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The diagnosis of the tomato variant of pepino mosaic virus: An IC-RT-PCR approach

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Abstract

An immunocapture-retrotranscription-PCR (IC-RT-PCR) procedure for the detection of the tomato-infecting variant of pepino mosaic potexvirus (PepMV) was developed, following an approach that did not require knowledge of viral sequence, availability of commercial antibodies to the virus, or purification of viral particles. Degenerate PCR primers, whose design was based on alignments of published potexviruses sequences to prime theoretically the amplification of a viral genomic fragment of any potexvirus, were used to synthesize cDNA of the potato virus X (PVX) and PepMV RNA polymerases. Tubes coated with antibodies against double-stranded RNA were used in the initial amplifications. Two different non-overlapping fragments of the PepMV polymerase gene were cloned and sequenced, and their putative positions in the viral RNA were determined relative to the PVX sequence. For the diagnosis procedure, new specific PepMV primers were designed based on the virus sequence obtained and their utility to amplify a unique diagnostic band of 835 bp after IC-RT-PCR with specific anti-PepMV antibodies was shown. The method developed should allow the rapid diagnosis of a virus that seriously threatens tomato cultivation in several European countries.

Introduction

The emergence of new viruses in crops hitherto unaffected in a given geographical area is increasingly becoming a common occurrence, possibly as a consequence of the constantly growing international interchange of plant material. In such situations, the demand for reliable methods of sensitive and rapid detection of these new viruses as a control tool of the spread of their associated diseases is also growing rapidly. Molecular methods of virus detection, highly specific and sensitive, often require a certain, sometimes detailed, knowledge of the viral genome. The need for this genome characterization does not facilitate quick developments of sensitive methods.

Pepino mosaic virus (PepMV) is an emerging pathogen in tomato. It has caused severe economic losses throughout Europe in the last two or three years (Van der Vlugt et al., 2000; Mumford and Metcalfe, 2001; Roggero et al., 2001). The virus has been described in most European tomato producing countries. It is considered a very dangerous pathogen threatening tomato production in Europe, to the point that specific legislation at the European level has been dictated to regulate it (EC directive 2000/325/EC). Infected tomato plants exhibit variable symptoms, ranging from very mild mosaics to leaf deformations and fruit loss; the most typical symptomatology includes marked yellow mosaics in leaves and fruits, making them commercially non-viable (Jordá et al., 2000). The only known transmission way of this virus is by contact or infected plant material (Jones et al., 1980). This virus disease is a typical example of the need for a quick and sensitive diagnostic procedure to detect the infected material, even before its genome sequence has been elucidated.

We report the development of a method for the molecular detection of PepMV, based on the immunocapture-retrotranscription-PCR (IC-RT-PCR). The only knowledge required for this was that the virus is a member of the potexvirus genus of plant viruses (Jones et al., 1980; Van der Vlugt et al., 2000). No specific sequence data was required.

Materials and methods

Comparative sequence analysis of members of the genus potexvirus

Potexvirus sequences in the GenBank database (*www.ncbi.nlm.nih.gov*) were compared and searched for conserved regions, using the software MacVector™ 7.0 (Oxford Molecular Group Inc.) in a Power Macintosh 5500/225 computer. Nine complete potexvirus sequences were selected for more detailed analysis, trying to represent the genetic variability present in the genus (name and GenBank accession number: bamboo mosaic virus (D26017), cassava common mosaic virus Brazilian strain (U23414), clover yellow mosaic virus (D29630), cymbidium mosaic virus Korean type 2 (AF016914), foxtail mosaic virus (M62730), narcissus mosaic virus (D13747), papaya mosaic virus (D13957), potato virus X (PVX) (M72416), and white clover mosaic virus (X06728)). These nine sequences were aligned with respect to PVX (type member of the potexvirus genus). The most conserved regions within the different potexviruses were identified with a view to design 'universal' primers for this genus.

