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A RT–PCR assay combined with RFLP analysis for detection and differentiation of isolates of *Pepino mosaic virus* (PepMV) from tomato

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Abstract

The partial nucleotide sequence of the RNA polymerase gene from one isolate of *Pepino mosaic virus* (PepMV) was determined. Phylogenetic and distance analysis indicated that this isolate was related to other isolates of PepMV previously reported. To develop a method for detecting PepMV by reverse transcriptase–polymerase chain reaction (RT–PCR), a pair of primers was designated from RNA polymerase sequences. RT–PCR with RNA from a large number of tomato samples with PepMV symptoms, positive controls of PepMV, weed samples containing PepMV, and other potexviruses as negative controls confirmed the specificity of the primers. Restriction endonuclease digestion of the RT–PCR products distinguished three restriction fragment length polymorphism (RFLP) types. The majority of isolates were included within type P1, which correspond with the PepMV isolates found in Europe. This type and type P2, which corresponded with the original PepMV isolated from *Solanum muricatum,* appear more closely related to each other than to type P3. Type P3 had completely different RFLPs from the other two types studied. It may represent a further line within the PepMV virus. The RT–PCR–RFLP assay is proposed as a rapid and easy method to detect and identify new isolates of the PepMV virus.

Introduction

Pepino mosaic virus (PepMV) is a potexvirus which was first described on Pepino (*Solanum muricatum*) in Peru (Jones et al., 1980). PepMV came to our attention because it was recently identified as the causal agent of a new disease occurring in protected tomato (*Lycopersicon esculentum*) crops in the Netherlands (van der Vlugt et al., 2000). It was also noted in the UK (Wright and Mumford, 1999), France (information withdrawn from Internet, Ministère de L'Agriculture), Germany (Lesemann et al., 2000), Spain (Jordá et al., 2000a; 2001a), Italy (information withdrawn from Internet, Instituto di Fitovirologia Applicata, Torino) and North America (French et al., 2001). Symptoms consist of distorted leaf development, chlorosis and a yellow mosaic. Other plants show a dark green mosaic and bubbling of the leaf surface. Green striations can also be observed on the stem, petioles and sepals. Diseased plants may have discoloured fruits. In southern Europe, symptoms decrease with increasing temperature, but it is not clear whether this effect occurs in northern Europe.

PepMV is highly contagious and has quickly become a serious problem for European tomato production. Yield losses have not been quantified but significant crop losses are likely if action is not taken to eliminate infections. Measures to control the disease include destruction of affected plants and prevention of virus transmission through hygiene precautions. Therefore, sensitive, rapid and accurate detection methods are needed for the development of effective management of PepMV disease epidemics.

Different approaches have been used to detect and identify the virus. They include immunosorbent electron microscopy (IEM) (van der Vlugt et al., 2000), enzyme-linked immunosorbent assay (ELISA) (Jordá et al., 2000b; Lázaro-Pérez et al., 2002) and molecular

methods including reverse transcriptase–polymerase chain reaction (RT–PCR) and DNA sequencing (van der Vlugt et al., 2000; Mumford and Metcalfe, 2001). The aim of this study was to develop a RT–PCR assay for specific detection of PepMV in tomato tissues and to evaluate the suitability of restriction analysis of the PCR fragment for rapid differentiation of isolates of the virus.

Materials and methods

Plant material and RNA preparation

One hundred and two samples of tomato plants showing a range of mosaic, yellowing, chlorosis, leaf distortion and leaf bubbling, were collected from different areas of Spain (Almeria, Murcia and Canary Islands). Five weeds (*Amaranthus* sp., *Malva parviflora*, *Nicotiana glauca*, *Solanum nigrum* and *Sonchus oleraceus*) were also included. Three typical PepMV isolates from different geographical origins obtained from tomato were included as positive controls: DSMZ PV-0557, from southern Europe, DSMZ PV-0558, from Germany and 1127-11 from the UK. The two first isolates were provided by the German Collection of Micro-organisms and Cell Cultures (DSMZ) and the last one by ADGEN Agrifood Diagnostics, Scotland UK. A typical PepMV isolate (DSMZ PV-0554, from Peru) obtained from *S. muricatum* and provided by the DSMZ was also studied. Five isolates of different potexviruses were included as negative controls: *Viola mottle virus*, VMV (DSMZ PV-0224); *Potato virus X*, PVX (DSMZ PV-0018); *Cactus virus* X, CVX (DSMZ PV-0559); *Potato aucuba mosaic virus*, PAMV (DSMZ PV-0007) and *Narcissus mosaic virus* (NMV) 1027-11 provided by ADGEN Agrifood Diagnostics, Scotland UK.

