysed the specificity of the reaction in either single or multiple infections. All the analysed samples infected with the previously characterised PepMV isolates rendered the expected DNA fragments with good discrimination among them, even between the EU and PE isolates that share a nucleotide identity of 95%. No extra bands were observed in single-infected plants indicating the specificity of the selected primers. The same results were obtained when we analysed simulated multiple infections. The reliability of the method was demonstrated by sequencing the obtained amplicons that were always in agreement with the corresponding sequences published in GenBank database. We decided to use the new approach to characterise the genotype of previously reported PepMV isolates from different European origins (The Netherlands, Italy, Poland, UK and Spain). All the samples rendered a unique genotype that corresponded to the EU and CH2 isolates, except for the Spanish PepMV-Can1, that was classified as CH1/US1. According to this result, the nucleotide sequence of the amplicon derived from the PepMV isolate PepMV-Can1 showed a nucleotide identity of 99% with the US1 and CH1. A similar result has been reported using another region of the polymerase gene (Alfaro-Fernández et al. 2008).

Another critical aspect for a detection procedure is the detection limit. Previous results obtained by the multiplex RT-PCR assay have shown that a cocktail of seven pairs of primers affect the detection limit (Sánchez-Navarro et al. 2005). Although the multiplex RT-PCR-*Sac*I procedure presented here contains a cocktail of six different primers, no differences in terms of detection limit were observed when a serially-diluted double-infected sample was analysed by either the multiplex (containing a cocktail of six primers) or the single (containing two primers) RT-PCR. When the similarly diluted samples were analysed by ELISA or dot-blot hybridisation, the detection limit of the multiplex reaction was 3,125 (5^5) times higher, a difference in the range of previously reported results (Saade et al. 2000; Sánchez-Navarro et al. 1998, 2005).

To check for the reliability and the robustness of the new multiplex RT-PCR-*Sac*I developed, a total of 42 tomato field samples from three different Spanish regions were analysed using DAS-ELISA, dot-blot hybridisation and the multiplex RT-PCR-*Sac*I and RT-PCR-Hanssen. Except for one sample, ELISA and dot-blot hybridisation were able to detect the same positive samples, confirming the previous observation that both procedures have a similar detection limit. The multiplex technique was able not only to identify the corresponding PepMV isolates, but also to detect twelve more positives than the serological and dot-blot methods. The multiplex procedure was not able to discriminate between the EU and PE isolates only in two double-infected samples, since the amplicon corresponding to the EU/PE group was not digested with the *Sac*I enzyme. The characterisation of the nucleotide sequence of the amplicon and the posterior phylogenetic analysis revealed that both isolates were grouped with the EU genotypes. Apparently, the presence of a 980 bp or 684 bp fragment after the *Sac*I restriction is representative of the EU genotypes. It should be taken in consideration that both EU and PE isolates share the highest identity percentage (95%) among all PepMV isolates discriminated by the multiplex procedure.

The multiplex RT-PCR-*Sac*I allowed the identification of several double- and triple-infected plants. Interestingly, the majority of positive samples were double-infected (75%), in which the presence of the

CH1/US1 with EU or the CH2 and EU genotypes was representative of the Canary Islands and Murcia Region, respectively. Field samples showed different combinations of isolates commonly found in Europe. The EU genotype was the most prevalent since it was found in 94% of the infected plants, followed by the CH2 (61%) and CH1/US1 (25%) genotypes. A high percentage of double-infected plants was also detected in Belgium (Hanssen et al. 2008) and Spain (Martínez-Culebras et al. 2002; Pagán et al. 2006), in which the only detected genotypes were CH2 and EU, although CH1/US1 has been reported recently in the Canary Islands, Spain (Alfaro-Fernández et al. 2008). Apparently, double-infected plants are more frequent than we expected, in which the CH2 and EU genotypes are representative of the European region. Since the EU genotype was the more prevalent PepMV genotype in European tomato crops (Aguilar et al. 2002; Cotillon et al. 2002; Ling 2007), the question that arises is how and when the CH2 and CH1/US1 genotypes were introduced. In this context, we are performing a survey of tomato samples harvested from 2000.

The multiplex one-step RT-PCR-*Sac*I procedure developed in this study represents a significant advance in the diagnosis of PepMV. The multiplex technology reduces costs, time and avoids the use of multiple digestions and/or cloning and sequencing steps to identify the PepMV isolate. Furthermore, since multiple infections are present in tomato crops, this technology will facilitate the characterisation of the phytosanitary status of tomato crops and the correlation between tomato disorders and the different PepMV isolate combinations.

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