PVX detection through IC-RT-PCR

A stepwise strategy was followed for the PepMV IC-RT-PCR development. A first step included the adjustment of the experimental conditions for PVX, as an example of a well-known potexvirus, whose sequence has already been determined. The second step was to apply these conditions to PepMV.

Development of the method for PVX

As an initial step, the functionality of the primers designed after the potexvirus computer analysis was tested on a plasmid containing the PVX sequence (pgR107), provided by Dr. D. Baulcombe (John Innes Centre, Norwich, UK) (Baulcombe et al., 1995). Five nanogram template DNA were resuspended in 80 μl of PCR mixture containing 75 mM Tris–HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.25 mM dNTPs, 0.625 μM 5′ and 3′ primers, 2 U Tth DNA polymerase (Biotools, B&M Labs). PCR conditions used were 5 min at 94 °C as an initial DNA denaturation step, followed by 35 amplification cycles of 30 s at 94 °C, 1 min at 50 °C and 1.5 min at 72 °C, and 10 min at 72 °C as a final elongation step. Samples were stored at 4 °C, prior to their electrophoretic analysis.

The immunocapture was performed in 200 μl tubes, that had been previous coated 2 h at 37 °C with 100 μl of an anti-double stranded RNA antibody (α-dsRNA antibody), provided by Dr. R.A. Valverde (Louisiana State University), diluted 1/2000 in 50 mM carbonate buffer (pH 9.6). The material used were extracts from tobacco plants (*Nicotiana tabacum* L. cv. Samsun) inoculated with PVX vector (Baulcombe et al., 1995) and maintained in a growth chamber (16 h photoperiod, light intensity 100 μE m^{-2} s^{-1}). Thirty days after inoculation, a systemically infected half-leaf was collected, ground in 500 μl of extraction buffer (Nolasco et al., 1993) and subjected to immunocapture at 4 °C overnight. The tubes were washed three times by flooding with PBS-Tween (Nolasco et al., 1993), and the RT-PCR mixture was added. Prior to the RT-PCR reaction, a denaturation step of 5 min at 90 °C was sometimes performed by adding 30 μl of 1.6 μM 5′ and 3′ primers resuspended in sterile water. This denaturation step could be escaped without significant changes in the quality of the results. For the RT-PCR, 50 μl of a mixture containing 120 mM Tris–HCl (pH 9.0), 80 mM KCl, 32 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 0.4 mM dNTPs, 2 U Tth DNA polymerase (Biotools, B&M Labs), 10 U RNase inhibitor (GeneAmp® RNA PCR, Perkin Elmer), 12.5 U reverse transcriptase (GeneAmp® RNA PCR, Perkin Elmer) were added. RT-PCR conditions used were 30 min at 42 °C of retrotranscription, followed by the PCR conditions described above.

Development of the method for PepMV

The same IC-RT-PCR conditions used to detect PVX in tobacco were applied to extracts of tomato plants from Mazarrón (Spain) showing symptoms of PepMV infection. The samples used were pieces of leaves and fruits.

Specific detection of PepMV potexvirus

Sequencing amplified PepMV genomic fragments
Products amplified from the PepMV infected samples were cloned in the pCR® 2.1-TOPO plasmid with TOPO-TA Cloning® kit (Version I, Invitrogen) using the manufacturer's conditions and their sequences were determined in an external sequencing service.

Development of a specific IC-RT-PCR for PepMV
Primers were designed for the amplification of a 835 bp (base pairs) fragment of the newly obtained PepMV sequences. Primer design was done after an alignment with the corresponding PVX sequence, selecting sequences of low similarity with this potexvirus.

For specific immunocapture of PepMV, a commercial antibody (PepMV monoclonal or polyclonal antibody ADGEN Agrifood Diagnostic) was sometimes used, obtaining results indistinguishable from the ones obtained with the α-dsRNA antibody.