RNA was isolated from 100 mg of leaf tissue from infected plants using the Tripure isolation reagent (Roche Molecular Biochemicals, Switzerland) according to the manufacturer's instructions. Total RNA was diluted to about 0.1–0.5 $\mu g\ \mu l^{-1}$ and 5 µl aliquots were used for RT–PCR.

Sequencing, phylogenetic inference and primer design

A RT–PCR DNA product obtained from one tomato sample, using the universal potexvirus primers potex 1 and potex 2 (Gibbs et al., 1998), was cleaned with the GeneClean Purification Kit (Bio 101) and directly sequenced using the amplification primers and a Taq dideoxy cycle sequencing kit (Applied Biosystems) in an Applied Biosystems model 373 Automatic DNA sequencer.

The resulting sequence was compared with (575 nt) RNA polymerase sequences obtained from the GenBank/EMBL database, and corresponding to PepMV and other potexviruses: AJ270991, *Pepino mosaic virus* (PepMV); AJ270992, *Pepino mosaic virus* (PepMV); AJ270985, *Cactus virus* X (CVX); AJ270986, *Cymbidium mosaic virus* (CymMV); AJ270987, *Hydrangea ringspot virus* (HRSV); AJ270988, *Narcissus mosaic virus* (NMV); AJ270989, *Potato aucuba mosaic virus* (PAMV); AJ270990, *Potato virus X* (PVX). The sequences were aligned using the multiple-sequence alignment program CLUSTAL W (Thompson et al., 1994). The genetic distances were calculated using the Jukes–Cantor model and phylogenetic inference was estimated by the neighbour-joining (NJ) method (Saitou and Nei, 1987). Development of the NJ tree and the statistical confidence of a particular group of sequences in the NJ tree, evaluated by bootstrap test (1000 pseudoreplicates), were performed using the computer program MEGA version 1.0 (Kumar et al., 1993).

Specific PepMV regions in the RNA polymerase gene sequences were identified, and degenerate PepMV primers were designed using OLIGO (National Biosciences, Plymonth, USA). The primers were pep1: d5′-CWG TKG AYT TYG AGC CAA GAA C-3′ and pep2: d5′-AGC HGT TCK RTT AAA RTT CCA ACC-3′.

RT–PCR reactions and RFLP analysis

RT–PCR reactions of the RNA polymerase gene were performed using a RT-Netzyme RNA kit (Molecular Netline Bioproducts, N.E.E.D, SL, Spain) according to the manufacturer's instructions. After reverse transcription reaction, PCR reaction mixtures were incubated in a thermocycler (Eppendorf Mastercycler Personal) for 35 cycles consisting of 1 min at 95 °C, 1 min at 52 °C and 1 min at 72°C.

RT–PCR products were digested with the restriction enzymes *Alu*I, *Dde*I, *Hae*III, *Hinc*II, *Taq*I and *Sau*3AI (MBI Fermentas, Vilnius, Lithuania). PCR products and their restriction fragments were separated on 1%

and 3% agarose gels, respectively, with 0.5X TBE buffer (44.5 mM Tris–borate, 1 mM EDTA, pH 8). After electrophoresis, gels were stained with ethidium bromide (0.5 μg ml^{-1}), and the DNA bands visualized under UV light. Sizes were estimated by comparison against a DNA length standard (GeneRuler™ 50 bp DNA ladder, MBI Fermentas, Vilnius, Lithuania).

Results

The universal potexvirus primers (Gibbs et al., 1998) were used to obtain a RT–PCR product of the RNA polymerase gene from one tomato sample denoted 3253. This RT–PCR product was sequenced, and submitted to DDBJ/EMBL/GenBank Nucleotide Sequence Database, accession number AJ430672. From the comparison of a portion (575 nt) of the RNA polymerase sequences, isolate 3253 was shown to be different from two PepMV sequences available in the GenBank database of isolates from tomato (AJ270992) and *S. muricatum* (AJ270991, from Peru). It had 80.5% identity with the isolate from tomato and 79.3% identity with the Peruvian isolate, while the tomato and *S. muricatum* isolates reached higher nucleotide identity (95%). Sequences of PepMV exhibited identities of 60–69% to sequences of other potexviruses obtained from the database. The relationships within PepMV isolates and the relationships between PepMV isolates and other potexviruses are illustrated in a cluster analysis (Figure 1). From the phylogenetic tree it is clearly evident that isolate 3253 is distinct from other PepMV isolates.

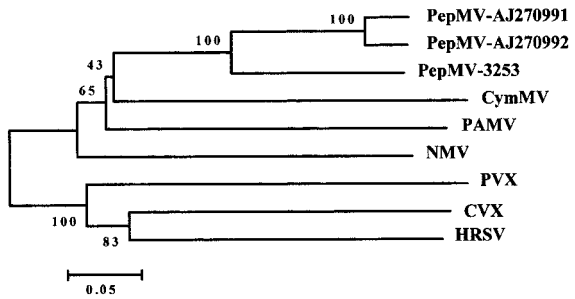

Figure 1. Neighbour-Joining tree based on nucleotide divergences, estimated according to Jukes–Cantor model, from partial RNA polymerase sequences of PepMV isolates and selected potexviruses. The viruses used in the analysis included *Pepino mosaic virus* (PepMV), *Cactus virus* X (CVX), *Cymbidium mosaic virus* (CymMV), *Hydrangea ringspot virus* (HRSV), *Narcissus mosaic virus* (NMV), *Potato aucuba mosaic virus* (PAMV) and *Potato virus X* (PVX). The number of the nodes are the frequency (in percent) with which a cluster appears in a bootstrap test of 1000 runs.

Despite the differences among PepMV RNA polymerase sequences found, specific regions in the RNA polymerase gene were identified and two primers designated pep1 and pep2, were selected. The predicted RT–PCR product using these two primers with RNA from the Peruvian isolate of PepMV as a template would be 375 bp in length, and of similar size with RNA from the other PepMV isolates analysed. One hundred and two tomato samples collected from different regions in Spain plus five weeds (*Amaranthus* sp., *M. parviflora*, *N. glauca*, *S. nigrum* and *S. oleraceus*), were analysed by RT–PCR using primers pep1 and pep2. To test the primers specificity, four reference isolates of PepMV from different geographical origins, including the Peruvian isolate obtained from *S. muricatum,* and five reference samples from different potexviruses were included in the analysis.

The electrophoretic analysis of RT–PCR fragments obtained showed the expected fragment of 375 bp (Figure 2). This RT–PCR product was obtained with all 102 tomato samples analysed, the five weeds and the four reference isolates of PepMV. No amplification products were detected in control experiments performed with RNA from any of the other potexviruses analysed. No fragments were detected in the negative control (RNA from a healthy tomato plant).

On the basis of the RNA polymerase gene sequences, the endonucleases *Alu*I, *Dde*I, *Hae*III, *Hinc*II, *Taq*I and *Sau*3AI were used in the restriction analysis in order to differentiate among isolates. The observed