For the IC-RT-PCR reaction, frozen samples of infected tomato plants were ground in extraction buffer, as described before, and subjected to immunocapture at 4 °C overnight. The immunocapture was performed in tubes previous coated with 100 μl polyclonal or monoclonal antibody, diluted using the manufacturer's indications in 50 mM carbonate buffer (pH 9.6). The tubes were washed three times by flooding with PBS-Tween (Nolasco et al., 1993), and the RT-PCR reaction was performed with or without denaturation step. For the reaction, 80 μl of a mixture containing 125 nM 5′ and 3′ primers, 75 mM Tris–HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.25 mM dNTPs, 1 U Tth DNA polymerase (Biotools, B&M Labs), 10 U RNase inhibitor (GeneAmp® RNA PCR, Perkin Elmer), 12.5 U reverse transcriptase (GeneAmp® RNA PCR, Perkin Elmer) was added. RT-PCR conditions used were 30 min at 42 °C of retrotranscription, 5 min at 94 °C as an initial DNA denaturation followed by 35 amplification cycles of 30 s at 94 °C, 1 min at 51.5 °C and 1 min at 72 °C, and 10 min at 72 °C as a final elongation step. Samples were stored at 4 °C, prior to their electrophoretic analysis.

Detection of PepMV isolates from several European areas
The same IC-RT-PCR conditions used to detect specifically a first Spanish isolate of PepMV were applied to two other Spanish isolates, a British isolate and a Dutch isolate.

Results

Comparative analysis of members of the genus Potexvirus

The most conserved regions within the sequences of the nine selected members of the genus potexvirus were found in the RNA-dependent RNA-polymerase (RdRp) open reading frame, specially the region comprising nucleotides 2000–4000 of PVX (not shown). Two areas of maximum similarity were chosen in this region for the design of the two potexvirus 'universal' primers (Figure 1). Sequences 5′ → 3′ were: 5′ primer, acn tay gcn ggh tgy car gg (six degenerations, 384 combinations), and 3′ primer, cca tng thc cyw ana mca tna c (seven degenerations, 1536 combinations).

IC-RT-PCR detection of PVX

The primers described above were designed for the amplification of an 835 bp fragment of the PVX genomic RNA (Figure 2). Such a fragment was indeed amplified by IC-RT-PCR both from a DNA template and from extracts of PVX infected tobacco plants (Figure 2). In addition to this specific fragment, we observed the presence of other 'non-specific' amplification products, more marked in IC-RT-PCR than in PCR (compare lanes 1 and 2 in Figure 2). Probably the considerable number of degenerations in the primers and the RT-PCR conditions used in the reaction can explain these additional bands.

IC-RT-PCR detection of PepMV

The same IC-RT-PCR conditions used to detect PVX in extracts of infected tobacco plants were applied to amplify an equivalent fragment of PepMV out of tomato plants showing symptoms of virus infection. The results obtained are shown in Figure 3, in comparison with PVX. The corresponding PepMV amplified fragment was larger than that of PVX (compare lanes 1, 2 and 3 in Figure 3). An electrophoretic estimate of the PepMV fragment size was of ca. 1.1 kbp.

This IC-RT-PCR fragment was cloned. Five clones were analyzed by insert size and restriction pattern, and sequenced. Four of these clones contained the same main fragment of ca. 1.1 kbp, and all of them were sequenced in order to look for a representation of possible sequence variability. The remaining clone contained another unexpected insert of a smaller size