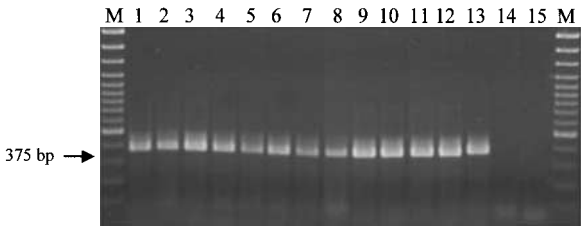

Figure 2. RT–PCR amplification of a specific fragment from PepMV isolates using the specific primers pep1 and pep2. Lanes 1–9 correspond to samples of tomato. Lanes 10–12 correspond to positive controls from tomato PV-0557, PV-0558 and 1027-11. Lane 13 corresponds to positive control from *S. muricatum* PV-0554. Lanes 14 and 15, negative controls using a healthy tomato plant and *Narcissus mosaic virus* (NMV) as representative of reference potexvirus used. Lanes M correspond to the molecular weight marker GeneRuler™ 50 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania).

restriction fragment length polymorphisms (RFLPs) are exemplified in Figure 3. The individual profiles designated A–C can be combined into composite restriction patterns or RFLP types (Table 1). Each one of the 107 samples analysed (102 from tomato plus 5 weeds) was then assigned to its RFLP type.

For the 107 samples analysed, three RFLP types (P1, P2 and P3) were observed (Table 1). The most common is type P1, represented by 87 samples including the five weeds analysed. The positive controls (DSMA PV-0557, DSMZ PV-0578 and 1127-11) were all P1 type (see Table 1). Type P2 was found in 15 samples, however, 12 of them showed banding patterns corresponding to a mixed infection between P1 and P2 types (Table 1). The positive control from *S. muricatum* (DSMZ PV-0554) was type P2. Finally, RFLP type P3 was found in five samples, but three of them showed banding patterns corresponding to a mixed infection between P1 and P3 types. Whereas the RFLP patterns obtained with five restriction enzymes proved very similar for types P1 and P2, the RFLPs obtained with *Sau*3A allowed the distinction of type P1 from the types P2 and P3 (Figure 3; Table 2).

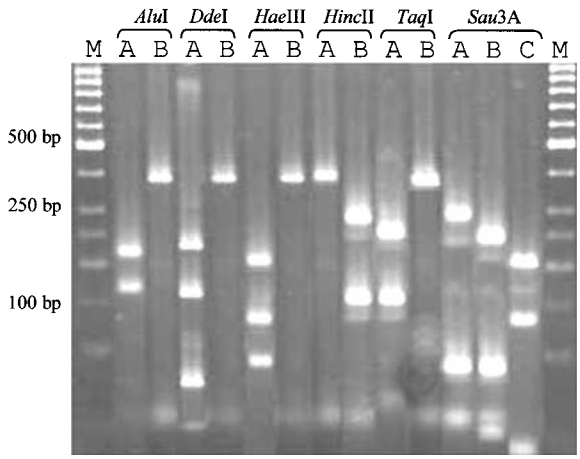

Figure 3. RNA polymerase restriction patterns exhibited by PepMV isolates after digestion with the restriction endonucleases *Alu*I, *Dde*I, *Hae*III, *Hinc*II, *Taq*I and *Sau*3A. The number of isolates in which these patterns were observed are listed in Table 1. Lanes M correspond to the molecular weight marker GeneRuler™ 50 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania).

Discussion

The phylogenetic tree, based on analysis of a portion (575 nt) of the RNA polymerase gene, showed that PepMV isolate 3253 was distinct from PepMV from tomato (AJ270992) and *S. muricatum* (AJ270991). However, we need to obtain full-length genomic RNA sequence data from these isolates to know the level of identity with each other.

By using the alignment of RNA polymerase sequences, PepMV-specific primers were designed. The specificity of the primers was predicted from comparison (alignment) of the PepMV sequences with the published sequences of different potexviruses. RT–PCR with RNA from a large number of tomato samples with typical PepMV symptoms, reference isolates of PepMV, weed samples containing PepMV and different potexviruses as negative controls or reference viruses confirmed the specificity of the primers. This indicated that pep1 and pep2 primers could be used for specific detection of all tested PepMV isolates. It is worth noting that the five weeds analysed in this work (*Amaranthus* sp., *M. parviflora*, *N. glauca*, *S. nigrum* and *S. oleraceus*) tested positive by ELISA (Jordá et al., 2001b). In the present work the RT–PCR analysis confirmed the natural infection of these weeds by PepMV.

The utility of restriction digestion analysis of the RT–PCR products from the RNA polymerase gene as a rapid method to identify different isolates of PepMV was tested. Three different RNA polymerase RFLPs patterns were detected among PepMV virus samples. Almost all samples analysed contained type P1.

Table 1. RNA polymerase restriction patterns and composite patterns or types exhibited by the PepMV isolates analysed

RFLP type	Reference isolates	Restriction patterns						Number of samples
		*Alu*I	*Dde*I	*Hae*III	*Hinc*II	*Taq*I	*Sau*3A	
P1	PV-0557, Southern Europe PV-0558, Germany 1027-11, UK	A	A	A	A	A	A	87
P2	PV-0554, Peru, *S. muricatum*	A	A	A	A	A	B	15[1]
P3		B	B	B	B	B	C	5[2]

[1]Twelve of these samples assigned to RFLP type P2 also showed the RFLP type P1.
[2]Three of these samples assigned to RFLP type P3 also showed the RFLP type P1.

Table 2. Length of the RT–PCR products and the restriction fragments generated by the endonuclease *Sau*3A used in identification of RFLP-types

RFLP types	RT–PCR (bp)	Fragment size (bp) *Sau*3A
P1	375	300 + 75
P2	375	250 + 75 + 50
P3	375	210 + 130 + 35

The positive controls from other European countries were all type P1 and the sequence of the PepMV isolate from tomato (GenBank AJ270992) indicated it was also type P1. It is likely that type P1 corresponds with an isolate of PepMV widely spread throughout Europe. In addition, many of the tomato samples assigned to types P2 and P3 were also infected with the virus corresponding to type P1. The presence of mixed infections, however, was not correlated to the observation of different disease symptoms.

Type P2 included 15 samples which showed the same RFLP patterns as the positive control obtained from *S. muricatum* (DSMZ PV-0554), and the fragments predicted from the *S. muricatum* isolate DNA sequence (GenBank AJ70991) also indicated it was type P2. As PepMV was originally described in Peru (Jones et al., 1980), it is tempting to speculate that the tomato isolates belonging to type P2 correspond with the original PepMV isolate from *S. muricatum*. The highly related RFLP patterns of the types P1 and P2 is in agreement with the phylogenetic tree, where the isolates from *S. muricatum* (GenBank AJ270991) and tomato (GenBank AJ270992) clustered closely. However, in spite of their closer phylogenetic relationship both the Peruvian and tomato isolates might be considered as different isolates (van der Vlugt et al., 2000). From the comparison of the RNA polymerase gene from both sequences, there is evidence to suggest that the Peruvian isolate is indeed different (sharing 95% identity) from the tomato isolate. These results compare well with the levels of homology found previously in the coat protein gene (CP), where the sequencing of 844 nt showed 99% identity between tomato isolates, but only 96% identity with the Peruvian isolate from *S. muricatum* (Mumford and Metcalfe, 2001).

The five samples included in type P3 had completely different RFLPs from the other two types studied. It is worth noting that type P3, with all the enzymes used, agreed with values estimated from the sequence 3253, which based on phylogenetic and distance analysis, was shown to be different from the other PepMV sequences previously reported. It is likely that type P3 represents one distinct isolate of PepMV. However, since the differentiation is based only on a portion of the sequence of the RNA polymerase gene, its significance for a differentiation of isolates in respect to pathogenicity, host range and geographical distribution remains unclear. It would be of great interest to analyse different solanaceous host plants to determine if a high level of genetic variability exists within PepMV virus.

In conclusion, the RT–PCR and RFLP analyses with *Sau*3A can be used as a rapid and easy method to detect and differentiate new PepMV isolates. Information of this nature would aid in development of understanding of the epidemiology and distribution of PepMV as well as to identify alternative virus hosts. Much work remains to be done in order to determine the significance of the differentiation reported here concerning the variability of PepMV isolates.

Acknowledgements

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