A

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PVX       2824 ACTGGGAGGAATGACACATTCACATACGCTGGATGTCAGGGGCTA 2868
CsCMV     2813 GCGGGACACAAAACTT---CAACATATGCGGGATGTCAGGGCATC 2854
PapMV     3097 ATGGGACATAAGGTGT---CCACCTACGCGGGCTGTCAGGGGATC 3138
ClYMV     3494 ATGGGATACAAGACCT---CAACATATGCAGGCTGCCAGGGCATC 3535
NMV       3228 ATGGGCCACCACAGCA---TGACGTATGCCGGCTGCCAAGGTCTT 3269
WClMV     2355 ATGGGCCAGAAAAGCA---TGACATACGCTGGATGCCAAGGTCTC 2396
BaMV      2537 ATGGGCAGGAAAACTT---CTACCTATGCGGGCTGTCAAGGGATA 2578
CymMV     2603 GTCGGCAACCGTTGCA---TGACTTACGCCGGTTGCCAGGGTCTC 2644
FoMV      2421 CTCGGGCAGCGAGCGT---CCACCTATGCGGGTTGTCAGGGGATC 2462
                  **                ** ** ** ** ** ** **  *
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B

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PVX       3637 GCTTTTTATCAGCAGACTGTGATGCTTTTTGGAACTATGGCCAGG 3682
CsCMV     3620 TCCTTCCAACAATCCGCTGTCATGCTATATGGCACAATGGCAAGA 3665
PapMV     3907 TCTTTTCATCAAGCCACTGTAATGCTCTTTGGAACTATGGCAAGA 3952
ClYMV     4304 TCATTTCAACAAGCCACAGTCATGCTCTACGGAACCATGGCACGC 4349
NMV       4041 TCTTTCATGCAAGAAACTGTCATGCTCTATGGCACCATGGCGCGC 4086
WClMV     3165 GCTTTTATGCAACAAACTGTCATGATTTATGGTACAATGGCCCGC 3210
BaMV      3347 GCGTTCTACCAGAGTACTGTCATGATCTTCGGCACGATGGCGCGC 3392
CymMV     3413 TCATTTATGCAAGAGACTGTTATGCTGTATGGCACCATGGCACGC 3458
FoMV      3231 GCCTTTTACCAACCAACTGTTATGCTGTTTGGAACCATGGCGAGA 3276
                * **    **     * ** *** * *  ** ** *****  *
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Figure 1. Nucleotide alignment in two regions of nine potexvirus sequences: PVX – Potato virus X; CsCMV – Cassava common mosaic virus; PapMV – Papaya mosaic virus; ClYMV-Clover yellow mosaic virus; NMV – Narcissus mosaic virus; WClMV – Whiteclover mosaic virus; BaMV – Bamboo mosaic virus; CymMV – Cymbidium mosaic virus Korean type 2; FoMV – Foxtail mosaic virus. High homology regions selected to design 'universal' primers for the genus are shadowed. Panel A: 5′ primer. Panel B: 3′ primer.

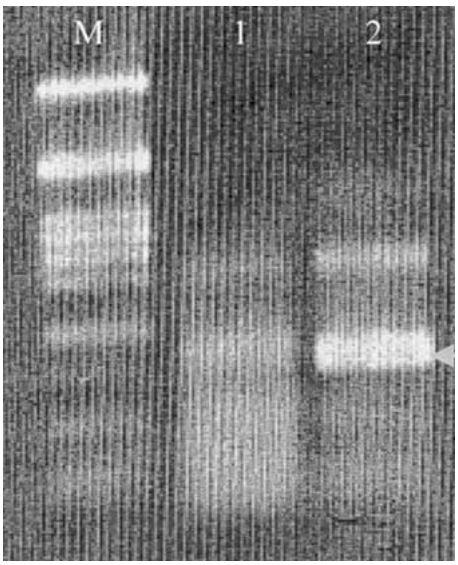

Figure 2. Amplification of PVX genomic fragments with 'universal' primers for the potexvirus genus. M – molecular weight markers; 1 – IC-RT-PCR performed on extracts of infected tobacco plants with α-dsRNA antibody; 2 – PCR performed on plasmid pgR107, containing the PVX genome, as a template. The arrow indicates the position in the gel of the expected 835 bp fragment.

(ca. 600 bp). The sequences of the five inserts were determined and compared with sequences present in GenBank, without including the primer sequences.

The similarities found strongly suggested that these clones contained inserts representing partial open reading frames of a potexvirus RdRp (Figure 4). The sequences did not match completely any of the sequences present in the database at the time.

The cDNA sequence of the four clones carrying inserts of similar sizes and restriction patterns were identical to each other, thus the four clones were actually the same, named clone B (accession number AJ308445). This clone represents the region of expected amplification according to the design of the primers. It carries an insert of 1024 bp (excluding primers) with an open reading frame all along the insert. The corresponding homology region in the PVX genome spans positions 2865–3894. Clone A had an insert of 610 bp (primers excluded), with an open reading frame (accession number AJ308446). It showed similarity with the PVX region 1017–1635 (Figure 4). The possible reasons for this clone are discussed later.

Having determined new specific RdRp PepMV sequences, we then designed virus specific primers

in genomic areas showing low similarity with PVX. Sequences 5′ → 3′ were: 5′ primer gag ctg tgg att cca tcc, and 3′ primer caa cct tgt tta aca aat tgg. Using these primers, an amplification product of 835 bp was obtained both from leaf and fruit samples of PepMV-infected tomato plants from Spain. The same result is obtained with other samples of PepMV-infected leaves from Spain, from England and from Holland (Figure 5). The PepMV primers failed to amplify PVX sequences from plasmid pgR107, containing the PVX genome, thus showing the specificity for PepMV.

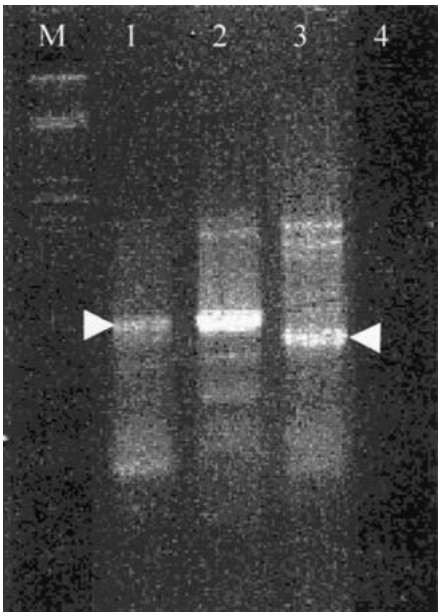

Figure 3. IC-RT-PCR amplification of PepMV and PVX genomic fragments using 'universal' primers for the potexvirus genus and α-dsRNA antibody. M – molecular weight markers; 1 – extracts of PepMV-infected tomato fruits; 2 – extracts of PepMV-infected tomato leaves; 3 – extracts of PVX infected tobacco; 4 – negative control (healthy tobacco plant). White arrows indicate the main amplification bands.

Discussion

The emergence of PepMV is increasingly reported as a limiting factor to the rapidly developing tomato cultivation business throughout Europe. Apparently, a new variant of this pepino virus has developed that is able to infect tomato and other important solanaceous crops (Jordá et al., 2000; Van der Vlugt et al., 2000; Mumford and Metcalfe, 2001; Roggero et al., 2001) producing intense visible symptoms. Tomato-infecting variants of this virus found in wild and cultivated *Lycopersicon* species in Peru (the area where tomato was originated) gave rise to symptomless or very mild infections (Soler et al., 2002), suggesting some form of genetic change in the European variant. Since this virus is new to tomato, no detailed sequence information or commercial antibodies were available when we started this development. Some limited sequence information is now deposited in databases, and during the course of our work, some commercial antibodies have appeared in the market. However, we have been able to develop the whole procedure without the need of these tools (which we have later used for confirmation protocols), using the information obtained to generate specific ones.

IC-RT-PCR has been applied to the detection, diagnosis and typing of plant and animal viruses for a decade now (Blancourgoiti et al., 1996; Estepa et al., 1995; Jansen et al., 1990; Nolasco et al., 1993; Rodríguez et al., 1994; Wetzel et al., 1992). In the case of plant viruses, its validity has been shown for members of most taxonomical groups for which it has been tested (Zabalgogeazcaga et al., 1997; Nolasco et al., 1993). Its obvious advantages over other well-established virus detection approaches, such as ELISA or regular (RT)-PCR, reside mostly in that it combines the ELISA characteristic of good adaptation to routine automatic analysis, with the superior sensitivity of PCR amplification of nucleic acids, avoiding the need for nucleic acid purification steps.

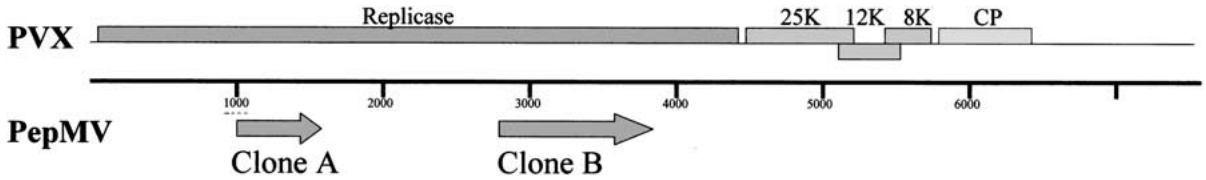

Figure 4. Relative position of PepMV amplified sequences reported in this paper with respect to the PVX sequence, based on nucleotide homology. The corresponding homology region for clone A in the PVX genome spans positions 1017–1635, and for clone B, positions 2865–3894.

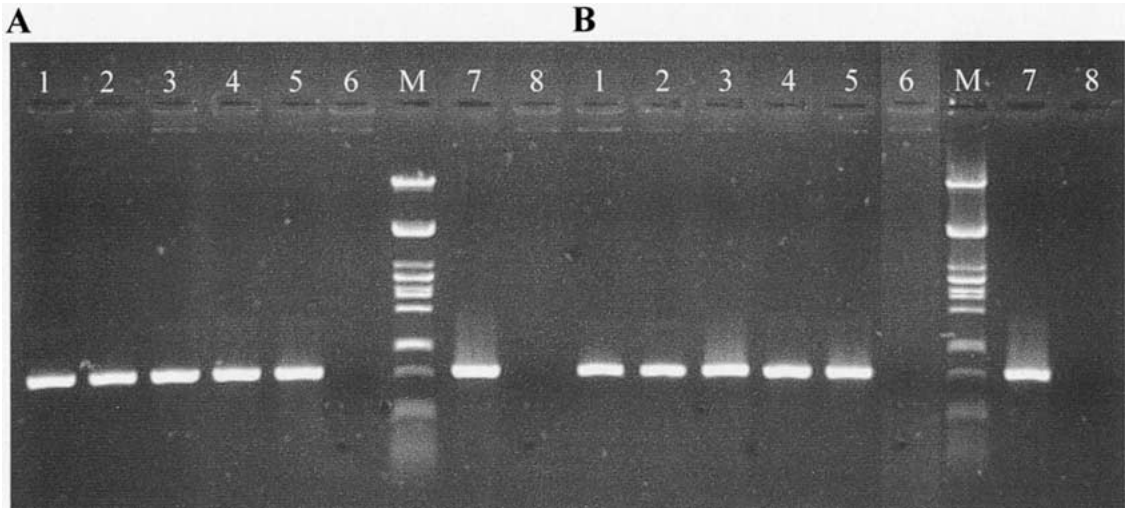

Figure 5. Specific amplification of an 835 bp fragment of samples infected with PepMV from different areas. 1 and 2 – IC-RT-PCR performed on extracts of Spanish samples infected with PepMV; 3 and 4 – IC-RT-PCR performed on extracts of British samples infected with PepMV; 5 – IC-RT-PCR performed on extracts of a Dutch sample infected with PepMV; 6 – negative control (healthy plant); M – molecular weight markers; 7 – PCR positive control (clone B DNA); 8 – PCR specificity control (PVX DNA). A – polyclonal PepMV antibody; B – monoclonal PepMV antibody.

The approach taken tried to exploit a maximal advantage of the minimum amount of molecular information available concerning PepMV. We first designed primers for the RT-PCR that could be general for potexvirus, based on sequence comparisons of members of this genus available in public databases. The strategy of designing primers that are suitable for the detection of viruses belonging to a given taxonomic group has been shown previously to be an efficient one in the case of potexviruses (Gibbs et al., 1998; Van der Vlugt and Berendsen, 2002). We chose a region of the polymerase gene that could be considered as reasonably conserved, if enough degenerate positions were included in the primer sequences. The validity of such primers was tested on a PVX DNA template. No further tests on other potexviruses were tried in this work. For plant material, the IC step was performed initially using an antibody against ds-RNA, that should bind to replicating forms of the virus present in the infected tissue, although it cannot be formally discarded that the RNA template for the RT reaction binds directly to the plastic in the solid phase. We have previously shown that amplifications based on materials captured in this manner are comparable in yield to the ones obtained using a specific one (Nolasco et al., 1993). The number of degenerate positions included in the general primers forced to use special amplification conditions, such as a low annealing temperature and high concentrations of primers and thermostable polymerase. The results obtained were not normally as clear-cut as the ones that are usually obtained using specific primers, but were selective enough to distinguish amplification bands in samples coming from plants showing virus symptoms and not from negative control plants. The whole procedure was tested first on PVX-infected tobacco and then on PepMV-infected tomato.

PepMV amplified fragments were cloned and sequenced. In order to study the variability of the amplification, we analyzed five clones. Four of the fragments (fragment B) had a size compatible with the expected one, whereas the other (fragment A) was smaller than expected. The sequence of these fragments allowed to find out its nature. Curiously, for both fragments A and B only the designed 5′ primer was found at both ends of each fragment. Likely, this was so because the number of combinations due to the degenerations present in the 3′ primer were too high, and the correct primer was in such a low concentration that diminished too much its annealing possibilities. The 5′ primer was also able to anneal on two other regions not selected by design, and served to amplify the internal region represented in fragment A, which is putatively another part of the PepMV polymerase gene.

The sequence information obtained allowed us to design new primers, now specific for PepMV. Once the sequences of both fragments were determined, the

new specific primers designed allowed a specific amplification of a selected internal subfragment of fragment B, using now normal PCR conditions. Under these conditions a unique amplification band was obtained, whose size was compatible with the designed one. The procedure was tested on tomato samples from different European areas to show its validity for more than one area. The result obtained confirmed that this was the case. The specificity of the primers was shown by their failure to amplify any PVX fragment from a PVX infectious clone. At this point in time in the year 2000, some commercial antibodies for serological diagnosis of PepMV appeared in the market. The results obtained using these antibodies were identical to the ones obtained with the antibody to ds-RNA. Also, at this time the first partial sequences of PepMV appeared in the public databases (accessions numbers AJ270991, AJ270992). These sequences are parts of our B fragment. Another sequence (AF340024) starts within our B fragment (Mumford and Metcalfe, 2001).

In conclusion, we have developed a specific IC-RT-PCR diagnosis procedure for PepMV starting from a situation in which the amount of information about this virus was minimal. Other researchers have reported the use of degenerated primers for a general PCR amplification of other groups of plant RNA viruses (e.g. Chen et al., 2001; Sabanadzovic et al., 2000; Saldarelli et al., 1998). Maybe the degenerated primers used in this work have the same potential application, but further work on other potexviruses would be required to assess this point.

In plant virology, we are constantly facing the emergence of new viruses, for which there is an urgent need for diagnosis tools, and approaches like the one described in this paper should help to develop them. In the particular case of PepMV, this virus is new to tomato, it is contact-transmissible and no good resistance sources have been yet identified. Thus, the only feasible control measures available today are based on the implementation of reliable diagnostic techniques.

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